Aquaculture Virology

Frederick S.B. Kibenge
Marcos G. Godoy
Editors

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Editors

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Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1800, San Diego, CA 92101-4495, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-801573-5

For Information on all Academic Press publications visit our website at https://www.elsevier.com/

Publisher: Nikki Levy
Acquisition Editor: Patricia Osborn
Editorial Project Manager: Karen R. Miller
Production Project Manager: Julie-Ann Stansfield
Designer: Greg Harris

Typeset by MPS Limited, Chennai, India

Cover photo credit: top: Electron micrograph of TO cells infected with infectious salmon anemia virus (ISAV) showing highly pleomorphic (filamentous, spherical, oval, donut-shaped, etc.) enveloped virus particles, each with a uniform fringe surrounding several electron-dense nucleocapsids. Courtesy of Frederick S.B. Kibenge (Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada).

Dedication

This book is dedicated to all current and future highly qualified personnel (graduate students, postdoctoral fellows, research associates, research assistants, diagnostic laboratory staff, clinical veterinarians, aquaculture disease practitioners, farmers and all teachers of virology) for whom it was written. Their inspiration was the seed for this text.

We also dedicate it to our families:

Molly, Stephen, Elizabeth and Patricia

Esterlin, Mirta, Leonel, Mónica, Daniela, Paula and Sebatian

I find that a great part of the information I have was acquired by looking up something and finding something else on the way.

— Franklin P. Adams, American columnist

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Preface

The textbook *Aquaculture Virology* covers the principles of clinical virology for viruses of the three major aquatic food animals: fish, crustaceans and mollusks. The increasing importance of these aquatic animals as an excellent source of quality protein for human consumption has resulted in increased interest in aquatic animal viral diseases, which are the principal natural limiting factors for aquaculture worldwide, causing massive losses in farmed and occasionally in wild populations. Due to the rarity of effective viral vaccines, and in the absence of antiviral drugs for use in aquaculture, prevention and effective control of viral diseases rely very heavily on biosecurity, hygiene and attempted eradication by depopulation. Such control measures depend on an understanding of all aspects of the virus and its relationship to the disease and epidemiology, including interaction between wild and farmed populations. This is an area that has desperately needed a comprehensive textbook for at least two decades; this book attempts to fill that knowledge gap and become the "go-to" book for this topic.

To date, textbooks on viruses infecting fish, crustaceans and mollusks have been on the whole "disease-centric" and focus on individual viral diseases selected based on "epizoo-centric" approaches, with little to no coverage of the basic biology of the viruses. This is in contrast to textbooks on viruses infecting terrestrial—farmed, pet and free-range (wild)—animals, as well as humans. Where aquatic animal (mostly fish) viruses are included in the various animal virology textbooks, they are consistently relegated to the last pages of the various chapters, in most cases with very limited information. Moreover, there is no context for aquaculture being described in a similar way as the terrestrial viruses impacting agriculture or human health. Despite considerable advances in animal virology in recent years, coupled with an economically important global aquaculture industry, knowledge of viruses of animal aquaculture is still sparse and in some cases outdated, although these viruses are closely related to well-known virus families.

The last book in fish virology (*Fish viruses and fish viral diseases,* 1988, Wolf, K.) was published in the 1980s. A lot of work has been done on fish viruses, and many new aquatic animal viruses continue to be discovered. The editors of *Aquaculture Virology* wanted to present the current state of knowledge of aquatic animal viruses within the current virus classification and taxonomic context, thereby allowing readers to draw on the principles of general virology.

The authors were encouraged to use the same format in all chapters to facilitate the reading and studying. Thus whereas each chapter includes the main specific diseases that affect the aquatic organism, the description of each disease includes disease name, structure and composition of virus, classification and virus replication, epidemiology, pathology and immunity, diagnostic methods (gross pathology, histopathology, cell culture, polymerase chain reaction, sequencing, enzyme-linked immunosorbent assay, etc.) and prevention and control. The information in the book provides the reader with readily applicable and updated knowledge, delivered in a systematic and succinct way. Those interested in aquatic animal viral diseases will find that the book provides comprehensive, high-quality information in a single source, detailing the diseases, viruses, pathology, laboratory methods for the detection and confirmation of the virus and the prevention and control of these diseases within aquaculture systems. This book targets a unique audience knowledgeable in aquaculture and fisheries with interest in animal pathogens, specifically viruses. This book will be used by researchers, teachers, students, diagnostic laboratory staff, clinical veterinarians, aquaculture disease practitioners, farmers and all people interested in viruses in general.

The authors for the different chapters are invited international experts recognized in their specific virus family or diseases. The editors gratefully acknowledge all the authors for their contributions and keeping to the deadlines.

Frederick S.B. Kibenge and Marcos G. Godoy
EDITORS

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Part I

General Aspects

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Chapter 1

Introduction to Aquaculture and Fisheries

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1.1 INTRODUCTION TO AQUACULTURE AND FISHERIES

The Food and Agricultural Organization of the United Nations (FAO) attributes aquatic organisms that are harvested by an individual or corporate body that has owned them throughout their rearing period to aquaculture, while aquatic organisms that are exploitable by the public as a common property resource, with or without appropriate licenses, are harvested by fisheries (FAO, 2015). The contribution of wild-catch fisheries as a protein source for human consumption plateaued in the mid-1980s, and since then, most natural stocks in marine waters have been harvested at or near maximum rates. According to the FAO, aquaculture remains one of the fastest-growing animal food-producing sectors worldwide. In fact, it is the only animal food-producing sector growing faster than the human population, and it provides an acceptable supplement to and substitute for wild fish. In fact, in 2014, aquaculture contributed more to aquatic animal food destined for human consumption than fisheries for the first time. This share of aquaculture is projected to rise to 62% by 2030, as catches from wild capture fisheries level off (FAO, 2014). Fish, crustaceans and mollusks represent the most economically important global aquaculture industry subsectors (Fig. 1.1), at 66.6 million tonnes in 2012, with an estimated total farmgate value of US$144.4 billion (FAO, 2014), and they are expected to reach US$202.96 billion by 2020 (Grand View Research Inc, 2014).

Most of the global aquaculture production (89% by volume) is located in countries in Asia (the most important being China, India, Vietnam and Indonesia). Table 1.1 summarizes the distribution of the three main aquaculture industry subsectors among the top 15 countries that account for 92.7% in global aquaculture production.

1.2 AQUATIC SPECIES IN AQUACULTURE

Among the three main aquaculture groups (fish, crustaceans and mollusks), there were 354 fish species, 59 crustacean species and 102 mollusk species recorded by FAO as cultured in 2012. Carp dominates production in both China and the rest of Asia; for Europe and South America, it is salmonids; African aquaculture production is almost exclusively tilapias; and for Oceania, shrimps and prawns dominate (Hall et al., 2011). The most significant farmed species, with more than 100,000 tonnes apiece in 2012 (FAO, 2012), are listed in Table 1.2. However, analysis of aquaculture production and details about farmed species remain only an approximation because many indigenous aquatic species are used in aquaculture without being registered individually in national statistics. For example, in China, more than 200 species are farmed commercially, but total production is registered under fewer than 90 species and species groups. Similarly, in India and Vietnam, the number of cultured species far exceeds the number included in statistics (FAO, 2014). Moreover, for the most widely farmed species, tilapia, the true number of producer countries is higher than the FAO tally of 135 countries because commercially farmed tilapia is not yet reflected separately in national statistics in Canada and some European countries (FAO, 2014).

1.3 AQUACULTURE TECHNIQUES, SYSTEMS AND FACILITIES

The techniques, systems and facilities used in aquaculture are as diverse as the species currently being raised and also vary by geographical region (Bostock et al., 2010), but normally encompass three stages: incubation/hatchery seed production, early rearing and on-growing (fish aquaculture briefly reviewed by Roberts and Shepherd, 1974). In salmon and trout aquaculture, on-growing has merged with husbandry breeding (Aarset and Borgen, 2015). For new farmed species like cod, two methods are mainly used: one is based on capturing wild cod for on-growing, while the other focuses on the production of cod from hatchery seed to market size. Currently, on-growing of wild cod is more economically efficient than using farmed

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00001-2
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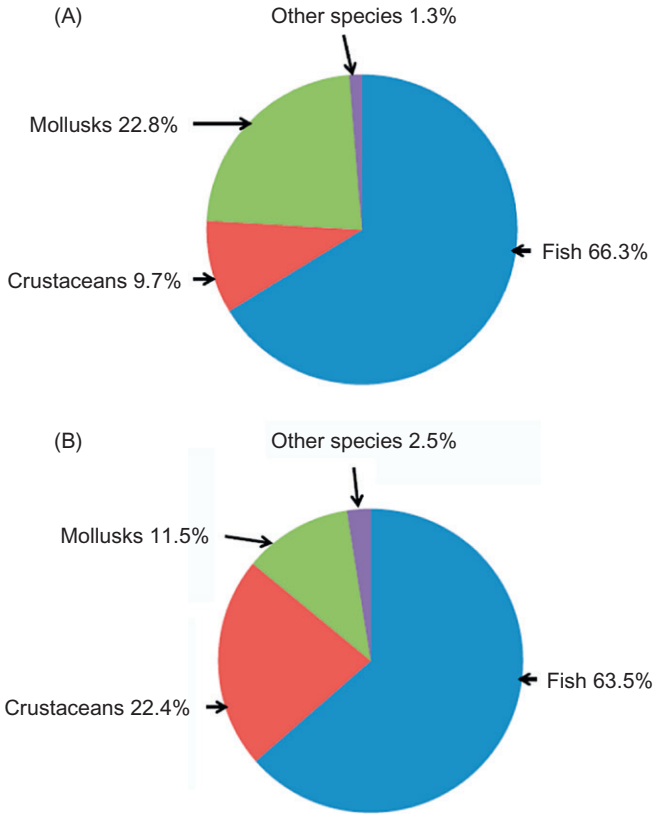

FIGURE 1.1 World aquaculture by farmed species sector, 2012. (A) Aquaculture industry subsectors by quantity 2012; (B) aquaculture industry subsectors by value. *Developed from FAO, 2014. The State of World Fisheries and Aquaculture 2014. FAO, Rome, 223 pp.*

TABLE 1.1 Distribution of the Aquaculture Industry Subsectors Among the Top 15 Countries in Global Aquaculture Production in 2012[a]

Country	Production by Aquaculture Subsector (in tonnes)				Share of World Total (%)
	Fish	Crustaceans	Mollusks	Other	
China	24,369,533	3,592,588	12,343,169	803,016	61.7
India	3,896,584	299,926	12,905	NL[b]	6.3
Vietnam	2,142,200	513,100	400,000	30,200	4.6
Indonesia	2,679,484	387,698	NL[b]	477	4.6
Bangladesh	1,588,892	137,174	NL[b]	NL[b]	2.6
Norway	1,319,118	NL[b]	2001	NL[b]	2.0
Thailand	400,980	623,660	205,192	4045	1.9
Chile	818,114	NL[b]	253,307	NL[b]	1.6
Egypt	1,016,629[b]	1109	NL[b]	NL[b]	1.5
Myanmar	824,457	58,981	NL[b]	1731	1.3
Philippines	671,764	72,822	46,308	NL[b]	1.2
Brazil	611,343[b]	74,415	20,699	1005	1.1
Japan	284,429	1596	345,914	1108	1.0
Republic of Korea	90,406	2838	373,488	17,672	0.7
United States	206,767	44,928	168,329	NL[b]	0.6
Rest of world	3,230,455	635,983	999,426	5288	7.3
Total	44,151,155	6,446,818	15,170,738	864,542	100

[a]*Developed from FAO, 2014. The State of World Fisheries and Aquaculture 2014. FAO, Rome, 223 pp.*
[b]*Some of the production data are not available (marine aquaculture), or the production volume is regarded as negligibly low (NL).*

juveniles (FAO, 2016). For a few species, such as eels (*Anguilla* spp.), farming still relies entirely on wild seed. Although the life cycle of the Japanese eel (*Anguilla japonica*) in captivity has been completed, techniques for mass production of glass eels have not yet been established because of various technical difficulties (Masuda et al., 2012; Bird, 2013).

World aquaculture production takes place in a number of locations: on land (using freshwater or saline water); in static water ponds (eg, for growing channel catfish and other warm-water species); concrete raceway systems; freshwater gravity tanks; large recirculation tanks; in floating net cages in a lake or river (eg, for growing salmonids and other cold-water species that require clean water with high oxygen levels); offshore (mariculture or marine aquaculture) in floating net cages in the sea and intertidal zones (Shepherd, 1993); in coastal lagoons or brackish-water ponds; and in onshore-based tanks using pumped seawater (eg, for flat fish farming of turbot or halibut that normally lie on the bottom and will not shoal within the water column inside a floating cage (Shepherd, 1993)).

1.3.1 Fish Aquaculture

The top 10 cultured fish species are carps, except tilapia, Pangasius catfish and Atlantic salmon (Table 1.2). China, the largest producer of farmed seafood (61.7% share of the world total in 2012; Table 1.1), grows carp mostly in ponds and rice paddies, with little or no effort to actively nurture the animals, and has been practicing this type of aquaculture for thousands of years. Most carp farming is pond-based, with several species stocked in the same pond (polyculture) (SPC, 2011). Tilapia, and sometimes catfish, are stocked along with carp. Single-species culture (monoculture) is rare, except in flow-through systems and cage cultures of common carp in streams or canals. There are different stocking models for polyculture, depending on the availability of the main source of feed. If grasses (aquatic or terrestrial) are abundant, grass carp

TABLE 1.2 Major Farmed Species

Aquaculture Sector	Species or Species Group[a]
Fish	
	Grass carp (*Ctenopharyngodon idellus*) (FW)
	Silver carp (*Hypophthalmichthys molitrix*) (FW)
	Common carp (*Cyprinus carpio*) (FW)
	Tilapias (*Oreochromis niloticus*) and other cichlids (FW)
	Bighead carp (*Hypophthalmichthys nobilis*) (FW)
	Major (Indian) carp (*Catla catla*) (FW)
	Crucian carp (*Carassius carassius*) (FW)
	Atlantic salmon (*Salmo salar*) (D)
	Rohu (*Labeo rohita*) (FW)
	Milkfish (*Chanos chanos*) (D)
	Rainbow trout (*Oncorhynchus mykiss*) (D)
	Wuchang bream (*Megalobrama amblycephala*) (FW)
	Black carp or Black Chinese roach (*Mylopharyngodon piceus*) (FW)
	Northern snakehead (*Channa argus*) (FW)
	Amur catfish or Japanese common catfish (*Silurus asotus*) (FW)
	Mrigal carp (*Cirrhinus mrigala*) (FW)
	Channel catfish (*Ictalurus punctatus*) (FW)
	Asian swamp eel or White ricefield eel (*Monopterus albus*) (D)
	Pond loach or Japanese weather loach (*Misgurnus anguillicaudatus*) (FW)
	Iridescent shark (*Pangasius hypophthalmus*) (FW)
	Mandarin fish or Chinese perch (*Siniperca chuatsi*) (FW)
	Yellowhead catfish or Korean bullhead (*Pelteobagrus fulvidraco*) (FW)

(*Continued*)

TABLE 1.2 Major Farmed Species (Continued)

Aquaculture Sector	Species or Species Group[a]
	Characins (FW)
	Coho salmon (*Oncorhynchus kisutch*) (D)
	Barramundi or Asian seabass (*Lates calcarifer*) (D)
	Jacks, pompanos and mackerels (M)
	Drums and croakers (M)
	Gilt-head (sea) bream (*Sparus aurata*) (M)
	Other seabreams (M)
	Mullets (M)
	European seabass (*Dicentrarchus labrax*) (M)
	Japanese seabass or Suzuki (*Lateolabrax japonicus*) (M)
Crustaceans	
	White leg shrimp (*Litopenaeus vannamei*, formerly *Penaeus vannamei*)
	Giant tiger prawn or Asian tiger shrimp (*Penaeus monodon*)
	Chinese mitten crab (*Eriocheir sinensis*)
	Red swamp crawfish (*Procambarus clarkii*)
	Other marine shrimps
	Mud crabs
	Other marine crabs
	Freshwater shrimp
Mollusks	
	Japanese carpet shell or Manila clam (*Ruditapes philippinarum*)
	Chinese razor clam or Agemaki clam (*Sinonovacula constricta*)
	Pacific oyster, Japanese oyster or Miyagi oyster (*Crassostrea gigas*)
	Blood cockle or Blood clam (*Anadara granosa*)
	Mussels
	Scallops, pectens
	Other marine mollusks
	Abalones, winkles, conchs

FW, freshwater; D, diadromous (these fish spawn in freshwater and migrate to the sea for the main growing period); M, marine.
[a]Major farmed species or species group (with production of 100,000 tonnes or more in 2012). Data from FAO, 2012: The State of World Fisheries and Aquaculture 2012. Food and Agriculture Organization of the United Nations, Rome.

can be stocked as the major species. The leftover feed and grass carp excreta would sufficiently fertilize the pond water for the growth of filter feeders. Pond-based carp culture has been traditionally integrated with crop farming (mulberry, fruit, vegetables, etc.) and animal husbandry (ducks, swine, chicken, etc.) in China. The practice has been widely introduced to many other parts of the world, with some modifications to suit local conditions (SPC, 2011).

In industrial aquaculture, farmed fish are reared at high population densities in floating open-net cages near shore (for marine aquaculture), or in a lake or river (for inland aquaculture), in the same water column as the wild fish reservoir, which is often at a relatively low population density (Hill, 2005). The cages are either square/hexagonal steel platform constructions linked together or 10–50-m-deep circular polyethylene rings with netting to hold the farmed fish. Expansion can simply be by cage volume or number of cages. Such a cozy aquatic environmental interaction is inevitably ideal for selecting and propagating more virulent variants of wild-type pathogens that exist benignly in local wild stocks. This so-called

"local effect" is an inherent and highly costly risk of aquaculture throughout the world (Hill, 2005), although for several viral diseases of economic importance to aquaculture, there is no clear evidence for transmission of viruses between wild and farmed fish (Johansen et al., 2011).

Today, in various countries, wild fish are collected from rivers for raising juvenile fish in hatcheries and then released for the restocking of natural habitat and under enhancement programs for recreational fishing purposes, which helps to develop large fisheries in some rivers and inland lakes (Dexter and O'Neal, 2004).

1.3.2 Crustacean Aquaculture

The most recent dramatic change in aquaculture has been the explosive growth in shrimp farming in Southeast Asia (Cressey, 2009). Production has increased almost exponentially since the mid-1970s. This rapid increase largely reflects the dramatic increase in white leg shrimp (*Penaeus vannamei*) culture in Asia (eg, China, Thailand and Indonesia) and Latin America (Ecuador and Mexico) (Bondad-Reantaso et al., 2012), where the penaeid shrimps have tended to dominate due to high-value and short production cycles and accessible technologies (Bostock et al., 2010). Crustacean farming, similar to marine fish aquaculture, requires a high-quality diet usually containing fish meal and often fish oil as well (Bostock et al., 2010).

1.3.3 Mollusk Aquaculture

For the aquaculture of mollusks (oysters, clams, scallops, abalone, mussels, cockles and related species), there are many varieties of culture and grow-out methods that are particular to specific species and locations (Elston, 1999; Elston and Ford, 2011; Dumbauld et al., 2009). Most mollusk culture requires no feed input (algae are used to feed the larvae and juveniles in hatcheries to provide spat for mollusk culturing). Seed bivalves are produced either in land-based hatcheries or from natural populations, and can be transported over considerable distances to grow-out sites in estuaries, and even to different countries (FAO, 2013). Farming mollusks is limited in several countries because of the limited capacity to produce seed from mollusk hatcheries and nurseries (FAO, 2014), relying on wild seed. The use of wild seed is impossible if the natural populations of mussels are not suited to culture. Intensification will require a reliable, plentiful and inexpensive supply of hatchery seed, requiring the selection and management of broodstocks and the controlled production of high-quality seedstocks (Elston and Ford, 2011).

1.4 AQUACULTURE PATHOGENS

The most common cause of infectious diseases in aquaculture is bacteria (54.9%), followed by viruses (22.6%), parasites (19.4%) and fungi (3.1%) (reviewed in Kibenge et al., 2012). Viral diseases have been more difficult to control due to the high susceptibility of aquatic animals to them at an early age, limited availability of therapeutics, insufficient knowledge of pathogenesis of virus infections and limited knowledge about natural resistance mechanisms in aquatic animals. Thus, viruses are the principal pathogens that are negatively affecting aquaculture worldwide.

1.5 AQUACULTURE HEALTH MANAGEMENT

The intensive rearing of fish, crustaceans and mollusks is highly vulnerable to infectious diseases, similar to captive livestock agriculture (eg, the poultry and swine industries). However, in contrast to terrestrial farmed animals, the animal strains used in aquaculture usually have been derived from wild strains in recent years (Stuart et al., 2007; Duarte et al., 2007; Rosenlund and Skretting, 2006; Svasand et al., 2004) and may not have had enough time to adapt to high-density confinement within the aquaculture environment (Rodriguez-Ramilo et al., 2011). This chronic stress (Snieszko, 1974; Wedemeyer, 1996; Weyts et al., 1999; Yada and Nakanishi, 2002) provides opportunities for the emergence of diseases caused by pathogens that may have been harmless under natural conditions. Several strategies that are used to successfully rear terrestrial farm animals in intensive production systems are also used to prevent and control viral diseases in aquaculture, including the selective breeding for increased resistance against diseases (Gjedrem, 2010) and vaccination (Gudding, 2014). However, despite the traditional use of antibiotics, there is no field experience with applying antiviral drug treatment to farmed fish, although prophylactic antiviral compounds, if available, would be best used in broodstock and valuable genetic stocks that are not used for human consumption (Kibenge et al., 2012). Vaccination is not an option with farmed crustaceans and mollusks because of their primitive immune systems. Thus, reducing the risk of disease in these cases will largely depend on the application of better husbandry and biosecurity management practices at the farm level and the use of quality, high-health, specific pathogen-free seed supported by strict biosecurity (Bondad-Reantaso et al., 2012). Additional biotechnology efforts are being directed at better understanding of "immune priming" in invertebrates and developing innovative antiviral drug treatment, such as by RNA interference (Rowley and Pope, 2012).

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Chapter 2

Classification and Identification of Aquatic Animal Viruses

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2.1 VIRUSES ARE ENTITIES AT THE EDGE OF LIFE

Viruses occupy a unique position in biology. Although they possess some of the properties of living systems (such as a genome), they are in fact nonliving infectious entities and should not be considered microorganisms. Viruses have two clearly defined phases in their life cycle. A virus particle outside a host cell is metabolically inert (functionally inactive). Inside their host cell, the viruses are metabolically active (functionally active); this is their replicative phase, in which the viral genome exploits the machinery of the host cell to produce progeny genome copies, viral messenger RNA and viral proteins that assemble to form new virus particles. Structural features of a virus are determined by requirements for virus multiplication in a host cell (ie, assembly, release, transmission, attachment, penetration and uncoating).

2.2 HISTORY OF VIRUS CLASSIFICATION

Schemes for virus classification have changed rapidly in the past few years and continue to evolve. Schemes used in the past included those based on common organ tropisms and common ecological and transmission characteristics of the virus infection, leading to names such as *enteric viruses*, *respiratory viruses*, *viscerotropic viruses*, *arboviruses* (*ar*thropod-*bo*rne *viruses*), etc. While these systems were useful (and are still useful when discussing diagnosis, pathogenesis and epidemiology), it quickly became clear that groups of viruses affecting the same organ in animals, or with similar epidemiological features, actually consisted of a diverse array of quite different viruses. Subsequent taxonomic systems have focused on the viruses themselves, and the advances in classification have generally followed advances in our knowledge of virus morphology, genomic composition and replication strategy.

2.3 MORPHOLOGY AND COMPOSITION OF ANIMAL VIRUSES

A virus particle (or *virion*) is a structure that has evolved to transfer nucleic acid from one cell to another (which is the function of the virion). The central structure of every virus is a nucleic acid (*genome*) surrounded intimately by a protein shell or coat (*capsid*). Together, they form a *nucleocapsid*.

Genetic economy dictates that the capsid/nucleocapsid structure be built from identical copies of a small number of viral proteins. These protein molecules are arranged to provide maximal contact and noncovalent bonding among *subunits* (polypeptides) and *structural units*. The repetition of such interactions among a limited number of proteins results in a regular structure of either *icosahedral* or *helical* symmetry. Thus during virus multiplication in an infected cell, viral *structural proteins* associate spontaneously (through random thermal movement and are bonded in place through weak chemical bonds) to form *capsomeres*. The capsomeres self-assemble in a defined manner, giving the capsid a characteristic symmetry (icosahedral or helical) (Fig. 2.1A). Viruses that do not fall into either category are referred to as being *complex* (eg, poxviruses and bacteriophages).

Virus capsids of icosahedral symmetry have 12 corners, 20 equilateral triangular faces and 30 edges with a 5:3:2-fold rotational symmetry, which passes through their corners, faces and edges, respectively (Fig. 2.1B). This symmetry is the most efficient of possible arrangements for repeating subunits in a closed shell, in the sense that it uses the smallest unit to build a strong structure enclosing a maximum volume. Thus, in the surface of any icosahedrally symmetrical structure, there are exactly 60 identical elements (and certain multiples of 60) related to each other by twofold, threefold and fivefold

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00002-4
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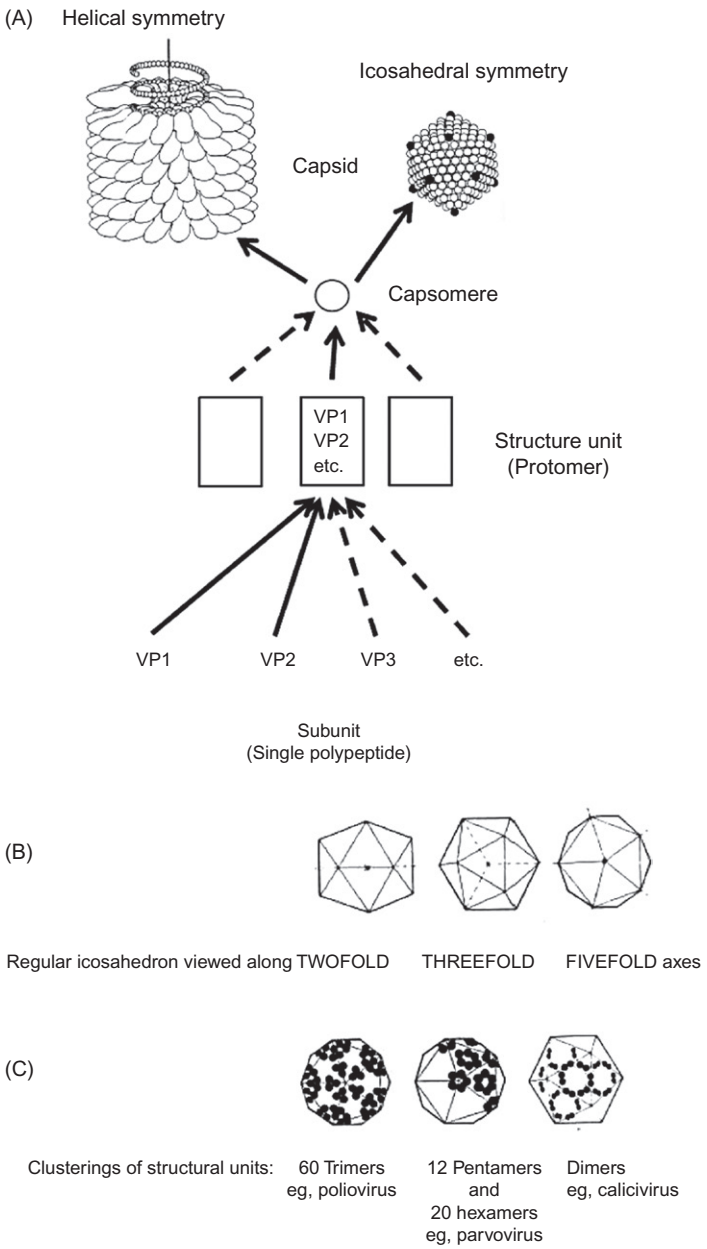

FIGURE 2.1 General structure of viral capsids. (A) Spontaneous association of viral structural proteins (VPs) and self-assembly of capsomeres into capsids with characteristic symmetry (icosahedral or helical); (B) Icosahedral symmetry with a 5:3:2-fold rotational symmetry; (C) Various clustering of structural units (trimers, pentamers, hexamers or dimers).

rotation axes. Viruses generally fit $60 \times N$ subunits into their capsids. N is sometimes called the *triangulation number*, also abbreviated T. Various clustering of structural units (trimers, pentamers, hexamers or dimers; Fig. 2.1C) give the characteristic appearance of capsomeres in electron microscopy (EM) and may be used to identify a virus. Viewed by EM with negative staining, icosahedral virions appear hexagonal or spherical in outline.

Virus capsids of helical symmetry have a single rotational symmetry. The length, width and pitch of the helix (ie, the distance covered by each complete turn of the helix), as well as the number of capsomeres in each turn of the helix, are constant for a given virus type. Viewed by EM with negative staining, helical virions appear pleomorphic (if they lack a matrix protein in the envelope) but in native state, they are spherical (except rhabdoviruses, which are rod- or bullet-shaped, and bafiniviruses and roniviruses, which are bacilliform).

Some viruses are unenveloped (or naked), and exist basically as nucleocapsids. Many other animal viruses acquire an *envelope* by passing through one of the cell membranes (nuclear membrane, plasma membrane, rough endoplasmic reticulum, Golgi complex). Thus, the actual content of a viral envelope depends on the host cell and the cellular membrane through which the particle buds, but is generally comprised of a lipid bilayer with transmembrane viral glycoproteins. In viral envelopes, the protein is viral coded and the carbohydrate is added by cellular glycosyl transferases. The glycoproteins are the surface structures (*spikes*, *peplomers*) on enveloped viruses; they therefore form the viral attachment proteins essential for both host specificity and viral infectivity, and *epitopes* targeted for virus neutralizing antibodies, as well as for the effector cells of cell-mediated immunity. The viral *matrix protein* (nonglycosylated envelope protein) adds rigidity to the enveloped virions. Enveloped viruses are *ether-sensitive* and *chloroform-sensitive* (ie, infectivity of enveloped viruses is destroyed by detergents and lipid solvents), which is important when determining the morphology of a virus.

The morphology of a virus particle includes the size (ranging from 12 to 430 nm in diameter) and shape of the virion (spherical, rod-shaped, ovoid, bacilliform, bullet-shaped, pleomorphic), number of capsomeres, symmetry of capsid (*icosahedral*, *helical*, *complex*) and presence or absence of an envelope. This is the primary criterion for virus classification (see also Fig. 2.4). The viral genome can be either RNA (and nonsegmented or segmented) or DNA (and linear or circular) and single-stranded (positive sense or negative sense or ambisense) or double-stranded (see also Tables 2.2 and 2.3).

Regardless of the structural complexity of a virion, proteins are its principal constituent. The viral proteins can be *structural* or *nonstructural* proteins. The structural viral proteins include surface proteins that make up the capsid and viral enzymes like neuraminidase, and internal proteins such as the viral enzymes DNA polymerase, RNA polymerase, proteases, helicases and ligases.

The surface viral proteins (viral attachment proteins) have an affinity for complementary *receptors* on the surface of susceptible cells and contain antigenic determinants (epitopes), which is important for vaccines as immunogens, for virus identification (serotypes) and for diagnosis (serology). Nonstructural proteins are not found in the virion; rather, they are only present in infected cells during virus replication and are involved in virion assembly. Such nonstructural proteins have been used to develop serological tests that *d*ifferentiate between *i*nfected and *v*accinated *a*nimals (DIVA).

2.4 OVERVIEW OF VIRUS REPLICATION

Virus replication occurs only in living cells, in sequential steps involving the attachment, penetration, replication of the parent virus and release of progeny viruses. Every animal virus family (refer to Tables 2.2 and 2.3) has a different strategy of replication (a useful parameter in virus classification). The strategy of virus replication depends on the characteristics of the virus genome and the pathway they use to produce their messenger RNA (mRNA) (ie, the Baltimore classification; see Section 2.7). The ability of the virus to multiply and the fate of an infected cell depend on the synthesis and function of the *viral proteins*. These proteins replicate viral genomes, package the genomes into capsids, alter the structure of infected cells and alter the function of infected cells to favor virus replication.

One-step growth curve is a common experimental procedure for studying virus replication. One-step conditions exist when all cells in a culture are infected simultaneously using a high *multiplicity of infection* (MOI; ie, the ratio of virus particles to cells) so as to prevent secondary cycles of infection. After the initial infection, the excess unabsorbed virus is rinsed off, and the amount of infectious virus over time is followed by sequential sampling and titration. When these titers are plotted as a function of time, a growth curve of a given virus in a given cell system is generated. A virus that is free in the cell culture medium can be titrated separately (for the yield of *extracellular virus*) from a virus that remains cell-associated (the yield of *intracellular/cell-associated virus*). We recognize four stages of a virus growth curve (Fig. 2.2): *attachment*, *eclipse period* (infectious virus particles cannot be demonstrated inside the cell), *maturation* and *release* (by cell lysis in naked viruses and by budding in enveloped viruses).

There are eight essential steps in the virus replication cycle. In order to initiate infection, the virus must bind to the cell surface via capsid or envelope proteins. It must then penetrate the cell and uncoat to make its genome accessible to viral and host machinery for transcription and/or translation, and transport to sites where virus-directed synthesis takes place. If replication occurs in the nucleus, viral proteins have to be transported to the nucleus for virion assembly.

1. *Attachment (adsorption) to cell surface:* Depends on the presence of specific cell receptors for complementary viral attachment proteins on the surface of the virion (ligands or antireceptors). This specificity determines the tissue tropism and host range of a virus. If a cell does not have receptors for the virus, then that cell and that animal will be resistant to that virus. Adsorption of virus may require calcium ions (to reduce electrostatic repulsion) but does not require energy or temperature.

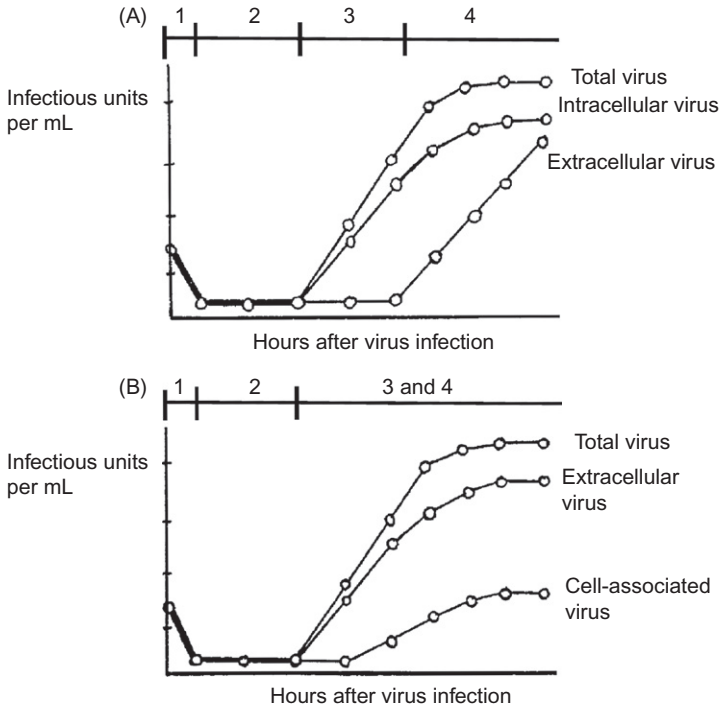

FIGURE 2.2 A one-step growth curve for an animal virus. (A) One-step growth curve for a naked animal virus; (B) One-step growth curve for an enveloped animal virus budding from the cytoplasmic membrane. Note the differences between intracellular and extracellular virus titers for both the naked virus (A) and the enveloped virus (B). The top bar demarcates the four stages of a virus growth curve: 1, attachment and penetration; 2, eclipse period; 3, maturation; 4, release. In enveloped viruses, stages 3 and 4 occur simultaneously. Stages 2 and 3 make up the latent period.

2. *Penetration (uptake):* Can be by at least one of four mechanisms; however, some viruses can use more than one mechanism. These processes require energy and are temperature-dependent. Therefore, the cell must be metabolically active for these mechanisms to occur:
- Translocation: The entire virus particle crosses the cell membrane intact. This is relevant *only* to naked viruses.
- Genome injection: Viral genome penetrates cytoplasm via a pore that has been created in the plasma membrane. This is relevant *only* to naked viruses.
- Receptor-mediated endocytosis (viroplexis): (a) for naked viruses, this occurs via clathrin-coated pits; (b) for enveloped viruses, this occurs via membrane fusion with the endosome membrane.
- Membrane fusion directly with plasma membrane of the cell. This is relevant *only* to enveloped viruses.

3. *Uncoating:* Makes viral genome accessible to viral or cell machinery for transcription or translation.

4. *Transcription (viral genome expression):* Eukaryotic cells lack enzymes for synthesizing mRNA off a viral RNA genome, either in the nucleus or in the cytoplasm; and they cannot transcribe viral DNA located in the cytoplasm. Therefore, only DNA viruses that replicate in the nucleus utilize the cellular machinery (cellular RNA polymerase II and other cellular enzymes) for transcription. All other viruses provide their own enzymes to produce mRNAs. The classification schemes used for animal viruses (refer to Tables 2.2 and 2.3 and Section 2.7) emphasize the different strategies of viral genome expression.

5. *Translation:* This is the key event in viral replication. The eukaryotic cell translation apparatus works only on monocistronic mRNA. Polycistronic mRNA is translated into a precursor polyprotein, which requires posttranslational cleavage into individual mature proteins. In DNA viruses, early proteins serve as viral enzymes and regulatory proteins, whereas late proteins are structural proteins. Viral proteins may undergo posttranslation modification (eg, glycosylation) and are then transported to sites where virion assembly occurs.

6. *Replication of viral nucleic acid:* Like transcription, it differs between virus families.

7 and **8.** Assembly, maturation (morphogenesis) and release. These four processes occur with the two types of viruses, as follows:
- Naked viruses: Virus release occurs by *cell lysis*. Structural proteins of naked icosahedral viruses associate spontaneously to form capsomeres, which self-assemble to form empty procapsids, into which viral nucleic acid is

specifically packaged (viral genomes contain a *packaging sequence* that directs its encapsidation. Virions accumulate within the cytoplasm/nucleus.

- Enveloped viruses: Virus release occurs by *budding*. Viral envelope proteins are transported and inserted into the cellular membrane at the site where virus buds. In many virus types, the final stage of virus maturation does not occur until after the virus particle is released from the host cell. Budding may not damage the cell, so many such viruses (eg, arenaviruses, retroviruses) are noncytopathogenic and are associated with persistent infections; others are cytolytic (eg, herpesviruses).

2.5 CURRENT VIRUS CLASSIFICATION SCHEME

The International Committee on Taxonomy of Viruses (ICTV) in 1973 established a single, universal taxonomic scheme for all viruses regardless of host, which is set arbitrarily at hierarchical levels of order, family, subfamily, genus and species, for example:

- Order is named with the suffix *virales* (eg, *Nidovirales*)
- Family has the suffix *viridae* (eg, *Coronaviridae*)
- Subfamily has the suffix *virinae* (eg, *Torovirinae*)
- Genus has the suffix *virus* (eg, *Bafinivirus*)
- Species are given by a full name (eg, *White Bream virus*).

The first letters of virus order, family, subfamily and genus terms are capitalized, and the names are printed in *italic* or underlined.

The ICTV currently lists 2618 virus species. The genomes of about 40,000 viral strains, across more than 900 species, have been sequenced (reviewed by Sharma et al., 2015). Based on the *Ninth Report of the International Committee on Taxonomy of Viruses* (King et al., 2012), the order is the highest taxon in virus classification. Virus *orders* represent groupings of families of viruses with good evidence of phylogenetic relationship among them; ie, they share a common evolution (ancestry), as evidenced by common nucleotide sequences between virus families, similar gene arrangements and gene products and similar mechanisms of gene expression, and similar virion morphology.

Virus *families* represent groupings of genera that share common characteristics and are distinct from the member viruses of other families. This level in the taxonomic hierarchy is stable, and it is the benchmark of the entire taxonomy system (Fig. 2.3). Today, classification of viruses into families is based principally upon four criteria:

- The structure of the virion—that is, size and shape of virion, number of capsomers, symmetry of capsid and presence or absence of envelope.
- Type and character of the viral genome—that is, type of nucleic acid (DNA or RNA), molecular weight, linear or circular, single- or double-stranded segmented or nonsegmented, polarity, nucleotide sequence, etc.
- Strategy of viral replication—this depends on type of genome, etc. Positive-sense single-stranded RNA (ssRNA) viruses behave like mRNA once inside a host cell. Negative-sense ssRNA viruses and double-stranded RNA (dsRNA) viruses have viral transcriptase. DNA viruses multiplying in the nucleus use cellular enzymes, and those multiplying in the cytoplasm carry their own enzymes.
- Possession of characteristic enzymes—for example, reverse transcriptase, neuraminidase, etc.

Some families are so complex that *subfamilies* have been introduced to break them into manageable divisions (Fig. 2.3).

Table 2.1 summarizes the distribution of the different virus families that occur in the three major aquatic food animals (fish, crustaceans and mollusks), which are discussed in the specific chapters in this book. Interestingly, only one virus family (*Birnaviridae*) occurs in all three major aquatic animal host species, and given the potential food-web among the aquatic animals, it follows that natural transmission pathways and interspecies (fish, crustaceans and mollusks) reservoirs of many of these viruses are limited. There is still a wide range of viruses that cannot be classified using this scheme (see Chapter 3: Unclassified and Unassigned Aquatic Animal Viruses). Many viruses, particularly those of crustaceans and mollusks, are inadequately identified, poorly described, and attributable sequences are available for only a small number of economically significant viruses, poorly reflecting the diverse nature of viruses of animal aquaculture. In some cases, presumptive viruses that are apparent in histological sections (Bonami et al., 1992; Edgerton et al., 1994) or in ultrathin sections for EM (Dunlap et al., 2013; Granzow et al., 2014) have not been further characterized. Examples of *unclassified* viruses in aquaculture and fisheries are listed in Section 2.8, later in this chapter. It is considered that the number of unclassified viruses will likely continue to increase as viral genomes with unique architectures are identified through increasing application of metagenomic approaches. Viral metagenomics has already challenged current classification schemes by revealing new viruses that blur the boundaries between groups once thought to have well-defined and unique characteristics (Rosario et al., 2009, 2012;

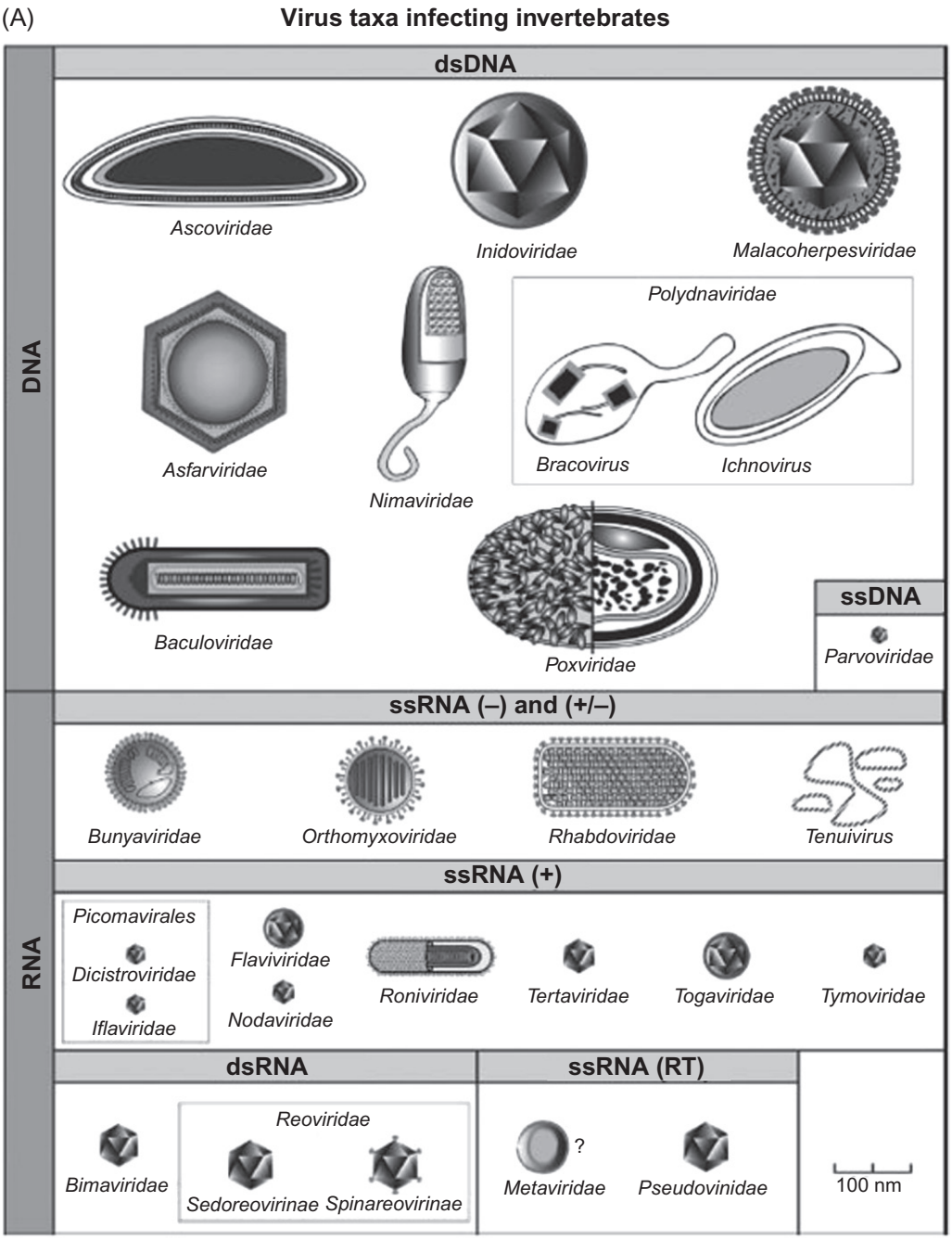

FIGURE 2.3 Diagrams of animal viruses arranged by type of nucleic acid and the presence or absence of a viral envelope. (A) Orders, families and subfamilies of viruses that infect invertebrates (including crustaceans and mollusks); (B) Orders, families and subfamilies of viruses that infect vertebrates (including fish). *From King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2012. Virus Taxonomy. Ninth Report of the International Committee on Taxonomy of Viruses. Academic Press, New York, NY. Copyright © Elsevier (2012), with permission.*

Reuter et al., 2015). On the other hand, there is at least one situation where a disease in mollusks (such as hemic neoplasia of the clam *Mya arenaria*) that previously was considered to have a viral etiology has been determined not to be the case (Arriagada et al., 2014). The neoplastic hemocytes are transmitted from individual to individual—so the transmissible element appears to be the cancer cell itself, rather than a virus (Metzger et al., 2015).

Tables 2.2 and 2.3 list the distinguishing features of aquatic food animal virus families based on the *Ninth Report of the International Committee on Taxonomy of Viruses* (King et al., 2012). This information is bound to change as the discovery of new viruses and the generations of sequence data on older virus isolates are reported. Moreover, new criteria being used

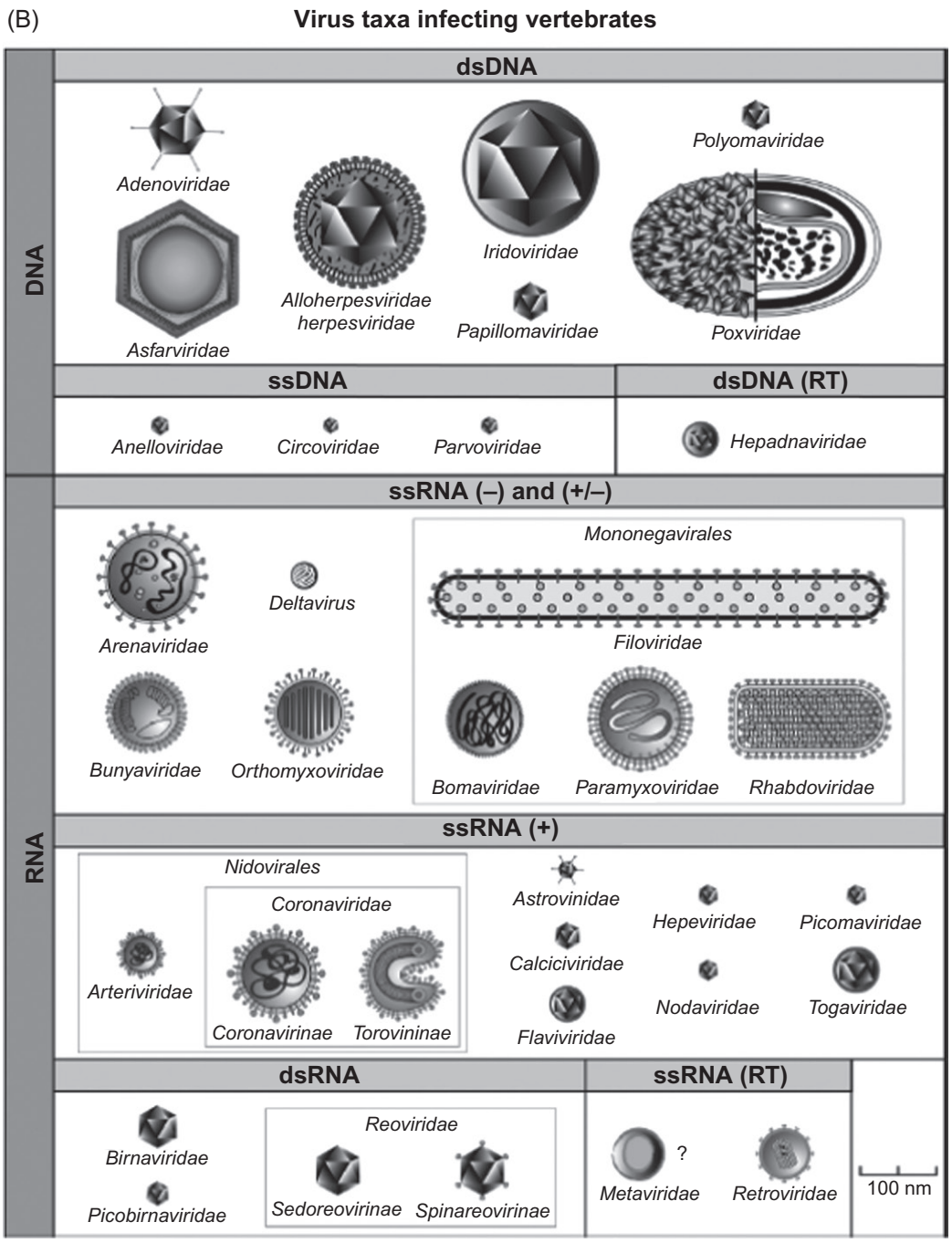

FIGURE 2.3 (Continued)

by the different ICTV study groups require extensive sequence data. For example, the current taxonomic hierarchy could not recognize phylogenetic grouping of the 11-segment fish reoviruses, grass carp reovirus (GCRV) isolates HZ08 (Wang et al., 2012), GD108 (Ye et al., 2012) and 104 (Fan et al., 2012), and the 10-segment fish reovirus, piscine orthoreovirus (PRV) (Palacios et al., 2010), into a larger, monophyletic taxon (Nibert and Duncan, 2013). Another discrepancy was noted with regard to grouping cutthroat trout virus (CTV) in *Hepeviridae* (Batts et al., 2011). When complete genome sequences of Hepatitis E virus (HEV) and other HEV-like viruses were compared, CTV fell out of the family group, as it has different positioning of open reading frame 3 (ORF3), resulting in gaps when aligned with other members of *Hepeviridae* (Oliveira-Filho et al., 2013).

TABLE 2.1 DNA and RNA Viruses Infecting the Three Major Aquatic Food Animals (Fish, Crustaceans and Mollusks)

DNA Virus Family[a]	Major Aquatic Food Animal Species		
	Fish	Crustaceans	Mollusks[b]
Circoviridae	+	+	–
Parvoviridae	+[c]	+	–
Hepadnaviridae	+[d]	–	–
Polyomaviridae	+	–	+
Papillomaviridae	+	–	+
Adenoviridae	+	–	–
Alloherpesviridae	+	–	–
Malacoherpesviridae	–	–	+
Iridoviridae	+	–	+[e]
Poxviridae	+	–	–
Nudiviridae[f]	–	+	–
Nimaviridae	–	+	–
Unclassified	+[g]		
Unclassified	–	+[h]	–
Unclassified	–	–	+[i]
RNA Virus Family[j]			
Picornaviridae	+	–	+
Dicistroviridae	–	+	–
Caliciviridae	+	–	+
Hepeviridae	+	–	–
Togaviridae	+	+	–
Roniviridae	–	+	–
Coronaviridae	+	–	–
Retroviridae	+	–	–[k]
Paramyxoviridae	+	–	–
Rhabdoviridae	+	–	–
Arenaviridae	+[l]	–	–
Bunyaviridae	–	+	–
Orthomyxoviridae	+	–	–
Reoviridae	+	+	+[b]
Birnaviridae	+	+	+
Nodaviridae	+	+	–
Totiviridae	+	+	–
Unclassified	–	+[m]	–
Unclassified	+[n]	–	–

TABLE 2.1 DNA and RNA Viruses Infecting the Three Major Aquatic Food Animals (Fish, Crustaceans and Mollusks) (Continued)

[a]*The following DNA virus families of vertebrates do not occur in fish species: Herpesviridae, Asfarviridae and Anelloviridae.*
[b]*Bivalve mollusks are filter feeders and consequently may bioaccumulate in their tissues viruses from humans and other vertebrates (Meyers, 1984). Only families of viruses reported to be infective for bivalves are included in this table, and discussed in the specific chapters. Indeed, Meyers (1980) and Meyers et al. (2009) have shown that the 11-segment American oyster isolate 13p2 does not productively infect oysters and have argued that the putative aquareoviruses obtained from oysters or clams are more likely to be fish viruses that simply accumulated in these shellfish upon filter-feeding of virus-contaminated water (Nibert and Duncan, 2013).*
[c]*A full genome sequence of a novel salmon parvovirus—the first parvovirus to be identified in a fish species—was obtained from sockeye salmon smolts from British Columbia–Canada, but it is not known yet if it is associated with disease (Miller et al., 2013).*
[d]*A novel Hepadnavirus that infects the white sucker Catostomus commersonii from the Great Lakes region of the United States was recently reported (Hahn et al., 2015).*
[e]*A novel iridovirus was reported in a mollusk (Gregory et al., 2006). Three other iridovirus infections have been reported in bivalve mollusks (CEFAS, 2011).*
[f]*Nudiviridae is a newly approved virus family (Jehle et al., 2013).*
[g]*JEECV is a novel DNA virus (with 15-kbp circular genome) isolated from samples showing endothelial cell necrosis in Japanese eel, Anguilla japonica (Mizutani et al., 2011).*
[h]*Panulirus argus virus 1 (PaV1), a novel dsDNA virus with morphological features of Herpesviridae and Iridoviridae, infects spiny lobsters (Shields and Behringer, 2004; Montgomery-Fullerton et al., 2007).*
[i]*Abalone shriveling syndrome-associated virus (AbSV) belongs to a novel unclassified virus family of bacteriophage-related chimeric marine virus infecting abalone (Zhuang et al., 2010).*
[j]*The following RNA virus families of vertebrates do not occur in fish species: Bunyaviridae, Deltavirus, Filoviridae, Bornaviridae, Nyamiviridae, Arteriviridae, Astroviridae, Flaviviridae and Picobirnaviridae.*
[k]*Previously, there had been several reports of reverse transcriptase activity in diseased clams, suggesting a retrovirus or retrotransposon; and there are also reports that the disease was transmissible, which suggested a virus. Arriagada et al. (2014) used high-throughput sequencing to look for a retroelement and found amplification of a long terminal repeat-retrotransposon, not a retrovirus, associated with disease. Most recently, it has been found that the genotypes of cancer cells in different individuals are nearly identical and do not match their hosts. This shows that the cancer cells are transmitted from individual to individual—so the transmissible element appears to be the cancer cell itself, rather than a virus (Metzger et al., 2015).*
[l]*Two isolates (DF 20/00 and DF 26/02) of novel virus obtained from common carp and koi carp with gill necrosis were ultrastructurally reminiscent of arenaviruses, and may represent the first fish viruses of the family Arenaviridae but may be members of a hitherto undescribed viral taxon (Granzow et al., 2014).*
[m]*LSNV is positive ssRNA virus, which on phylogenetic analysis formed a distinct clade between members of Luteoviridae and Togaviridae (Flegel, 2006), and associated with MSGS in shrimp (Sritunyalucksana et al., 2006).*
[n]*Fisavirus 1 (fish stool-associated RNA virus) is a novel positive ssRNA virus identified from a freshwater carp (Cyprinus carpio) using viral metagenomics; complete genome sequence is 8712 nucleotides long and shares the same genome organization and distant phylogenetic relationship to the currently unclassified posaviruses that may belong to a novel family of the order Picornavirales (Reuter et al., 2015).*

TABLE 2.2 Distinguishing Viral Properties of DNA Virus Families[a] Containing Veterinary Aquatic Pathogens

DNA Family	DNA Genome		Virion		Nucleocapsid		
	Size (kb or kbp for ssDNA and dsDNA, respectively)	Nature[b]	Diameter (nm)	Envelope	Symmetry, shape	Capsomers	Transcriptase[c]
Circoviridae	1.7–2.3	−ve or +/−ve ss, circular	12–27	–	Icosahedral	32?	–
Parvoviridae	4–6.3	+/−ve ss, linear	18–26	–	Icosahedral	32	–
Hepadnaviridae[d]	3.5	partially ds, circular	40	+	Icosahedral	N/A	+(RT)
Polyomaviridae	4.7–5.4	ds, circular	40–45	–	Icosahedral	72	–
Papillomaviridae	6.8–8.4	ds, circular	55	–	Icosahedral	72	–
Adenoviridae	26–48	ds, linear	70–90	–	Icosahedral	252	–
Alloherpesviridae	134–295	ds, linear	160–300	+	Icosahedral	162	–
Malacoherpesviridae	207	ds, linear	160–300	+	Icosahedral	162	–
Iridoviridae	140–303	ds, linear	120–350	+[e]	Icosahedral, spherical	1892	+#

(*Continued*)

TABLE 2.2 Distinguishing Viral Properties of DNA Virus Families[a] Containing Veterinary Aquatic Pathogens (Continued)

DNA Family	DNA Genome		Virion		Nucleocapsid		
	Size (kb or kbp for ssDNA and dsDNA, respectively)	Nature[b]	Diameter (nm)	Envelope	Symmetry, shape	Capsomers	Transcriptase[c]
Poxviridae	130–375	ds, linear	250 × 200 × 200	+	Complex	–	+ #
Nudiviridae[f]	80–180	ds, circular supercoiled	80 × 300–400	+	Rod-shaped	N/A	–
Nimaviridae	300	ds, circular	120–150 × 270–290	+	Ovoid, bacilliform	N/A	–

N/A, not available.
[a]*Summarized from the Ninth Report of the International Committee on Taxonomy of Viruses (King et al., 2012).*
[b]*Genome of DNA virus is invariably nonsegmented; ss, single-stranded; ds, double-stranded. +ve, positive sense; −ve, negative sense. In Parvoviridae, virions may contain a negative or positive sense.*
[c]*RT, reverse transcriptase; +#, replicates in cytoplasm.*
[d]*Additional information specific to fish hepadnavirus from Hahn et al. (2015).*
[e]*Vertebrate iridoviruses are enveloped.*
[f]*Summarized from Wang and Jehle (2009). Nudiviridae is a newly approved family (Jehle et al., 2013).*

2.6 CLASSIFICATION OF AQUATIC ANIMAL VIRUSES OF VETERINARY IMPORTANCE

Aquatic food animals are cold-blooded (poikilothermic) animals and are either vertebrates (eg, fish) or invertebrates (eg, crustaceans and mollusks). Aquatic animal virology started in the early 1960s using EM morphological data for crustacean viruses (Vago, 1966) and with the establishment of fish cell lines for the isolation of piscine viruses (Wolf and Quimby, 1962; Gravell and Malsberger, 1965). According to the Food and Agricultural Organization of the United Nations (FAO), aquaculture is set to remain one of the fastest-growing animal food-producing sectors in the world. In fact, it is the only sector growing faster than the human population. Fish, crustaceans and mollusks represent the most economically important global aquaculture industry sectors, at 66.6 million tonnes in 2012 with an estimated total farmgate value of US$144.4 billion (FAO, 2012) and expected to reach US$202.96 billion by 2020 (Grand View Research Inc., 2014). Information on viruses of these aquatic animal species of economic importance is lagging behind that of viruses of homeothermic vertebrates (ie, terrestrial viruses affecting agriculture) (Sano, 1995; Essbauer and Ahne, 2001). The increasing demand for seafood around the world has resulted in increased interest in aquatic viral diseases, which are the principal natural limiting factors for fish aquaculture (Kibenge et al., 2012a) and shrimp aquaculture (Flegel et al., 2008; Johnson et al., 2008; Stentiford et al., 2012) worldwide, causing massive losses in farmed and wild populations.

Improved diagnostic and surveillance efforts, which have accompanied the burgeoning aquaculture industry, have been accompanied by the discovery of new and emerging viral diseases that are naturally endemic in wild aquatic animal populations. Often, new viruses have been discovered only after a serious disease has occurred, although proof of etiology (fulfilling Koch's postulates of disease causation) and study of pathogenesis are often lacking because the viruses have not yet been cultured. The development of high-throughput sequencing methodology (or next-generation sequencing) (Shendure and Ji, 2008; Metzker, 2010; Munroe and Harris, 2010) and the enabling of high-throughput metagenomics to commercially important fisheries and aquaculture samples have resulted in the discovery of unknown viruses in newly emerging diseases (Batts et al., 2011, 2012; Haugland et al., 2011; Lőrincz et al., 2011; Murray and Peeler, 2005; Palacios et al., 2010; Walker and Winton, 2010; Alavandi and Poornima, 2012; Fichtner et al., 2013; Gjessing et al., 2015; Hahn et al., 2015; Reuter et al., 2015; Bacharach et al., 2016), albeit the taxonomic position and pathogenicity of some of these new viruses remain unresolved (Suttle, 2005, 2007; Sritunyalucksana et al., 2006; Zhuang et al., 2010; Ng et al., 2013; Dunlap et al., 2013).

With the improved bioinformatics tools for nucleic acid sequence analysis and sophisticated databases in virus research (ie, a new field called *viroinformatics* (Sharma et al., 2015)), it is now necessary in most cases only to obtain nucleotide sequence data on a few viral genes in order to place an unknown virus in its proper taxon (Benkő et al., 2002; Palacios et al., 2010; Lőrincz et al., 2011; Batts et al., 2011, 2012; Haugland et al., 2011), and from there to identify it specifically. The practical result of the current virus classification scheme discussed previously is a system of groups of viruses that have common biological properties, as given in Table 2.4 for aquatic animal viruses of veterinary importance. Viruses

TABLE 2.3 Distinguishing Viral Properties of RNA Virus Families[a] Containing Veterinary Pathogens

RNA Family	RNA Genome		Virion			Nucleocapsid		
	Size (kb or kbp)	Nature[b]	Diameter (nm)	Shape	Envelope	Symmetry	Capsomers	Transcriptase
Picornaviridae	7–9	+ve ss, 1	30	Spherical	–	Icosahedral	60	–
Dicistroviridae	8.5–10	+ve ss, 1	30	Spherical	–	icosahedral	N/A	–
Caliciviridae	7–8	+ve ss, 1	35–40	Spherical	–	Icosahedral	32	–
Hepeviridae	6.6–7.2	+ve ss, 1	27–34	Spherical	–	Icosahedral	32?	–
Togaviridae	9.7–11.8	+ve ss, 1	60–70	Spherical	+	Icosahedral	N/A	–
Roniviridae	26	+ve ss, 1	40–60 × 150–200	Bacilliform	+	Helical	–	–
Coronaviridae	26–32	+ve ss, 1	120–220	Pleomorphic	+	Helical	–	–
Retroviridae	Dimer × (7–13)	+ve ss, 1 (diploid)	80–100	Spherical	+	Icosahedral	N/A	+(RT)[c]
Nodaviridae	4.5	+ve ss, 2	33	Spherical	–	Icosahedral	N/A	–
Paramyxoviridae	13–19	−ve ss, 1	>150	Pleomorphic	+	Helical	–	+
Rhabdoviridae	11–15	−ve ss, 1	45–100 × 100–430	Bullet-shaped	+	Helical	–	+
Arenaviridae	11	Ambisense, 2	50–300	Pleomorphic	+	Helical	–	+
Bunyaviridae	11–19	−ve ss or ambisense, 3	80–120	Pleomorphic	+	Helical	–	+
Orthomyxoviridae[d]	10–15	−ve ss, 6–10	80–120	Pleomorphic	+	Helical	–	+
Reoviridae	19–32	ds, 9–12	60–85	Spherical	–	Icosahedral	32, 92[e]	+
Birnaviridae	6.5	ds, 2	65	Spherical	–	Icosahedral	132	+
Totiviridae	4–7	ds, 1	33–40	Spherical	–	Icosahedral	N/A	+

N/A, not available.
[a]*Summarized from the Ninth Report of the International Committee on Taxonomy of Viruses (King et al., 2012).*
[b]*All RNA is linear; 1, nonsegmented; 2–12, number of segments in genome (segmented); ss, single stranded; ds, double stranded; −ve or +ve, negative or positive sense of ssRNA.*
[c]*RT, reverse transcriptase.*
[d]*Additional information specific to Tilapia lake virus (TiLV) with 10 genome segments from Bacharach et al. (2016).*
[e]*Double capsid.*

TABLE 2.4 Major Taxonomic Virus Groups (and Approved/Tentative Species) of Veterinary Importance in Aquaculture

DNA Virus Family[a]	Subfamily/Genus	Virus Species in Respective Host and Acronym or Synonym[b]		
		Fish	**Crustaceans**	**Mollusks[c]**
Circoviridae	Unassigned	Barbel circovirus (BaCV1 and 2)		
	Unassigned	European catfish circovirus (CfCV)		
	Unassigned		*Acartia tonsa* circovirus (AcCopCV) *Labidocera aestiva* circovirus (LaCopCV)	
Parvoviridae	*Densovirinae/ Penstyldensovirus*		*Penaeus stylirostris penstyldensovirus* (*Pst*DV 1 and 2) *Penaeus monodon penstyldensovirus* (*Pmo*PDV 1 and 2) [the viruses were known previously as infectious hypodermal and hematopoietic necrosis virus (IHHNV) of prawns and shrimp]	
	Hepandensovirus		*Penaeus monodon hepandensovirus* (*Pmo*HDV1-4); *Penaeus merguiensis hepandensovirus* (*Pme*DV); *Penaeus chinensis hepandensovirus* (*Pch*DV) *Fenneropenaeus chinensis hepandensovirus* (*Fch*DV) (the viruses were known previously as hepatopancreatic parvovirus (HPV) of prawns and shrimp)	
	Unassigned		*Cherax quadricarinatus* parvo-like virus (*Cq*PlV) *Cherax destructor systemic* parvo-like virus (*Cd*SPV) Spawner-isolated mortality virus (SMV)	
	Unassigned	Novel salmon parvovirus[d]		
Hepadnaviridae	Unassigned	*White sucker hepatitis B virus* (WSHBV)		
Polyomaviridae		+		+
Papillomaviridae		+		+
Adenoviridae	*Ichtadenovirus*	*Sturgeon ichtadenovirus A*		
	Unassigned	Cod adenovirus Dab adenovirus		

Alloherpesviridae	*Cyprinivirus*	*Anguillid herpesvirus 1* (AngHV1) *Cyprinid herpesvirus* (CyHV1-3)		
	Ictalurivirus	*Acipenserid herpesvirus* (AciHV1 and 2) *Ictalurid herpesvirus* (IcHV1 and 2)		
	Salmonivirus	*Salmonid herpesvirus* (1–3)		
	Unassigned[e]	*Gadid herpesvirus 1* (GaHV1) Pilchard herpesvirus Tilapia herpesvirus *Percid herpesvirus 1* (PeHV1)		
Malacoherpesviridae	*Ostreavirus*			*Ostreid herpesvirus 1* (OsHV-1) Chlamys acute necrobiotic virus
	Aurivirus			*Haliotid herpesvirus 1* (Abalone herpesvirus)
Iridoviridae	*Lymphocystivirus*	*Lymphocystis disease virus 1* (Flounder Lymphocystis disease virus)		
	Megalocytivirus	*Infectious spleen and kidney necrosis virus* (ISKNV) *Dwarf gourami iridovirus* (DGIV) *African lampeye iridovirus* (ALIV) *Red sea bream iridovirus* (RSIV) *Rock sea bream iridovirus* (RBIV) *Turbot reddish body iridovirus* (TRBIV) *Large yellow croaker iridovirus* (LYCIV) *Red tux swordtail iridovirus* (RTSIV)		
	Ranavirus	*Epizootic hematopoietic necrosis virus* (EHNV) *European catfish virus* (ECV) *Grouper iridovirus* (GIV) *Singapore grouper iridovirus* (SGIV) *Pike-perch iridovirus* (PPIV) *Short-finned eel ranavirus* (SERV) *Santee-Cooper ranavirus* (Largemouth bass virus, LMBV)		
	Unassigned	White sturgeon iridovirus disease Erythrocytic necrosis virus (ENV)		
	Unassigned			+[f]
Poxviridae	Unassigned	*Carp edema virus* (same as Koi sleepy disease virus, KSD-virus) *Salmon gill poxvirus* (SGPV)		

(*Continued*)

TABLE 2.4 Major Taxonomic Virus Groups (and Approved/Tentative Species) of Veterinary Importance in Aquaculture (Continued)

DNA Virus Family[a]	Subfamily/Genus	Virus Species in Respective Host and Acronym or Synonym[b]		
		Fish	**Crustaceans**	**Mollusks[c]**
Nudiviridae	Unassigned		*Penaeus monodon nudivirus* (PmNV)[g] Numerous members infect shrimp, crabs and crayfish	
Nimaviridae	*Whispovirus*		*White spot syndrome virus* (WSSV)	
RNA Virus Family[h]				
Picornaviridae	*Piscevirus*[i]	*Fathead minnow picornavirus* (FHMPV) *Bluegill picornavirus* (BGPV) *Eel picornavirus 1* (EPV-1)		+
	Limnipivirus[j]	*Cyprovirus*[j] (Carp picornavirus 1, CPV-1)		
	Unassigned			
Dicistroviridae	*Aparavirus*		*Taura syndrome virus* (TSV) *Mud crab virus* (MCV)	
Caliciviridae	*Salovirus*[k]	Atlantic salmon calicivirus (ASCV)		
	Unassigned			+
Hepeviridae	*Piscihepevirus*[l]	*Piscihepevirus A*		
Togaviridae	*Alphavirus*	*Salmon pancreas disease virus*		
	Unassigned	Erythrocytic inclusion body syndrome (EIBS)		
	Unassigned		*Lymphoid organ vacuolization virus* (LOVV)[m]	
Roniviridae	*Okavirus*		*Gill-associated virus* (GAV) *Yellow head disease virus* (YHV) *Eriocheir sinensis* Ronivirus (*Es*RNV)	
Coronaviridae	*Torovirinae/ Bafinivirus*	*White bream virus* (WBV) *Fathead minnow nidovirus* (FHMNV)		
Retroviridae	*Orthoretrovirinae/ Epsilonretrovirus*	*Walleye dermal sarcoma virus* (WDSV) *Walleye epidermal hyperplasia virus* 1 (WEHV-1) *Walleye epidermal hyperplasia virus* 2 (WEHV-2)		
	Unassigned	*Perch epidermal hyperplasia virus* 1 (PEHV-1) *Perch epidermal hyperplasia virus* 2 (PEHV-2) *Salmon swimbladder sarcoma virus* (SSSV) *Salmon leukemia virus* (SLV) *Snakehead retrovirus* (SnRV) *Zebrafish endogenous retrovirus* (ZFERV)		

Paramyxoviridae	*Aquarespirovirus*[n]	Atlantic salmon paramyxovirus Pacific salmon paramyxovirus		
	Unassigned	Black sea bream paramyxovirus		
Rhabdoviridae	*Novirhabdovirus*	*Viral hemorrhagic septicemia virus* (VHSV) *Infectious hematopoetic necrosis virus* (IHNV) *Snakehead virus* (SHRV) *Hirame rhabdovirus* (HIRRV)		
	Perhabdovirus	*Perch rhabdovirus* (PerRV) *Anguillid rhabdovirus* (eel virus) *Sea trout rhabdovirus*		
	Sprivivirus	*Spring viraemia of carp virus* (SVCV) *Pike fry rhabdovirus* (PFRV)		
Arenaviridae	Unassigned	Novel carp and koi carp isolates[o]		
Bunyaviridae	*Phlebovirus*[p]		*Mourilyan virus* (MoV) *Crab hemocytopenic virus* (CHV) *Cancer pagurus* systemic bunya-like virus (*Cp*SBV) *Eriocheir sinensis* bunya-like virus (*Es*BV)	
Orthomyxoviridae	*Isavirus*	*Infectious salmon anemia virus*		
	Unassigned	Koi carp orthomyxovirus[q]		
	Unassigned	Tilapia lake virus (TiLV)[r]		
Reoviridae	*Spinareovirinae/ Aquareovirus*	*Aquareovirus A* (Chum salmon reovirus CS) *Aquareovirus B* (Chinook salmon reovirus B) *Aquareovirus C* (Golden shiner reovirus) *Aquareovirus D* (Channel catfish reovirus) *Aquareovirus E* (Turbot reovirus) *Aquareovirus F* (Chum salmon reovirus PSR) *Aquareovirus G* (American grass carp reovirus)	*Reo-like virus*[s]	American oyster reovirus 13p2[c]
	Orthoreovirus	Piscine orthoreovirus (PRV)		
	Sedoreovirinae/ Cardoreovirus		*Eriocheir sinensis* reovirus 905 (EsRV905) *Macropipus depurator reovirus* (P virus) *Carcinus mediterraneus reovirus* (CMRV; W2 virus)	
	Unassigned		*Eriocheir sinensis* reovirus 816 (EsRV816) Mud crab reovirus (MCRV) Blue crab reovirus (BCRV)	

(*Continued*)

TABLE 2.4 Major Taxonomic Virus Groups (and Approved/Tentative Species) of Veterinary Importance in Aquaculture (Continued)

DNA Virus Family[a]	Subfamily/Genus	Virus Species in Respective Host and Acronym or Synonym[b]		
		Fish	Crustaceans	Mollusks[c]
Birnaviridae	*Aquabirnavirus*	*Infectious pancreatic necrosis virus* (IPNV) *Tellina virus* *Yellowtail ascites virus* (YTV)	IPN-like virus[t]	*Tellina virus 1* (TV-1)
	Blosnavirus	*Blotched snakehead virus*		
Nodaviridae	*Betanodavirus*	*Striped jack nervous necrosis virus* (SJNNV) *Barfin flounder nervous necrosis virus* (BFNNV) *Redspotted grouper nervous necrosis virus* (RGNNV) *Tiger puffer nervous necrosis virus* (TPNNV)		
	Unassigned		*Macrobrachium rosenbergii* nodavirus (*Mr*NV) *Penaeus vannamei* nodavirus (*Pv*NV) Wuhan nodavirus (WhNV)	
Totiviridae	*Giardiavirus*	Piscine myocarditis virus (PMCV)	Infectious myonecrosis virus (IMNV)	

[a]*The following DNA virus families of vertebrates do not occur in fish species: Herpesviridae, Asfarviridae, and Anelloviridae.*
[b]*"+" denotes that an unnamed virus in the family has been reported.*
[c]*Bivalve mollusks are filter feeders and consequently may bioaccumulate in their tissues viruses from humans and other vertebrates (Meyers, 1984). Only families of viruses reported to be infective for bivalves are included in this table, and discussed in the specific chapters. Indeed, Meyers (1980) and Meyers et al. (2009) have shown that the 11-segment American oyster isolate 13p2 does not productively infect oysters and have argued that the putative aquareoviruses obtained oysters or clams are more likely to be fish viruses that simply accumulated in these shellfish upon filter feeding of virus-contaminated water (Nibert and Duncan, 2013).*
[d]*A full genome sequence of a novel salmon parvovirus—the first parvovirus to be identified in a fish species—was obtained from sockeye salmon smolts from British Columbia–Canada, but it is not known yet if it is associated with disease (Miller et al., 2013).*
[e]*Reviewed in herpesviruses that infect fish (Hanson et al., 2011).*
[f]*A novel iridovirus was reported in a mollusk (Gregory et al., 2006). Three other iridovirus infections have been reported in bivalve mollusks (CEFAS, 2011).*
[g]*The family Nudiviridae includes Penaeus monodon nudivirus (PmNV) (formerly occluded shrimp Monodon Baculovirus, the cause of spherical baculovirosis) and other members that were formerly called "nonoccluded" baculoviruses (Herniou et al., 2012; Wang and Jehle, 2009). There are numerous "nonoccluded" baculoviruses infecting crabs—eg, Tau virus, Baculo-A, Baculo-PP, B2 virus, etc. (Bonami and Zhang, 2011) and crayfish, eg, AaBV, ApBV, CdBV, CqBV, PlBV (Longshaw, 2011).*
[h]*The following RNA virus families of vertebrates do not occur in fish species: Bunyaviridae, Deltavirus, Filoviridae, Bornaviridae, Arteriviridae, Astroviridae, Flaviviridae and Picobirnaviridae.*
[i]*Piscevirus is a proposed new genus in the family Picornaviridae (Phelps et al., 2014).*
[j]*Limnipivirus is a proposed new genus and Cyprovirus is the proposed species name in family Picornaviridae (Lange et al., 2014).*
[k]*Salovirus is a proposed new genus in family Caliciviridae (Mikalsen et al., 2014).*
[l]*Piscihepevirus is a proposed new genus in a new classification scheme for Hepeviridae (Purdy et al., 2014).*
[m]*Lymphoid organ vacuolization virus (LOVV) from white shrimp Penaeus vannamei is the first togavirus in any marine invertebrate animal (Bonami et al., 1992).*
[n]*Aquarespirovirus is the tentative genus for Atlantic salmon pamyxovirus (Nylund et al., 2008).*
[o]*Two isolates (DF 20/00 and DF 26/02) of novel virus obtained from common carp and koi carp with gill necrosis were ultrastructurally reminiscent of arenaviruses, and may represent the first fish viruses of the family Arenaviridae but may be members of a hitherto undescribed viral taxon (Granzow et al., 2014).*
[p]*Phlebovirus is the tentative genus for Mourilyan virus (Cowley et al., 2005).*
[q]*A complete sequence of PB1 gene of a putative koi carp orthomyxovirus was obtained from koi carp in California with 43% amino acid sequence identity with ISAV (Anonymous, 2013). In addition, there is reference to an orthomyxovirus from koi carp (Granzow et al., 2014).*
[r]*Tilapia lake virus (TiLV) with 10 genome segments is a novel orthomyxo-like virus causing mass die-offs of tilapia, and will ultimately be classified as representing a new genus in the family Orthomyxoviridae (Bacharach et al., 2016).*
[s]*Genus Aquareovirus is speculated to include Reovirus-like isolates found in white shrimp Penaeus vannamei (Krol et al., 1990) and blue crab Callinectes sapidus (Johnson, 1977), but these isolates have not been genetically characterized.*
[t]*IPN-like virus is a birnavirus found in the crab Macropipus depurator (Clotilde, 1984).*

within groups share characteristics that help in our understanding of the epidemiology and pathogenesis of infections they produced. If you know the member viruses of a particular family and understand the group properties of each family, it is possible to obtain some vocabulary about the particular viral disease. The information presented in Table 2.4 is bound to change as the discovery of new viruses and the generation of sequence data on older virus isolates are reported. Recently, a novel fish hepadnavirus associated with hepatic neoplasia in the white sucker *Catostomus commersonii* from the Great Lakes region of the United States was characterized (Hahn et al., 2015). Even more recently, a novel orthomyxo-like virus named Tilapia lake virus (TiLV) causing mass die-offs of tilapia in Israel and Ecuador has been characterized; the virus as 10 genome segments of negative-sense ssRNA, with 5′ and 3′ noncoding regions characteristic of influenza viruses, and will ultimately be classified as representing a new genus in the family *Orthomyxoviridae* (Bacharach et al., 2016).

2.7 BALTIMORE CLASSIFICATION OF VIRUSES

The Baltimore classification of viruses (Baltimore, 1971), which complements the ICTV virus families, is based on the nature of the genomes and replication strategies of viruses. It has (+)RNA as its central point (ie, that all viruses must produce mRNA, or (+)sense RNA that can be read by the cell's translational machinery), and the unique pathways the various viral genomes are expressed to generate mRNA define specific virus classes. The principles of the Baltimore classification are fundamental to an understanding of virus classification and genome replication and expression, but it is rarely used as a classification system in its own right. The viral genomes are divided into seven (admittedly arbitrary) classes. The viral genomes of viruses that infect the three major aquatic food animals (fish, crustaceans and mollusks) are found in all the seven classes (Fig. 2.4):

I. dsDNA viruses (*Adenoviridae*; *Alloherpesviridae*; *Iridoviridae*; *Malacoherpesviridae*; *Nimaviridae*; *Nudiviridae*; *Papillomaviridae*; *Polyomaviridae*; *Poxviridae*). Some replicate in the nucleus (eg, adenoviruses using host DNA-dependent RNA polymerase). The mRNA transcripts are spliced into gene-length mRNAs for translation into individual proteins. Poxviruses replicate in the cytoplasm and make their own enzymes for nucleic acid replication and transcription.

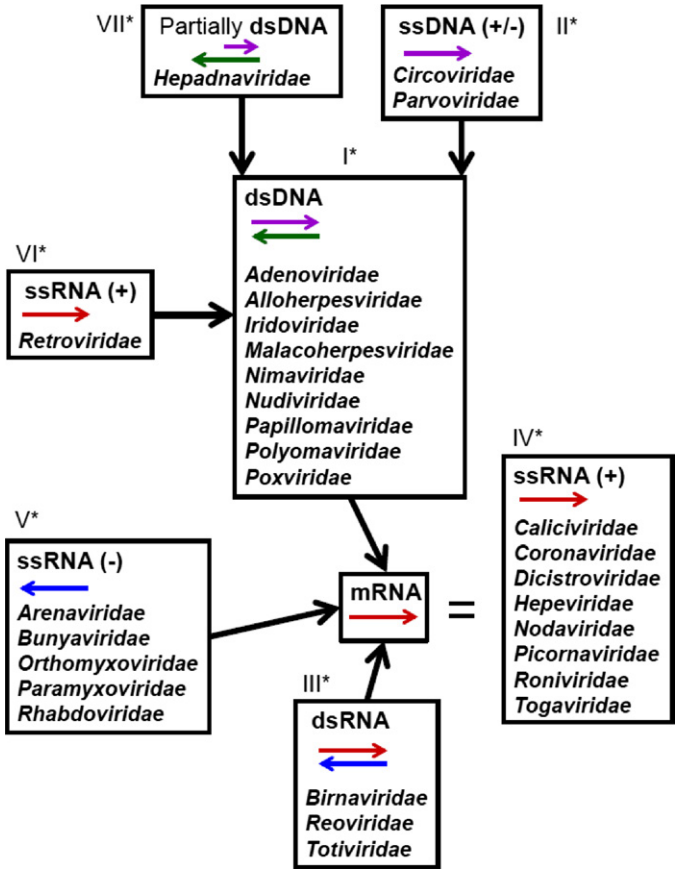

FIGURE 2.4 Pathways for the production of viral mRNA utilized by animal viruses. The Baltimore classification "classes" are denoted by asterisk. All the virus families listed in Tables 2.2 and 2.3 are shown.

II. ssDNA viruses (*Circoviridae*; *Parvoviridae*). Replication occurs in the nucleus using host enzymes, involving the formation of a negative-sense strand, which serves as a template for positive-strand RNA and DNA synthesis. Similar to Class I (nuclear dsDNA viruses), mRNA transcripts are spliced into gene-length mRNAs for translation into individual proteins.

III. dsRNA viruses (*Birnaviridae*; *Reoviridae*; *Totiviridae*). These viruses have segmented genomes. Each genome segment is transcribed separately by viral RNA-dependent RNA polymerase to produce monocistronic mRNAs.

IV. positive-sense ssRNA viruses (*Caliciviridae*; *Coronaviridae*; *Dicistroviridae*; *Hepeviridae*; *Nodaviridae*; *Picornaviridae*; *Roniviridae*; *Togaviridae*). Genomic RNA is similar to mRNA. Therefore naked positive-sense ssRNA is infectious. There are several variations of gene expression, ranging from translation of precursor polyprotein and cleavage into individual mature proteins to production of monocistronic nested subgenomic mRNA transcripts.

V. negative-sense ssRNA viruses (*Arenaviridae*; *Bunyaviridae*; *Orthomyxoviridae*; *Paramyxoviridae*; *Rhabdoviridae*). (*Arenaviridae* and *Bunyaviridae* have ambisense segments as well.) These viruses must have a virion particle RNA directed RNA polymerase. The first step in replication is transcription of the negative sense RNA genome by the virion RNA-dependent RNA polymerase to produce monocistronic subgenomic mRNAs, and full-length (+)sense RNA that serve as the template for genome replication. For segmented viruses, each genome segment is transcribed separately by viral RNA-dependent RNA polymerase to produce monocistronic mRNAs.

VI. positive-sense ssRNA viruses with DNA intermediate in their replication cycle (*Retroviridae*) (*RNA reverse-transcribing viruses*). The genome is (+)sense ssRNA but unique among viruses in that it is diploid, and does not serve as mRNA, but as a template for reverse transcription once uncoated in the cytoplasm. This becomes dsDNA, which is integrated into the host chromosome from where mRNA is produced as for Class I (nuclear dsDNA viruses).

VII. partially dsDNA viruses (*Hepadnaviridae*) (*DNA reverse-transcribing viruses*). The genome of these viruses is partially dsDNA, having a full-length (-)sense DNA strand and partial length (+)sense DNA strand. These viruses are unique among DNA viruses in requiring an RNA intermediate step to replicate the genome. The reverse transcription occurs inside the virus particle on maturation.

2.8 IDENTIFICATION OF AQUATIC ANIMAL VIRUSES OF VETERINARY IMPORTANCE

Aquatic animal viruses are isolated and identified according to standard virological procedures (Essbauer and Ahne, 2001), but culture-dependent characterization is not always possible. Of the three major aquatic food animals (fish, crustaceans and mollusks), virus isolation is only possible for some fish viruses, where several cell lines derived from fish are available (American Type Culture Collection (ATCC), http://www.atcc.org; European Collection of Authenticated Cell Cultures (ECACC), http://ecacc.org.uk). There is a lack of crustacean and molluskan cell lines, which limits isolation of crustacean and mollusk viruses, although some mollusk viruses (birnaviruses and reoviruses) have been isolated in fish cell lines (Hill, 1976; Meyers, 1979), and some crustacean viruses might be isolated in primary cell cultures (Nadala et al., 1993) and insect cell lines. Virus amplification for detection is possible by polymerase chain reaction (PCR)/reverse transcription polymerase chain reaction (RT-PCR) but for experimental infections, in the absence of a viral isolate, the preferred method is infection of known susceptible host species (which are free of infection by the agent in question) using tissue homogenates (as suggested by Du et al., 2007, for white spot syndrome virus). In case of reoviruses isolated from mollusks, Meyers (1980) and Meyers et al. (2009) have shown that the 11-segment American oyster isolate 13p2 does not productively infect oysters and have argued that the putative aquareoviruses obtained from oysters or clams are more likely to be fish viruses that simply accumulated in these shellfish upon filter feeding of virus-contaminated water (Nibert and Duncan, 2013).

The World Organization for Animal Health (OIE), which provides international standards and guidelines for safe trade in animals and their products (Mohan, 2009; Kibenge et al., 2012b), has published standard protocols in the *OIE Aquatic Manual* (OIE, 2014) for detection and identification of pathogens on the OIE list of notifiable aquatic animal diseases (those with a risk of spread through trade in aquatic animals and their products), which in 2015 included eight fish viral diseases (epizootic hematopoietic necrosis, infection with HPR-deleted or HPR0 infectious salmon anemia virus, infection with salmonid alphavirus, infectious hematopoietic necrosis, koi herpesvirus disease, Red Sea bream iridoviral disease, spring viraemia of carp and viral hemorrhagic septicemia), six crustacean viral diseases (infection with yellow head virus, infectious hypodermal and hematopoietic necrosis, infectious myonecrosis, Taura syndrome, white spot disease and white tail disease) and one mollusk viral disease (infection with abalone herpesvirus) (OIE, 2015).

Table 2.5 summarizes the methods used for isolation and propagation in cell culture and molecular detection by PCR/RT-PCR (virus-specific primers) of these viruses and other important/common aquatic animal viruses. Sequence analysis of the PCR product to confirm the virus nucleic acid sequence provides further support for the taxonomic position of the virus, as sequence similarity values are key elements in modern virus taxonomy. It must be noted, specifically for crustaceans, that sequence analysis of PCR products is essential because the PCR primers for virus detection may also cross-react with components of host crustacean genomes (Claydon et al., 2004; Tang et al., 2007), resulting in false positives.

TABLE 2.5 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses

Virus Family	Virus Genus	Virus Species or Isolate[a] in Host			Virus Isolation and Propagation		Conventional PCR/RT-PCR
		Fish	Crustaceans	Mollusks	Cell Lines[b]	Incubation Temperature[c]	Virus-Specific Primers and Reference[f]
Circoviridae	Unassigned	BaCV1 and 2			–	–	MTL 5′-GTGATTACGCCAACGTGATG-3′ MTR 5′-GCCCTTTGTGCAGTATTCGT-3′ (Lőrincz et al., 2011)
	Unassigned	CfCV			–	–	HT-F 5′-CAGACCATGCTTCCGGTACT-3′ HT-R 5′-GGGCTTCCTCGAAGGTTATC-3′ (Lőrincz et al., 2012)
Parvoviridae	*Penstyldensovirus*		IHHNV		–	–	77012F 5′-ATCGGTGCACTACTCGGA-3′ 77353R 5′-TCGTACTGGCTGTTCATC-3′ (Nunan et al., 2000)
	Hepandensovirus		HPV		–	–	HPV600F 5′-TGTCTAAGCGCCAGTAACCAA-3′ HPV600R 5′-TATACAGTTACTTGGCATGAC-3′ (La Fauce et al., 2007)
Hepadnaviridae	Unassigned	WSHBV					Hahn et al. (2015)
Adenoviridae	*Ichtadenovirus*	WSAdV			WSS-2	15–30°C	AdVE2B-F 5′-TCMAAYGCHYTVTAYGGBTCDTTTGC-3′ AdVE2B-R 5′-CCAYTCHSWSAYRAADGCBCKVGTCCA-3′ (Benkő et al., 2002)
Alloherpesviridae	*Cyprinivirus*	CyHV-3			KF-1; CCB	25–26°C	FP 5′-GGGTTACCTGTACGAG-3′ RP 5′-CACCCAGTAGATTATGC-3′ (Bercovier et al., 2005)
	Ictalurivirus	CCV			CCO, BB	25–30°C	FP 5′-TCATCCGAATCCGACAACTGA-3′ RP 5′-CCAAGATCGCGGAGAAAC-3 (OIE, 2009)
	Salmonivirus	OMHV			RTG-2; CHSE-214	10–15°C	FP (F10) 5′-GTACCGAAACTCCCGAGTC-3′ RP (R5) 5′-AACTTGAACTACTCCGGGG-3′ (Aso et al., 2001)
Malacoherpesviridae	*Ostreavirus*			OsHV-1	–	–	A3: 5′-GCCAACCGTTGGAACCATAACAAGCG-3′ A4: 5′-GGGAATGAGGTGAACGAAACTATAGACC-3′ (Renault et al., 2000)
	Aurivirus			AbHV	–	–	AbHV-16 5′-GGCTCGTTCGGTCGTAGAATG-3′ AbHV-17 5′-TCAGCGTGTACAGATCCATGTC-3′ (OIE, 2014)
Unclassified			PaV1		–	–	45aF 5′-TTCCAGCCCAGGTACGTATC-3′ 543aR 5′-AACAGATTTTCCAGCAGCGT-3′ (Montgomery-Fullerton et al., 2007)
Iridoviridae	*Ranavirus*	EHNV			BF-2; EPC; FHM; CHSE-214	15–24°C	FP 5′-CGCAGTCAAGGCCTTGATGT-3′ RP 5′-AAAGACCCGTTTTGCAGCAAAC-3′ (Hyatt et al., 2000)
	Megalocytivirus	RSIV			GF	25°C	1-F 5′-CTCAAACACTCTGGCTCATC-3′ 1-R 5′-GCACCAACACATCTCCTATC-3′ (Kurita et al., 1998)
	Lymphocystivirus	LCDV			SAF-1[d]	20°C	F 5′-TTTGAATGGGAGGATCAC-′3 R 5′-TCCGTAAATGCTGTTAGC-′3 (Kvitt et al., 2008)

(*Continued*)

TABLE 2.5 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses (Continued)

Virus Family	Virus Genus	Virus Species or Isolate[a] in Host			Virus Isolation and Propagation		Conventional PCR/RT-PCR
		Fish	Crustaceans	Mollusks	Cell Lines[b]	Incubation Temperature[c]	Virus-Specific Primers and Reference[f]
Poxviridae	Unassigned	CEV					Oyamatsu et al. (1997)
		SPGV					Gjessing et al. (2015)
Nudiviridae	Unassigned		PmNV		–	–	261F 5′-AATCCTAGGCGATCTTACCA-3′ 261R 5′-CGTTCGTTGATGAACATCTC-3′ (Surachetpong et al., 2005)
Nimaviridae	*Whispovirus*		WSSV		–	–	146F1 5′-ACTACTAACTTCAGCCTATCTAG-3′ 146R1 5′-TAATGCGGGTGTAATGTTCTTACGA-3′ (Lo et al., 1996)
Picornaviridae	*Piscevirus*	EPV-1			EK-1	26°C	
		BGPV			BF-2	15–25°C	FP 5′-CTCGATAGTGTATGACTCGGACC-3′ RP 5′-CATGGGGTTCAACACTCACA-3′ (Barbknecht et al., 2014)
		FHMPV			EPC, FHM, CHSE-214, BF-2, RTG-2	15–22°C	
	Limnipivirus	CPV-1			FHM		
Dicistroviridae	*Aparavirus*		TSV		–	–	9992F 5′-AAGTGAACAGCCGCGCTT-3′ 9195R 5′-TCAATGAGAGCTTGGTCC-3′ (Nunan et al., 1998)
Caliciviridae	*Salovirus*	ASCV			GF-1	15°C	Mikalsen et al. (2014)
Hepeviridae	*Piscihepevirus*	CTV			CHSE-214	15°C	(+) primer 5′-ACTGTTACACCTCATGTAGC-3′ (–) primer 5′-GACTTTACTAGCAGTGTGGAT-3′ (Batts et al., 2011)
Togaviridae	*Alphavirus*	SAV			CHSE-214, BF-2, FHM, SHK-1, EPC, CHH-1	15°C	E2F: 5′-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3′ E2R: 5′-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3′ (Fringuelli et al., 2008)
Roniviridae	*Okavirus*			YHV GAV	–	–	YC-F1ab pool: 5′-ATCGTCGTCAGCTACCGCAATACTGC-3′ 5′-ATCGTCGTCAGYTAYCGTAACACCGC-3′ YC-R1ab pool: 5′-TCTTCRCGTGTGAACACYTTCTTRGC-3′ 5′-TCTGCGTGGGTGAACACCTTCTTGGC-3′ (Wijegoonawardane et al., 2008)
Coronaviridae	*Bafinivirus*	FHMNV			FHM	15°C	Sense primer 5′-TTTTGTTGAATTTATAGCTCTT-3′ Antisense primer 5′-TGGCCATATCCTTAAGGG-3′ (Batts et al., 2012)
Retroviridae	*Epsilonretrovirus*	WDSV			–	–	Primer 3: 5′-TGAAGCAGGAATACCTACCT-3′ Primer 4: 5′-CTGTAAGTCCGTTCTCTTGT-3′ (Poulet et al., 1996)

Paramyxoviridae	*Aquarespirovirus*	PSPV			CHSE-214	20°C	Sense primer 5′-GCAGAGATATTTTCCTTCTT-3′ Antisense primer 5′-TGTTTGATTTCCTTCTCCTT-3′ (Batts et al., 2008)
Rhabdoviridae	*Novirhabdovirus*	VHSV			BF-2, EPC, FHM	15°C	VN forward 5′-ATGGAAGGAGGAATTCGTGAAGCG-3′ VN reverse 5′-GCGGTGAAGTGCTGCAGTTCCC-3′ (Snow et al., 2004)
		IHNV			EPC, FHM	15°C	Upstream Primer 5′-AGAGATCCCTACACCAGAGAC-3′ Downstream Primer 5′-GGTGGTGTTGTTTCCGTGCAA-3′ (Emmenegger et al., 2000)
		SHRV			EPC, ZF4	28°C	Sense primer 5′-ATTTATCCGCTGGAGAGGGATTGG-3′ Antisense primer 5′-GTTGAGCCCATAGGCCTTGAAGTA-3′ (Phelan et al., 2005)
		HIRRV			BF-2, EPC, FHM, RTG	15°C	oPVP278 5′-ACTACAATCAACAAATCGCA-3′ oPVP279 5′-GTTGGCGAGTGGGATGTTG-3′ (Borzym et al., 2014)
	Perhabdovirus	PRV			BF-2, EPC	16°C	oPVP116 5′-ACWTGTGAYTWCMGWTGGTATGG-3′ oPVP118 5′-CTGTTAGCTGTTTTTTTCATA-3′;
							oPVP126 5′-GATATGAAAAAAACTGCAACAG-3′ Rha-G-seqR2 5′-GAGGAGTCCTCTATGTTGGTC-3′ (Talbi et al., 2011; Gadd et al., 2013)
	Sprivivirus	SVCV			EPC, FHM	20°C	F1 5′-TCTTGGAGCCAAATAGCTCARRTC-3′ R2 5′-AGATGGTATGGACCCCAATACATHACNCAY-3′ (Stone et al., 2003)
Bunyaviridae	*Phlebovirus*[e]			MoV	–	–	MoV24F 5′-GGGATGGTGTTGCCATACAAAGG-3′ MoV25R 5′-GTCATTAGCTGGTCTTAGTTTTCAC-3′ (Cowley et al., 2005)
Orthomyxoviridae	*Isavirus*	ISAV			SHK-1, ASK	15°C	HPR Fwd 5′-GCCCAGACATTGACTGGAGTAG-3′ HPR Rev 5′-AGACAGGTTCGATGGTGGAA-3′ (Kibenge et al., 2009)
Reoviridae	*Aquareovirus*	"All species"			CHSE-214, EPC	22°C	GARV-F 5′-TAAAGCTTGCGACGCCTCCATCAC-3′ GARV-R 5′-TGCTCGGTGGAGGTGACAGT-3′ (Zainathan, et al., 2014)
	Orthoreovirus	PRV			GF-1	15°C	PRV-S1-F1 5′-GATAAAGACTTCTGTACGTGAAAC-3′ PRV-S1-R1 5′-GATGAATAAGACCTCCTTCC-3′ (Kibenge et al., 2013)
	Cardoreovirus			BCRV	–	–	BCRV For 5′-TCAGTGTCTTCAGCTTTAGGTTG-3′ BCRV Rev 5′-TCTCTTGAGGCCTAGATTCG-3′ (Bowers et al., 2010)
Birnaviridae	*Aquabirnavirus*	IPNV			CHSE-214	15°C	P1 5 ′-GGTCAACAACCAACTAGTGACC-3′ P2 5′-GTTGGGATTGACTGCGTAAAC-3′ (Lopez-Lastra et al., 1994)
				TV-1	BF-2		Nobiron et al. (2008)
Nodaviridae	*Betanodavirus*	SJNNV			SSN-1, E-11, GF-1	20–25°C	VNNV1 5′-ACACTGGAGTTTGAAATTCA-3′ VNNV2 5′-GTCTTGTTGAAGTTGTCCCA-3′ (Dalla Valle et al., 2000)
			*Mr*NV				
Totiviridae	*Giardiavirus*	PMCV					Haugland et al. (2011)
			IMNV		–	–	4587F 5′-CGACGCTGCTAACCATACAA-3′ 4914R 5′-ACTCGGCTGTTCGATCAAGT-3′ (Poulos and Lightner, 2006)

(*Continued*)

TABLE 2.5 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses (Continued)

[a]*Virus abbreviations: BaCV1 and 2, Barbel circovirus 1 and 2; CfCV, European catfish circovirus; IHHNV, Infectious hypodermal and hematopoietic necrosis virus; HPV, Hepatopancreatic parvovirus; WSHBV, White sucker hepatitis B virus; WSAdV, White sturgeon adenovirus; CyHV-3,* Cyprinid herpesvirus 3 *(Koi herpesvirus); CCV, Channel catfish virus (*Ictalurivirus herpesvirus 1*); OMHV, Oncorhynchus masou herpesvirus; OsHV-1, Ostreid herpesvirus 1 (Oyster herpesvirus); AbHV, Abalone herpesvirus (*Haliotid herpesvirus 1*); PaV1,* Panulirus argus *virus 1; EHNV, Epizootic hematopoietic necrosis virus; RSIV, Red sea bream iridovirus; LCDV, Lymphocystis disease virus; CEV, Carp edema virus; SGPV, Salmon gill poxvirus; WSSV, White spot syndrome virus; EPV-1, Eel picornavirus 1; BGPV, Bluegill picornavirus; FHMPV, Fathead minnow picornavirus; CPV-1, Carp picornavirus 1; TSV, Taura syndrome virus; ASCV, Atlantic salmon calicivirus; CTV, Cutthroat trout virus; SAV, Salmonid alphavirus; YHV, Yellow head disease virus; GAV, Gill-associated virus ; WDSV, Walleye dermal sarcoma virus; ASPV, Atlantic salmon paramyxovirus; PSPV, Pacific salmon paramyxovirus; FHMNV, Fathead minnow nidovirus; VHSV, Viral hemorrhagic septicemia virus; IHNV, Infectious hematopoetic necrosis virus; SHRV, Snakehead rhabdovirus; HIRRV, Hirame rhabdovirus; PerRV, Perch rhabdovirus; SVCV, Spring viraemia of carp virus; MoV, Mourilyan virus; ISAV, Infectious salmon anemia virus ; PRV, Piscine orthoreovirus; BCRV, Blue crab reovirus; IPNV, Infectious pancreatic necrosis virus; TV-1, Tellina virus 1; BSNV, Blotched snakehead virus; SJNNV, Striped jack nervous necrosis virus;* MrNV, Macrobrachium rosenbergii *nodavirus; PMCV, Piscine myocarditis virus; IMNV, Infectious myonecrosis virus.*

[b]*Cell line abbreviations: WSS-2, White sturgeon spleen; KF-1, Koi fin; CCB, Common carp brain; CCO, Channel catfish ovary; BB, Brown bullhead; RTG-2, Rainbow trout gonads; CHSE-214, Chinook salmon embryo; BF-2, Blue gill fry; EPC, Epithelioma papulosum cyprini; FHM, Fathead minnow; GF, Grunt fin; SAF-1, Gilthead seabream fibroblasts; EK-1, eel embryonic kidney; GF-1, Grouper fin; SHK-1, Salmon head kidney; CHH-1, Chum salmon heart; ZF4, zebrafish embryo fibroblast; ASK, Atlantic salmon kidney; SSN-1, striped snakehead; E-11, cloned from SSN-1 cell line.*

[c]*Fish viruses are typically isolated on cultured fish cell lines at 15–25°C.*

[d]*LCDV grows in SAF-1 cell line; CPE consisted of the rounding and enlargement of infected cells and the formation of cytoplasmic inclusions. The yields of isolated viruses ranged from* 1×10^4 *TCID50/mL to* $> 1 \times 10^9$ $TCID_{50}$*/mL (Garcia-Rosado et al., 2004).*

[e]Phlebovirus *is the tentative genus for Mourilyan virus (Cowley et al., 2005).*

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2.9 UNCLASSIFIED VIRUSES IN AQUACULTURE

Examples of unclassified and unassigned viruses in aquaculture (Table 2.1) include the following:

- Extra small virus (XSV), a nonautonomous satellite-like virus associated with white tail disease of freshwater prawn *Macrobrachium rosenbergii* (Sri Widada and Bonami, 2004).
- Several members of *Parvoviridae* in crustaceans (Longshaw, 2011) remain unassigned due to lack of sequence information.
- *Panulirus argus* virus 1 (PaV1), a novel dsDNA virus with morphological features of *Herpesviridae* and *Iridoviridae*, infects spiny lobsters (Shields and Behringer, 2004; Montgomery-Fullerton et al., 2007).
- Abalone shriveling syndrome-associated virus is a novel marine bacteriophage-related dsDNA virus in abalone (Zhuang et al., 2010).
- *Cherax albidus* picorna-like virus (*Ca*PV), also known as *Yabby virus*, is a new virus with a taxonomic position of either *Circoviridae* or *Picornaviridae* that infects yabbies (a type of freshwater crayfish) (Jones and Lawrence, 2001).
- Laem-Singh virus (LSNV) is a positive ssRNA virus, which on phylogenetic analysis formed a distinct clade between members of *Luteoviridae* and *Togaviridae* (Flegel, 2006), and associated with monodon slow growth syndrome (MSGS) in shrimp (Sritunyalucksana et al., 2006).
- *Cherax quadricarinatus* Giardia-like virus (CGV) is a putative totivirus of freshwater crayfish (has not yet been isolated or characterized) (Edgerton et al., 1994).
- Japanese eel endothelial cells-infecting virus (JEECV) is a novel DNA virus isolated from samples showing endothelial cell necrosis in Japanese eel, *Anguilla japonica* (Mizutani et al., 2011). One ORF is homologous to the large T-antigen of polyomavirus but other ORFs have no homology with any nucleic acid or amino acid sequences of polyomavirus.
- Two isolates (DF 20/00 and DF 26/02) of novel virus obtained from common carp and koi carp with gill necrosis were ultrastructurally reminiscent of arenaviruses and may represent the first fish viruses of the family *Arenaviridae* but also may be members of a hitherto-undescribed viral taxon (Granzow et al., 2014).
- Fisavirus 1 (fish stool-associated RNA virus) is a novel positive ssRNA virus identified from a freshwater carp (*Cyprinus carpio*) using viral metagenomics; complete genome sequence is 8712 nucleotides long and shares the same genome organization and distant phylogenetic relationship to the currently unclassified posaviruses that may belong to a novel family of the order *Picornavirales* (Reuter et al., 2015). Posaviruses (porcine stool-associated viruses) are from pig fecal samples (Shan et al., 2011; Zhang et al., 2014) and are distantly related to the virus families *Dicistroviriridae* and *Picornaviridae*.

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Chapter 3

Unclassified and Unassigned Aquatic Animal Viruses

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3.1 INTRODUCTION

Within the three main aquaculture groups (fish, crustaceans, mollusks), many viruses, particularly of crustaceans (ie, crabs, crayfish, lobsters and shrimp) and mollusks (ie, abalone, clams, mussels, oysters and scallops), are inadequately identified and poorly described, and attributable sequences are available for only a small number of economically significant viruses, which poorly reflects the diverse nature of viruses of animal aquaculture and fisheries. Crustaceans are arthropods, and most crustacean viruses either are related to those previously known to infect insects or are completely new to science. In some cases, presumptive viruses apparent in histological sections (Bonami et al., 1992; Edgerton et al., 1994) or in ultrathin sections for electron microscopy (Dunlap et al., 2013; Granzow et al., 2014) have not been further characterized. For the purposes of this chapter, *unassigned viruses* are considered to be ones characterized (based on electron microscopic observations, nucleic acid sequence data, or in vivo experimental studies) to the level of virus family, but have not yet been assigned either to a known genus or a new genus within the family (see Chapter 2: Classification and Identification of Aquatic Animal Viruses, in this book, especially Table 2.4). For example, several members of the Parvoviridae family in freshwater crayfish (*Cherax* spp.) (Longshaw, 2011) and the giant freshwater prawn (*Macrobrachium rosenbergii*) (Anderson et al., 1990), several members of the Iridoviridae, Alloherpesviridae and Picornaviridae families of fish (Smail and Munro, 2012), and bunyavirus-like particles in crabs (Corbel et al., 2003; Bonami and Zhang, 2011) remain unassigned due to lack of sequence information; Carp edema virus (CEV), a pox-like virus that causes koi sleepy disease (KSD) at low water temperatures (13–23°C), belongs to the Poxviridae family (Miyazaki et al., 2005); and *Cherax quadricarinatus* Giardia-like virus (CGV) of freshwater crayfish is a putative member of the Totiviridae family that has not yet been isolated or characterized (Edgerton et al., 1994). Such viruses are described in the respective chapters of this book concerning the known virus families, so they will not be further discussed here. This chapter will be limited to viruses in aquaculture and fisheries that have not yet been assigned to a virus family, primarily because of their unique features. These viruses are designated as *unclassified* aquatic animal viruses (Table 3.1).

Genome structure is highly conserved within viral groups (King et al., 2012), and although new viruses are constantly being uncovered, the discovery of a novel family (or even a genus) among human viruses (including zoonotic viruses from mammals and birds) remains relatively uncommon (Ladner et al., 2014; Woolhouse et al., 2012). For the purpose of this discussion, the search of the research literature was not exhaustive, but rather focused on particular studies that attempted to determine the presence of aquatic animal viruses with unique features that did not allow their assignment to the current taxonomic classification schemes described in Chapter 2 "Classification and Identification of Aquatic Animal Viruses." It is considered that the number of unclassified aquatic animal viruses will likely continue to increase as viral genomes with unique architectures are identified through increasing applications of metagenomic approaches. Viral metagenomics has already challenged current classification schemes by revealing new viruses that blur the boundaries between groups once thought to have well-defined and unique characteristics (Rosario et al., 2009, 2012; Reuter et al., 2015).

Reports of presumptive viruses based on histological analysis or electron microscopy without further characterization or proper description are categorized as *unidentified virus infections* and are not discussed in this chapter.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00003-6
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TABLE 3.1 Unclassified Aquatic Animal Viruses

Virus	Abbreviation	Genome[a]	Host	Disease	Year First Reported	Known Geographical Distribution
Shrimp hepatopancreas-associated circular DNA virus	ShrimpCDV	ssDNA	Northern pink shrimp (*F. duorarum*)		2013	Gulf of Mexico
Japanese eel endothelial cells-infecting virus	JEECV	dsDNA	Japanese eel (*A. japonica*)	Viral endothelial cell necrosis of eel	1989	Japan
Panulirus argus virus 1	PaV1	dsDNA	Caribbean spiny lobsters (*Panulirus argus*)			Florida (United States), St. Croix, St. Kitts, Yucatan (Mexico), Belize, Cuba
Abalone shivering syndrome-associated virus	AbSV	dsDNA	Abalone (*H. diversicolor aqualitis*)	Abalone shivering syndrome	1985	California Channel Island, China
Cherax albidus picorna-like virus	*Ca*PV	ssRNA	Freshwater crayfish (Yabbies) (*C. albidus*)		1998	Western Australia
Isolates DF 20/00 and DF 26/02		ssRNA	Common carp and Koi carp	Gill necrosis	2000	Germany, Belgium
Fisavirus 1	HAL1/ fisa-ssRNAV	ssRNA	Freshwater carp, *Cyprinus carpio*		2015	Hungary
Laem-Singh virus	LSNV	ssRNA	Farmed black tiger shrimp (*Penaeus monodon*)	Monodon slow growth syndrome	2001–03	Thailand, Malaysia, Indonesia, India, Vietnam, Sri Lanka, East Africa
Extra small virus -like particles	XSV	ssRNA	Freshwater prawn (*M. rosenbergii*)	White tail disease	1995	India, China, Taiwan, Thailand, Australia, Caribbean

[a]*Genome is DNA or RNA; ss, single-stranded; ds, double-stranded.*

3.2 UNCLASSIFIED VIRUSES OF FISH

3.2.1 Japanese Eel Endothelial Cells-Infecting Virus

3.2.1.1 Disease

The Japanese eel endothelial cells-infecting virus (JEECV) is a novel DNA virus that has been shown to be the etiological agent of viral endothelial cell necrosis of eel (VECNE) of Japanese eel (*Anguilla japonica*) (Mizutani et al., 2011). VECNE has been recognized in the Japanese aquaculture industry since 1989 (Egusa et al., 1989) and caused a mass mortality of the species in 2008. Affected fish show reddening of fins with a swollen abdomen, as well as congested gills, liver and intestine. The histopathology of VECNE is characterized by congestion of the internal venous sinuses of the gill filaments and degeneration of swollen nuclei of the endothelial cells of blood vessels (Mizutani et al., 2011).

3.2.1.2 Virus Characteristics

The JEECV particles in the nuclei appeared hexagonal, measuring about 75 nm in diameter by transmission electron microscopy (TEM) with positive staining (Ono and Nagai, 1997). The virus that was isolated using primary Japanese eel endothelial (JEE) cell culture caused VECNE in eels and was inhibited by 5-iodo-2′-deoxyuridine and was resistant to chloroform treatment. It was initially characterized as an adenovirus-like virus (Ono et al., 2007). The JEECV sequences were determined using a next-generation sequencing system of viral DNA fragments obtained from the supernatant of virus-infected JEE cells by a method called *rapid determination system for viral nucleic acid sequences (RDV)* without molecular cloning (Maeda et al., 2008; Mizutani et al., 2007).

The JEECV genome consists of circular double-stranded DNA (dsDNA), 15,131 bp (GenBank accession no. AB543063), although a longer linear form may also exist in virus-infected cells. One open reading frame (ORF) of the genome was homologous to the large T-antigen gene of avian polyomaviruses at the adenosine triphosphate (ATP)–binding region, but it was distinct from the usual configuration of polyomaviral T-antigen genes in that it did not have introns. Moreover, other ORFs had no homology with any polyomavirus genes, suggesting that JEECV likely belongs to a novel virus family (Mizutani et al., 2011).

3.2.1.3 Diagnostic Methods

Diagnosis of VECNE can be made by histology (Egusa et al., 1989). A conventional polymerase chain reaction (PCR) was developed for monitoring JEECV infection (Mizutani et al., 2011). A primary cell culture of JEE cells has been established for virus isolation (Ono et al., 2007).

3.2.2 Virus Isolates DF 20/00 and DF 26/02

3.2.2.1 Disease

Two isolates (DF 20/00 and DF 26/02) of a novel enveloped RNA virus were obtained from common carp and koi carp with gill necrosis (Granzow et al., 2014). Isolate DF 20/00 was obtained from a diseased common carp from a fish farm in Germany that suffered high morbidity and mortality. Affected fish had reddening of the skin with a rough texture, focal loss of epidermis, overproduction of mucus on skin and gills, necrosis and inflammation of gills and swelling of the kidney and spleen and reddening of intestines. Isolate DF 26/02 was obtained from a diseased koi carp imported from Belgium that displayed petechial in the gills and skin and yellow foci in the liver, kidney and spleen (Granzow et al., 2014).

3.2.2.2 Virus Characteristics

Granzow et al. (2014) described a unique ultrastructure of the two isolates (DF 20/00 and DF 26/02) and were unable to assign the viruses to any established virus family. Initial analysis suggested that the viruses were arenaviruses, as they contained host-cell-derived ribosomes. Both virus isolates produced large syncytia in different cyprinid cell lines. The mature virus particles had a spherical to pleomorphic shape, with diameters of 100–350 nm by TEM with negative staining (Fig. 3.1), and had a 100–300-nm diameter with positive staining. The envelope had short projections (8–10 nm in length). Virus maturation

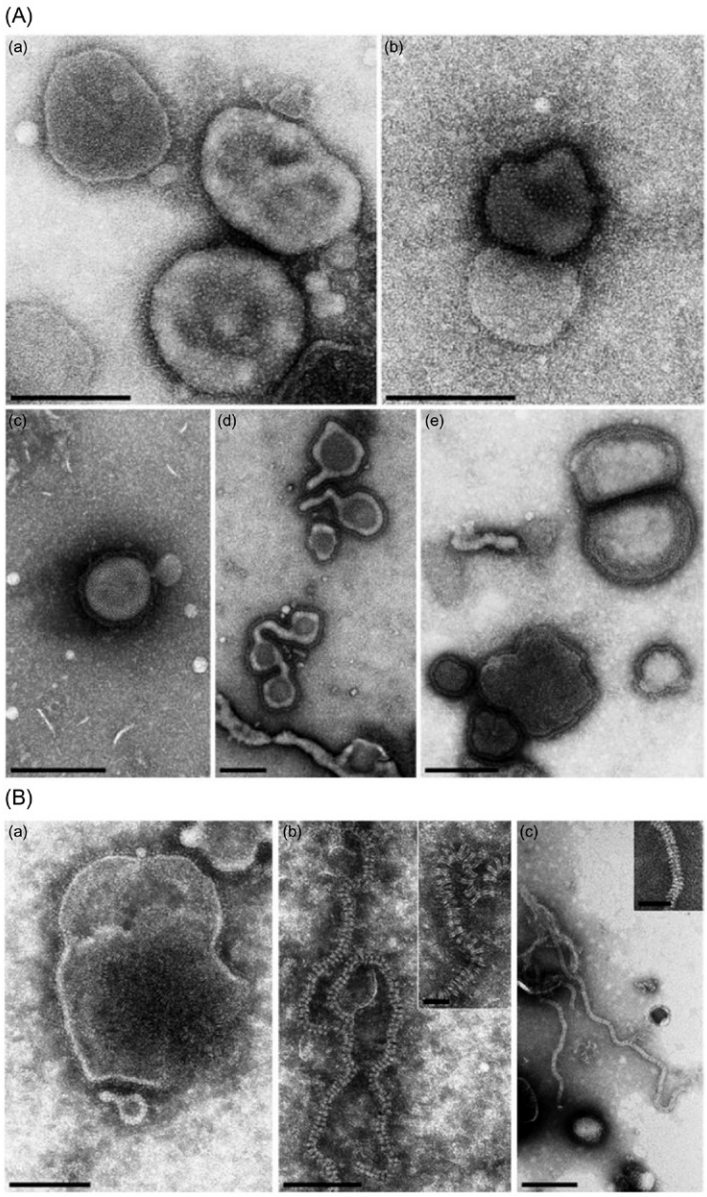

FIGURE 3.1 Transmission electron micrographs of virus isolate DF 20/00. (A) Negative staining of the virus isolate DF 20/00 (a), LCMV (b), ISAV (c, d) and NDV (e) from cell culture supernatant. Bar represents 200 nm in (a–e). (B) Nucleocapsid strands of virus isolate DF 20/00 and NDV after negative staining. A virus particle of DF 20/00 with partially visible nucleocapsid strand inside the virion (a). Strands of nucleocapsid were composed of subunits with clearly detectable parallel striations (b, inset). NDV with ruptured envelope lost the typical herring-bone nucleocapsid (c). At higher magnification, the angulated subunits of 18-nm diameter are visible (c, inset). Bar represents 200 nm; for the inset in B and C, 50 nm. *From Granzow, H., Fichtner, D., Schütze, H., Lenk, M., Dresenkamp, B., Nieper, H., Mettenleiter, T.C., 2014. Isolation and partial characterization of a novel virus from different carp species suffering gill necrosis – ultrastructure and morphogenesis. J. Fish Dis. 37, 559–569. Figures 1 and 2. Copyright © John Wiley & Sons Ltd (2013), with permission.*

occurred by budding and envelopment of filamentous nucleocapsids at the cell surface, a feature that was not detectable in arenaviruses. Previous virus isolates from koi carp from Belgium with gill necrosis were proposed to be paramyxoviruses based on their ultrastructural analysis (Body et al., 2000), a feature not supported by data presented by Granzow et al. (2014) for the two novel isolates. Granzow et al. (2014) concluded that the two virus isolates may be members of a hitherto undescribed viral taxon, but molecular biology and genetic data are required to establish this with certainty.

3.2.3 Fisavirus 1

3.2.3.1 Disease

Fisavirus 1 (*fi*sh *s*tool-*a*ssociated RNA virus) is a novel positive sense ssRNA virus identified from a freshwater carp (*Cyprinus carpio*) using viral metagenomics (Reuter et al., 2015). The fish were caught by line fishing from a fishpond in Hungary (Reuter et al., 2015), with no associated disease reported. In fact because of its sequence relatedness to *po*rcine *s*tool-*a*ssociated viruses (posavirus) identified in a pig nematode *Ascaris suum* isolated from a pig in the United States (Shan et al., 2011), it is possible that the natural host of fisavirus 1 is an unidentified nematode species in the gut of fish (Reuter et al., 2015).

3.2.3.2 Virus Characteristics

The complete genome of fisavirus 1 (strain HAL1/fisa-ssRNAV, GenBank accession no. KM434233) is 8712 nucleotides long and shares the same genome organization and distant phylogenetic relationship to the currently unclassified posaviruses that may belong to a novel family of the order Picornavirales but in a different genus (Reuter et al., 2015). Posaviruses are from pig fecal samples (Shan et al., 2011; Zhang et al., 2014) and are distantly related to the virus families Dicistroviridae and Picornaviridae (Reuter et al., 2015). Viruses of the order Picornavirales (currently families Dicistroviridae, Iflaviridae, Marnaviridae, Secoviridae and Picornaviridae and several unclassified ssRNA viruses) share similar properties, such as a common three-domain replication block (Hel-Pro-Pol domain including a superfamily III helicase, a chymotrypsin-like proteinase and an RNA-dependent RNA polymerase-RdRp) and a small non-enveloped icosahedral virion architecture (Le Gall et al., 2008).

3.3 UNCLASSIFIED VIRUSES OF CRUSTACEANS

3.3.1 Shrimp Hepatopancreas-Associated Circular DNA Virus

3.3.1.1 Disease

Shrimp hepatopancreas-associated circular DNA virus (ShrimpCDV) was identified along with *Farfantepenaeus duorarum* nodavirus (*Fd*NV) by viral metagenomic approaches in the hepatopancreas of overtly healthy northern pink shrimp (*F. duorarum*) captured from the Gulf of Mexico (Ng et al., 2013). Since the hepatopancreas is a digestive organ of shrimp, and bacteriophage phi29 DNA polymerase (rolling circle amplification) was used in the preparation of DNA for metagenomic sequencing (which preferentially amplifies small circular ssDNA templates (Kim and Bae, 2011)), it could not be discounted that ShrimpCDV represents an ingested environmental contaminant resilient to degradation by the shrimp digestive system. More studies are needed to further demonstrate this virus in other shrimp tissues and its ability to infect *F. duorarum* (Ng et al., 2013).

3.3.1.2 Virus Characteristics

ShrimpCDV is a novel virus with similarities to members of the family Circoviridae (genera *Circovirus* and *Cyclovirus*) that possess circular ssDNA (Ng et al., 2013). The ShrimpCDV genome is 1956 nucleotides long (GenBank accession no. KC441518), of ambisense organization, and it encodes two genes, putative Rep and Cap proteins. A putative DNA hairpin structure containing a sequence (AAG TAT TAC) similar to the conserved nonanucleotide motif of Circoviridae (TAG TAT TAC) exists in the short intergenic region (Ng et al., 2013). The canonical nonanucleotide motif (NAN TAT TAC) is at the apex of the potential stem-loop structure of Rep protein–encoding ssDNA viruses (Rosario et al., 2012). However, the ShrimpCDV Rep protein was only 21–34% identical to the cognate protein of Circoviridae and other unclassified ssDNA viruses, and in phylogenetic analysis, it was clustered separately from the genera *Circovirus* and *Cyclovirus* (Ng et al., 2013). Thus, the Rep protein, a conserved feature of many ssDNA viruses, does not provide adequate taxonomic resolution for classification (Rosario et al., 2012). Moreover, according to the classification scheme for ssDNA viruses based on genomic features that have been detected through metagenomic studies (Rosario et al., 2012), ShrimpCDV is a Type IV genome (Ng et al., 2013) in contrast to Circoviridae members of genera *Circovirus* and *Cyclovirus*, which are Type I and Type II genomes, respectively (Rosario et al., 2012). The Type IV genome is one of five novel genome architectures

not previously classified by the International Committee for Taxonomy of Viruses (ICTV) (King et al., 2012). The Type IV genome displays ambisense organization, but the intergenic region containing a potential stem-loop structure is found at the 3′ end of the major ORFs. Type I and Type II genomes also have ambisense organization, but the intergenic region containing the potential stem-loop structure is found at the 5′ end of the major ORFs. In Type II genome, the conserved nonanucleotide sequence is not found on the Rep-encoding strand (Rosario et al., 2012).

3.3.2 *Panulirus argus* Virus 1

3.3.2.1 Disease

Panulirus argus virus 1 (PaV1) is the first naturally occurring pathogenic virus reported from the lobster fishery. It was discovered in 2000 in juvenile Caribbean spiny lobster (*Panulirus argus*) from the Florida Keys in the United States (Shields and Behringer, 2004). This virus is of significant interest because the *Panulirus argus* fishery is the most economically valuable one in the Caribbean (FAO, 2006). The virus primarily infects benthic juvenile lobsters (with a 20–55-mm carapace length), but this declines with increasing lobster size, with adults being asymptomatic carriers of the virus. Heavily infected lobsters become sedentary, cease feeding and die of metabolic exhaustion within 5–7 days. Moribund lobsters had characteristically thin and milky or chalky hemolymph that did not clot, and sometimes a discolored, heavily fouled carapace (Shields and Behringer, 2004). Histopathology showed nuclear hypertrophy with diffuse eosinophilic Cowdry Type-A viral inclusions in infected hemocytes and complete destruction of circulating hyalinocytes and semigranulocytes (Shields and Behringer, 2004). Li et al. (2008) described the histopathology following experimental infection of the Caribbean spiny lobster with PaV1, and based on pathological changes, established four categories of severity: 0 (healthy), 1 (light infection), 2 (moderate infection) and 3 (heavy infection). The fixed phagocytes that were eventually lysed and connective tissues of the hepatopancreas were the primary sites of PaV1 infection (Li et al., 2006). In heavy infections, the hepatopancreas tubules became atrophied and spongy connective tissues were heavily infiltrated with hemocytes (Fig. 3.2).

3.3.2.2 Virus Characteristics

PaV1 is a novel dsDNA virus with morphological features of Herpesviridae and Iridoviridae (Shields and Behringer, 2004; Montgomery-Fullerton et al., 2007; Behringer et al., 2011). The PaV1 particles free in lobster hemolymph (Behringer et al., 2011) and in the nuclei of infected hemocytes appeared via TEM with positive staining as unenveloped, nonoccluded, icosahedral nucleocapsids that are approximately 182 ± 9 nm (standard deviation, SD) in diameter, and nucleoids of about 118 ± 4 nm (SD) in diameter, surrounded by a bilayered capsid wall, by TEM (Fig. 3.3) (Shields and Behringer, 2004). The nucleoids were described to possess a subcentric toroid structure similar to the classical toroid of Herpesviridae, but unlike herpesviruses, there was no tegument or viral envelope (Shields and Behringer, 2004). The PaV1 was described as being similar to members of Iridoviridae with respect to the large icosahedral virions, but unlike iridoviruses, PaV1 replicates

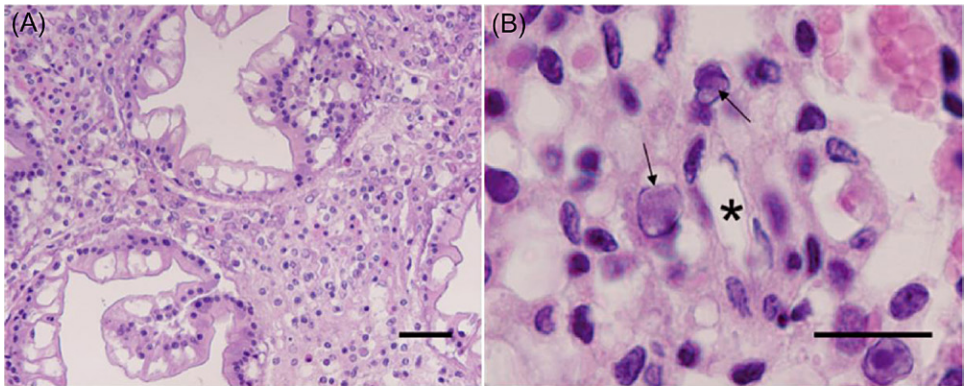

FIGURE 3.2 PaV1 pathology in *Panulirus argus*. PaV1 infecting *P. argus*. (A) Atrophied hepatopancreas showing infiltration of hemocytes into the spongy connective tissues as a result of infection by PaV1. Scale bar = 50 µm. (B) PaV1 infection in the fixed phagocytes (*arrows*) surrounding an arteriole in the hepatopancreas. *: lumen of the arteriole. Scale bar = 50 µm. *From Behringer, D. C., Butler, M. J. I. V., Shields, J. D., Moss, J., 2011. Review of Panulirus argus virus 1- a decade after its discovery. Dis. Aquat. Org. 94, 153–160. Figure 1. Copyright © Inter-Research (2011), with permission.*

entirely in the nucleus (Shields and Behringer, 2004). Members of Iridoviridae replicate in both the nucleus and cytoplasm with intracytoplasmic assembly and budding from the host cell membrane (ie, they are enveloped) (Jancovich et al., 2012).

The PaV1 genome consists of dsDNA, but little genetic information currently exists. The data that do exist are limited to the following: a 892-bp fragment (GenBank accession no. EF206313); a 177-bp fragment of a DNA-directed polymerase (GenBank accession no. DQ465025); and a 462-bp fragment of a hypothetical protein gene (GenBank accession no. JN786976).

3.3.2.3 Geographical Distribution

The Caribbean spiny lobster *Panulirus argus* occurs throughout the Caribbean Basin and western Atlantic Ocean from Brazil to Bermuda (Holthuis, 1991). PaV1 is widespread in this region, with infections reported in Florida, St. Croix, St. Kitts, the Yucatan in Mexico, Belize and Cuba (Huchin-Mian et al., 2008; Cruz-Quintana et al., 2011). The mean prevalence of PaV1 in the Florida Keys in 2000–2010 was 5% (reviewed by Behringer et al., 2011). Virus transmission is by contact,

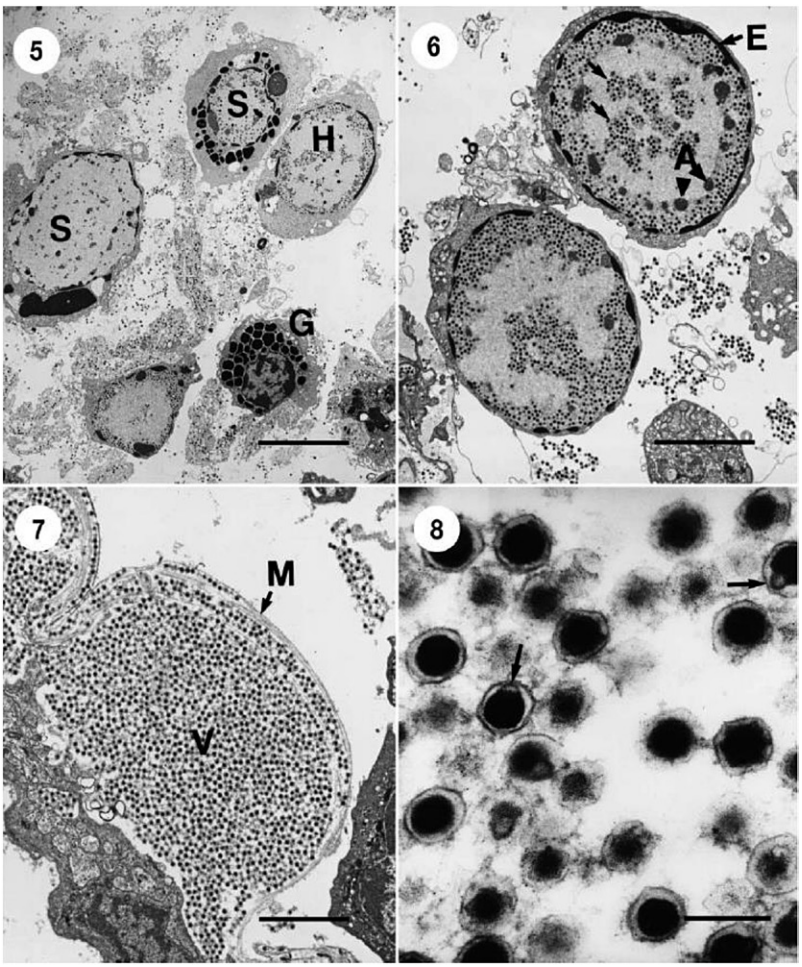

FIGURE 3.3 Electron microscopy of *Panulirus argus* virus 1 (PaV1) in *P. argus* hyalinocytes and semigranulocytes. This image is reproduced from Figures 5, 6, 7 and 8 of Shields and Behringer (2004), with permission; the respective figure numbers are listed on the image as 5, 6, 7 and 8. Fig. 5. Infected hyalinocytes (H) and semigranulocytes (S) exhibiting hypertrophied nuclei, emarginated chromatin and juxtanuclear mitochondria. Uninfected granulocyte (G) shown with numerous electron-dense granules and normal nucleus. Scale bar = 10 µm. Fig. 6. Infected hemocytes showing emarginated chromatin (E), loose aggregates of virions in the nucleoplasm (*arrows*) and electron-dense whorls within the virogenic stroma (A). Scale bar = 5 µm. Fig. 7. Matrix of virions (V) aggregated within the perforated membrane of the fixed phagocytes overlying endothelial cells of a hemolymph vessel. Scale bar = 3 µm. Fig. 8. Detail of virions from Fig. 7 showing icosahedral form, cylinder within the toroid (*arrows*) of the nucleoid surrounded by a bilayered capsid wall. Scale bar = 3 µm. *From Shields, J. D., Behringer, D. C., 2004. A new pathogenic virus in the Caribbean spiny lobster Panulirus argus from the Florida Keys. Dis. Aquat. Organ. 59, 109–118. Figures 5–8. Copyright © Inter-Research (2004), with permission.*

through food and over short distances (<1 m) through water (Behringer, 2003). Healthy lobsters can sense and avoid diseased lobsters, which limits the spread of the virus in the lobster population (Behringer et al., 2006). In PaV1 infection trials using three potential alternate hosts (ie, the spotted lobster, *Panulirus guttatus*; the stone crab, *Menippe mercenaria*; and the channel crab, *Mithrax spinosissimus*), none showed signs of disease; thus, PaV1 appears to be highly specific to *Panulirus argus* (Butler et al., 2008).

3.3.2.4 Diagnostic Methods

PaV1 infection can be diagnosed by histology and TEM of several organs, including hepatopancreas, hindgut, gill, heart, cuticle epidermis, nerve tissue, compound eyes and antennal gland (Shields and Behringer, 2004; Li et al., 2008), and confirmed using fluorescence in situ hybridization (Li et al., 2006). In 2006, a PCR assay was developed with sensitivity to 1.2 fg of purified viral DNA (Montgomery-Fullerton et al., 2007) and the primer annealing temperature was subsequently increased from 51°C to 63°C (Huchin-Mian et al., 2008). A primary cell culture of semigranulocytes and hyalinocytes has been developed to titrate PaV1 in hemolymph samples (Li and Shields, 2007). Uninfected cell cultures remain viable (about 80%) for 18 days, whereas in infected cells, CPEs were evident within 12–24 h postinoculation (Li and Shields, 2007; Shields, 2011).

3.3.2.5 Prevention and Control

Current commercial fishing practices where live juvenile lobsters are used as bait (ie, a social attractant for adult lobsters) in lobster traps may accelerate the transmission and spread of PaV1 (reviewed by Behringer et al., 2011).

3.3.3 *Cherax albidus* Picorna-Like Virus or Yabby Virus

3.3.3.1 Disease

Cherax albidus picorna-like virus (*Ca*PV), or Yabby virus, is a new virus associated with significant pathology and mortalities in farmed freshwater crayfish *C. albidus* in Western Australia (Jones and Lawrence, 2001). The virus was discovered in 1998 (Edgerton, 1999). CaPV has widespread distribution with low prevalence (<5%) in yabbies in farm dams in southwestern Western Australia (Jones and Lawrence, 2001).

3.3.3.2 Virus Characteristics

*Ca*PV or Yabby virus infections are characterized by large basophilic and small eosinophilic intranuclear inclusions. Nuclear changes occurred in the interstitial tissues of the digestive gland, labyrinth epithelium of the antennal gland and the hindgut. Ultrastructurally, nonenveloped virions in paracrystalline arrays measured 13–19 nm in diameter. Although Jones and Lawrence (2001) suggested that the new virus belonged to Circoviridae or Picornaviridae, its intranuclear replication and relatively small size make it more similar to Circoviridae. However, the virus was Feulgen-negative, indicating it has an RNA genome (Jones and Lawrence, 2001).

3.3.4 Laem-Singh Virus (LSNV)

3.3.4.1 Disease

Monodon slow growth syndrome (MSGS) is an important emerging disease in shrimp aquaculture in South and Southeast Asia, characterized by stunted growth and size variation in farmed black tiger shrimp (*Penaeus monodon*). The MSGS condition was first noticed in Thailand in 2001 (Withyachumnarnkul, 2005). In affected ponds, there was a 65% variation in growth compared to the usual 20%. Centex Shrimp (Mahidol University, Bangkok, Thailand) defined the criteria for MSGS as shrimp that are reverse transcription-polymerase chain reaction (RT-PCR) positive for the Laem-Singh virus (LSNV), reaching an average size of 5–10 g instead of the regular size of 24–40 g after 4 months and very high (>35%) coefficient of variation in weight, together with freedom from the hepatopancreatic parvovirus (HPV), now called *Penaeus monodon densovirus*, or any other severe hepatopancreas infection, plus any three of the following five characteristics: (i) unusually dark color, (ii) average daily weight gain of <0.1 g/day at 4 months, (iii) unusually bright yellow markings, (iv) bamboo-shaped abdominal segments and (v) brittle antennae (Flegel, 2006, 2008a). These features distinguish MSGS from stunted growth caused by monodon baculovirus or HPV (Flegel et al., 1999, 2004).

3.3.4.2 Virus Characteristics

The virus name Laem-Singh virus (LSNV) was derived from where the first positive sample was collected (Laem-Singh district, Chanthaburi Province, Thailand). The LSNV is the probable etiological agent of MSGS in farmed black tiger shrimp (Sritunyalucksana et al., 2006). This contention was supported by the rapid spread of the problem and preliminary laboratory challenge tests showing that bacteria-free tissue extracts from MSGS shrimp were able to cause MSGS with retinopathy when injected into *Penaeus monodon*, but not when injected into *Penaeus vannamei* (also called *Litopenaeus vannamei*) (Withyachumnarnkul et al., 2004). *Penaeus vannamei* might act as an asymptomatic carrier of LSNV (Poornima et al., 2012).

Subsequently, it was shown that LSNV occurs commonly in healthy *Penaeus monodon* in parts of Asia (Sittidilokratna et al., 2009). More recently, a novel integrase-containing element (ICE) (probably a satellite-like virus) has been shown to occur together with LSNV in shrimp from MSGS ponds, and only in shrimp showing gross signs of MSGS, unlike LSNV, which was sometimes found in normal shrimp in the absence of ICE (Panphut et al., 2011).

The LSNV particles in shrimp tissues are approximately 25–27 nm in diameter by TEM with positive staining. Negatively stained purified LSNV particles from CsCl density gradients were of icosahedral symmetry, nonenveloped and 25–33 nm in diameter, together with 15 nm-diameter particles considered as the source of ICE (Fig. 3.4) (Panphut et al., 2011).

The LSNV genome consists of positive-sense ssRNA, but it has not yet been completely sequenced. Phylogenetic analysis of its RNA-dependent RNA polymerase (RdRp) sequence formed a distinct clade between members of Luteoviridae and Togaviridae (Flegel, 2006), Flaviviridae, Reoviridae and Birnaviridae (Sritunyalucksana et al., 2006). Subsequent work and additional sequence information revealed a protease gene and other structural motifs suggestive of the insect-associated plant virus family Sobemoviridae; LSNV still did not fit with Luteoviridae and Sobemoviridae on phylogenetic analysis (Srisala, 2010). More genome information is needed for the proper classification of LSNV.

Panphut et al. (2011) used shotgun cloning and sequencing of ultrapurified tissue homogenates from MSGS shrimp to identify the ICE sequence, which is 2233 nucleotides long. This ICE sequence shared no significant identity to the extra small virus-like particle (XSV) genome sequence or any other known satellite virus sequences.

3.3.4.3 Geographical Distribution

From 2001–2003, MSGS caused severe economic losses for Thai shrimp farmers, ranking third after white spot syndrome virus (WSSV) (Sánchez-Martínez et al., 2007) and yellow head virus (YHV) in farmed black tiger shrimp (Chayaburakul et al., 2004; Flegel, 2006); it was responsible for the major switch from the higher-value native shrimp species to the exotic

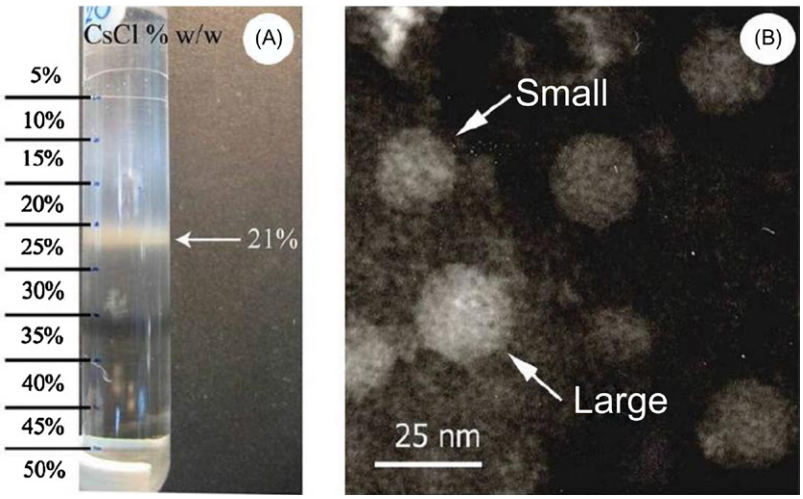

FIGURE 3.4 Transmission electron micrograph of negatively stained purified particles from a CsCl density gradient. (A) Photograph of third-round gradient CsCl density gradient separation showing a band at 21% CsCl (1.1843 g/cm^3). (B) TEM of a negatively stained sample from the band in (A) showing viral-like particles of approximately 25- and 15-nm diameter (*arrows*) with some angular sides. *From Panphut, W., Senapin, S., Sriurairatana, S., Withyachumnarnkul, B., Flegel, T. W., 2011. A novel integrase-containing element may interact with Laem-Singh virus (LSNV) to cause slow growth in giant tiger shrimp. BMC Veterinary Research 7, 18. Figure 1. Copyright © Panphut et al. (2011), with permission* http://www.biomedcentral.com/download/license.pdf.

farmed Pacific white shrimp (*Penaeus vannamei*). LSNV has been reported in association with MSGS in both healthy and infected *Penaeus monodon* from Thailand, Malaysia, Indonesia, India and Vietnam, suggesting a geographical distribution restricted to South and Southeast Asia (Sittidilokratna et al., 2009). The virus was found for the first time in Sri Lanka in 2010 (Poornima et al., 2012). However, unique MSGS lesions and virus-like particles consistent with LSNV were found in *Penaeus monodon* in East Africa in 2004 (Anantasomboon et al., 2006).

3.3.4.4 Diagnostic Methods

LSNV infections have been confirmed in various organs of infected shrimp, particularly the lymphoid organ, by various diagnostic methods such as TEM, RT-PCR and in situ hybridization (Sritunyalucksana et al., 2006; Pratoomthai et al., 2008), quantitative real-time RT-PCR and reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick (RT-LAMP-LFD) (Poornima et al., 2012). It is thought that progression of infection in the optic nerve, resulting in retinopathy, may be the cause of slow growth. Thus, histopathological studies of growth-retarded shrimp from MSGS ponds have shown that lesions in the eyestalk (retinopathy) are pathognomonic (Pratoomthai et al., 2008). The retinopathy comprised of abnormally enlarged hemolymphatic vessels, hemocytic infiltration and rupture of the membrane that separated the fasciculated zone from the overlying row of reticular cells (Pratoomthai et al., 2008). In situ hybridization testing of experimentally challenged shrimp showed positive reactions in the lymphoid organ, eyes and gills of the shrimp for both ICE and LSNV (Panphut et al., 2011), supporting the link between retinopathy and LSNV (Pratoomthai et al., 2008).

3.3.4.5 Prevention and Control

Since the discovery of LSNV in 2006, it has been added to the list of viruses to be excluded from domesticated specific pathogen-free (SPF) stocks of *Penaeus monodon* in Thailand, and it has been recommended that shrimp farmers avoid stocking LSNV-positive postlarvae to avoid MSGS (Poornima et al., 2012). It is recommended that in countries where *Penaeus vannamei* has already been introduced, *Penaeus vannamei* and *Penaeus monodon* should be reared separately, particularly during the maturation and hatchery phases (Flegel, 2008b). Quarantine protocols are recommended to limit the potential spread of MSGS through the movement of live shrimp stocks for aquaculture (Anantasomboon et al., 2006). In general, shrimp virus diseases are managed by exclusion using a combination of biosecurity and culturing domesticated SPF stocks or specific pathogen-resistant stocks.

3.3.5 Extra Small Virus-Like Particles

3.3.5.1 Disease

XSV is one of two viruses associated with white tail disease (WTD) (Romestand and Bonami, 2003; Bonami et al., 2005; Bonami and Sri Widada, 2011) or "whitish muscle disease" of the giant freshwater prawn *M. rosenbergii*. The cause of the disease is a nodavirus, *M. rosenbergii* nodavirus (*Mr*NV) (Arcier et al., 1999), in association with XSV (Qian et al., 2003). *M. rosenbergii* nodavirus and WTD are described in Chapter 34 "Nodaviruses of Crustaceans." Details of the interactions between XSV and *Mr*NV are not clearly known, and it has not yet been established whether XSV influences the severity of WTD (Sri Widada and Bonami, 2004; Zhang et al., 2006).

3.3.5.2 Virus Characteristics

XSV is a nonautonomous satellite-like virus (Qian et al., 2003). The virus particle is unusually small (14–16 nm in diameter, with an average of 15 nm, found by negative staining electron microscopy), hence the name of the virus (Qian et al., 2003).

Sri Widada and Bonami (2004) used virus particles purified from tissue extracts of WTD-infected animals on CsCl gradients to molecularly clone and sequence the XSV genome. The virus genome consists of a linear positive-sense ssRNA, 796 nucleotides long, with a poly (A) tail of 15–20 nucleotides (GenBank accession no. AY247793), and encodes a single structural protein of 174 amino acids (namely, the capsid CP-17). A second protein of 16 kDa (CP-16) is also present in purified XSV particles, and both proteins could be demonstrated by sodium dodcyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sri Widada and Bonami, 2004; Bonami et al., 2005). The XSV does not contain any RNA-dependent RNA polymerase (RdRp) genes and consequently needs the *Mr*NV polymerase to replicate (Bonami and Sri Widada, 2011). The XSV was not affiliated to any known satellite viruses (Sri Widada and Bonami, 2004).

3.4 UNCLASSIFIED VIRUSES OF MOLLUSKS

3.4.1 Abalone Shivering Syndrome-Associated Virus

3.4.1.1 Disease

Abalone shivering syndrome (AbSS) is a highly fatal wasting disease of abalone (*Haliotis diversicolor aqualitis*). The condition, called *withering syndrome*, first appeared in 1985 in the California Channel Island populations of black abalone (*Haliotis cracheroddi*) (Lafferty and Kuris, 1993). It was associated with Rickettsiales bacterial infection (Gardner et al., 1995). A condition with similar clinical signs was named *abalone shivering syndrome* when it first occurred in abalone culture fisheries in Fuzhou, China, in November 2005 (Zhuang et al., 2010). Abalones in all life stages can suffer from the disease. Affected abalones are off feed, the foot muscle appears shrunken, the visceral tissues are retracted, and most abalone, unable to tightly adhere to the substrate, fall from the reef and die, resulting in large numbers of empty shells. The infection, which was characterized by atrophy of the foot muscle (pedal muscle) and necrosis and aberrant modifications of hemocyanins and ferritin of mantle tissue (Fig. 3.5), could be experimentally reproduced by injection into the pedal muscle of healthy abalone with a 0.22-μm-pore-diameter filtered material from naturally infected abalone or purified viral DNA from virus suspension purified from homogenized infected abalone using polyethylene glycol (PEG)–mediated centrifugation or sucrose cushion/sucrose gradient ultracentrifugation (Zhuang et al., 2010).

FIGURE 3.5 Typical clinical sign of sick abalone. Infected abalone *H. diversicolor aquatilis* with shrunken mantle and pleopod. *From Zhuang, J., Cai, G. Q., Lin, Q. Y., Wu, Z. J., Xie, L. H., 2010. A bacteriophage-related chimeric marine virus infection abalone. PLoS One 5, e13850. Figure S1. Copyright* © Zhuang et al. (2010)*, with permission.* https://creativecommons.org/licenses/by/4.0/.

3.4.1.2 Virus Characteristics

The virus used to reproduce AbSS experimentally in healthy abalones is an unclassified, novel bacteriophage-related chimeric virus named AbSS-associated virus (AbSV) (Zhuang et al., 2010). Electron microscopy of negatively stained purified virus particles from PEG-mediated ultracentrifugation appear as squares of 30 × 30 nm and rectangles of 30 × 150 nm (and even longer) from the side and as circles of approximately 30 nm in diameter from the top (Fig. 3.6) (Zhuang et al., 2010).

The AbSV genome consists of a circular dsDNA, with 34,952 bp (GenBank accession no. EU350361). It encodes 28 putative ORFs covering 94% of the total genomic DNA. Sequence analysis revealed that AbSV has universal proteins of bacteriophages and the genomic organization of tailed bacteriophages. The AbSV genome also encodes a typical gene of vertebrate viruses, thymidylate kinase, which makes AbSV representative of an unknown group of viruses in the marine virosphere (Zhuang et al., 2010).

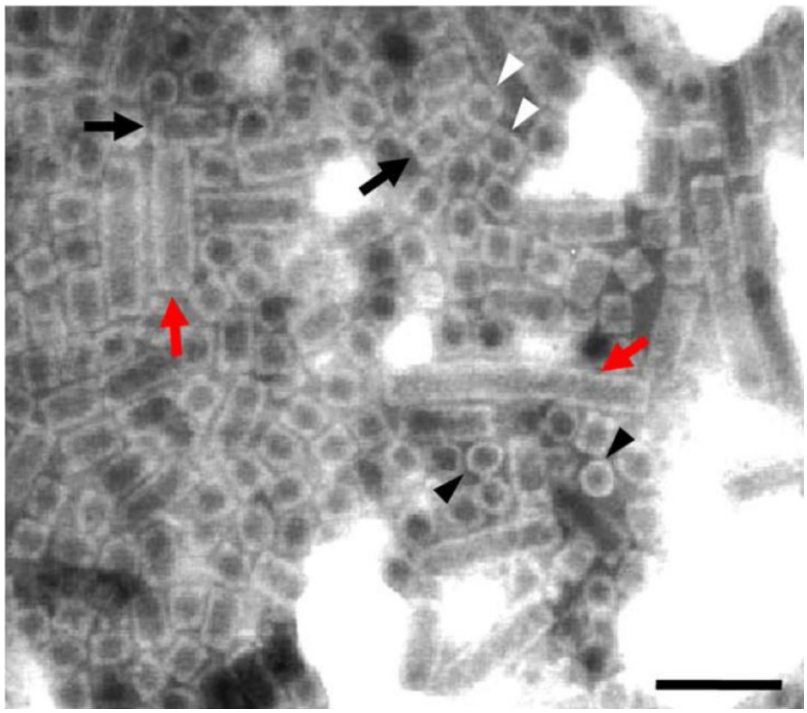

FIGURE 3.6 TEM of particles in an AbSV suspension. Novel particles are shown from the side view as long flexible/rigid rectangles (*red* (dark gray in print versions) *arrows*) and short rectangles (*black arrows*). The circles (*black arrowheads*) and cubes (*white arrowheads*) from the top view and side views, respectively, are all similar to typical hemocyanin architectures. Scale bar, 100 nm. *From Zhuang, J., Cai, G. Q., Lin, Q. Y., Wu, Z. J., Xie, L. H., 2010. A bacteriophage-related chimeric marine virus infection abalone. PLoS One 5, e13850. Figure 2. Copyright* © Zhuang et al. (2010), *with permission* https://creativecommons.org/licenses/by/4.0/.

3.4.2 Retrotransposons and Transmissible Cancer in Clams

In the past, there have been several reports of reverse transcriptase activity in diseased clams, suggesting a retrovirus or retrotransposon; and there are also reports that the disease was transmissible, which suggested a viral aetiology. Arriagada et al. (2014) used high-throughput sequencing to look for a retroelement and found amplification of an LTR-retrotransposon, *Steamer* (which is not a retrovirus), associated with disease. These authors have found that the genotypes of cancer cells in different individuals are nearly identical and do not match their hosts. This shows that the cancer cells are transmitted from individual to individual, so the transmissible element appears to be the cancer cell itself, rather than a virus (Metzger et al., 2015). Thus, to date, there are no known retroviruses in mollusks.

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Chapter 4

Diagnosis of Aquatic Animal Viral Diseases

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4.1 INTRODUCTION

A viral diagnosis involves three action points: (1) use of specialized virology techniques (ie, virus isolation, fluorescent antibody test, agar gel immunodiffusion test (AGID), electron microscopy (EM), polymerase chain reaction (PCR), serum neutralization test, enzyme-linked immunoabsorbant assay (ELISA), etc.), performed in virology diagnostic laboratories (which can be university-based, federal- or state/provincial-sponsored or private); (2) a diagnostician (eg, veterinarian), who (a) collects specimens and submits them to a virology diagnostic laboratory with a clinical history and request for specific tests (for detection of virus or viral antibody) and (b) interprets laboratory results for (i) differential diagnosis (rule-outs)/confirmatory diagnosis, (ii) disease management and (iii) client education; (3) a virology diagnostic laboratory that (a) receives submissions, (b) performs tests (often for a fee) and (c) sends results to the diagnostician. It is not uncommon to have diagnostic laboratories that specialize for example in testing only aquatic animal specimens because of the large number of viral pathogens and animal hosts. In fact the burgeoning international aquaculture expansion has been accompanied by a proliferation of aquatic animal diagnostic laboratories in regions with intense aquaculture. Moreover, the needs for laboratory testing for aquatic animal viral diseases go beyond disease management and pathogen surveillance to include health certification of aquatic animals and their products for transport/trade purposes.

The emergence and spread of serious diseases is a major threat to aquaculture, and robust methods for laboratory diagnosis and pathogen surveillance are needed to reduce the risks. The World Organization for Animal Health (OIE) (OIE, 2015a) has standard protocols for directed detection and identification of pathogens with a risk of spread through trade in aquatic animals and their products. The American Fisheries Society, Fish Health Section Blue Book (AFS-FHS, 2014) is an excellent source of high quality and relevant information on the detection and diagnosis of aquatic animal viral diseases of regulatory or reporting concern. Current trends in laboratory diagnosis and pathogen surveillance involve automation, miniaturization and nucleic acid-based assays. Such assays allow early detection of infection before clinical signs develop (because differential diagnosis is difficult in early infection before the appearance of clinical signs) and detection of low virus loads that may be present in carriers and egg-transmission; these assays are essential for effective control of any disease and especially reportable and/or emerging aquatic animal viral diseases. The main driver for changes in diagnostic tests for aquatic animal viral diseases is to shorten turnaround time on large sample submissions (ie, increase throughput). Moreover, newer technologies such as next-generation sequencing (NGS) and high-throughput metagenomics are providing robust methods for discovering new viruses and may address some of the limitations in identification of aquatic animal viruses: (1) crustacean and molluskan viruses, where a vast amount of viruses remain either undiscovered or uncharacterized—"unclassified" or "unidentified"—because of a lack of crustacean and molluskan cell lines for virus isolation and propagation and (2) bias of traditional virus detection and identification methods, requiring prior knowledge of the identity of potential viruses in order to select the test/primer to use. Shrimp, for example, can be infected simultaneously or sequentially with multiple viruses (Flegel et al., 2004) or even different strains of the same virus (Hoa et al., 2005), presenting significant challenges for diagnostic pathogen detection.

4.1.1 Objectives for Laboratory Viral Diagnosis in Aquaculture and Fisheries

There are largely three objectives of a laboratory viral diagnosis: (1) to confirm clinical diagnosis on the basis of either virus or antibody detection or both in clinical specimens; (2) assistance in making a differential diagnosis (rule-outs)/confirmatory diagnosis (ie, when clinical signs and/or necropsy findings are nonspecific and virus is suspected) and (3) provision

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00004-8
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of up-to-date client information on disease management. These are essentially the current needs of a diagnostician (eg, veterinarian). Basically, tests for the specific diagnosis of a viral infection in an animal are of two general types: (1) those that demonstrate presence of the virus (the direct methods)—which are of two types (a) direct demonstration of virions, viral proteins or viral nucleic acids (the rapid methods that provide results within 24–48 hours) and (b) virus isolation; (2) those that demonstrate the presence of specific viral antibody in cases where viral infectivity can be inhibited and/or viral proteins are bound by specific antibody (the indirect methods). The applicable tests may vary depending on the aquatic animal. For example, diagnoses for mollusk disease rely predominantly on histological evaluation of tissues (AFS-FHS, 2014), and use of virus isolation is limited to fish disease because of a lack of crustacean and molluskan cell lines for virus isolation and propagation. Crustaceans and mollusks do not produce antibodies and therefore, a viral diagnosis cannot be reached by detection of a specific viral antibody in these aquatic animals.

Fig. 4.1 provides a generic flowchart for establishing a specific diagnosis of a viral infection using tissues, secretions, excretions or serum samples. Whereas the direct visualization of virus particles by EM and virus isolation can only be performed one sample at a time, the rapid methods for detection of viral proteins or viral nucleic acids or virus-specific antibodies, have been miniaturized, not only allowing assays of multiple samples, but also screening simultaneously for multiple viruses in a single sample and thus have advantages in terms of speed, cost and number of samples that can be examined. Such assays are amenable to automation and computerized analyses, which make interpretation of results objective and the tests capable of being standardized objectively. Now, monoclonal antibodies with defined specificities are replacing hyperimmune sera for use and can enable specific diagnosis to the level of subtype, strain or variant of virus, and also now form the basis of several commercially available kits. A corollary to this has been the development of rapid diagnostic kits for on-site (point-of-care) detection of viral proteins or viral nucleic acids or virus-specific antibodies in clinical specimens (Table 4.1).

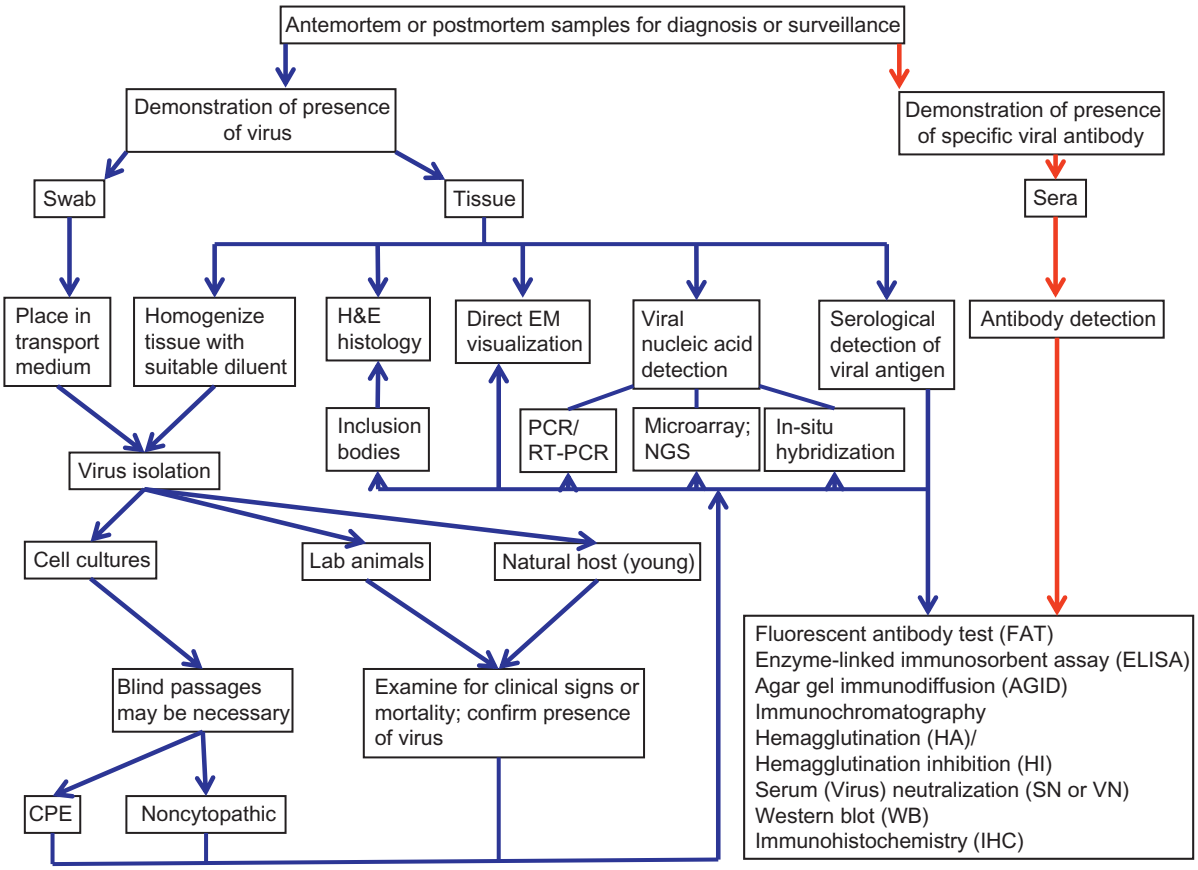

FIGURE 4.1 Flowchart for the laboratory confirmation of a viral diagnosis. Lines for demonstration of presence of virus in a specimen are in blue (dark gray in print versions), whereas those for demonstration of presence of specific viral antibody are in red (gray in print versions).

TABLE 4.1 Common Rapid Diagnostic Kits for On-Site Detection of Aquatic Animal Viral Infections

Name of Kit (Manufacturer)	Virus Test	Viral Disease
IdentiFEIA Test System (DiagXotics Inc., Wilton, CT)	Antigen ELISA	Infectious hematopoietic necrosis virus (IHNV)
IdentiFEIA Test System (DiagXotics Inc., Wilton, CT)	Antigen ELISA	Infectious pancreatic necrosis virus (IPNV)
ShrimProbe ImmunoDot	Antigen ELISA	Taura syndrome virus or IHHNV
Rapid ISAV Ag Kit (Aquatic Diagnostics Ltd, Stirling, Scotland)	Lateral flow chromatography	Infectious salmon anemia virus (ISAV)
Shrimple (Takahashi et al., 2003)	Lateral flow chromatography	White spot syndrome virus (WSSV)
WSSV Rapid Test Kit[a] (Profound Kestrel Laboratories, Selangor Darul Ehsan, Malaysia)	Lateral flow chromatography	White spot syndrome virus (WSSV)
WSSV Rapid Test Kit	LAMP[b]	White spot syndrome virus (WSSV)

[a]*Other kits include Infectious Myonecrosis Virus (IMNV) Rapid Test Kit, Yellow Head Virus (YHV) Rapid Test Kit, and Monodon Baculovirus (MBV) Rapid Test Kit.*
[b]*Loop-mediated isothermal amplification (LAMP) (http://advocate.gaalliance.org/on-site-diagnostic-kit-identifies-wssv-in-shrimp/).*

4.1.2 Rationale for Laboratory Viral Diagnosis in Aquaculture and Fisheries

Many viral diseases can be diagnosed in the field by a clinician, others with the assistance of a pathologist and/or requiring the use of a diagnostic virology laboratory. The following are situations and circumstances when laboratory testing is essential:

1. Suspected reportable aquatic animal viral diseases, such as diseases listed by the OIE. The 2015 OIE list of notifiable aquatic animal diseases (those with a risk of spread through trade in aquatic animals and their products) includes eight fish viral diseases, one mollusks viral disease and eight crustacean viral diseases (OIE, 2015b). It is of utmost importance that the clinical diagnosis of a suspected reportable virus should be confirmed quickly and accurately. Movement restrictions (quarantine) and test and removal programs (involving depopulation, cleaning and disinfection of premises) followed by enhanced surveillance control these diseases. Laboratory diagnosis is essential for the effective implementation of such measures.
2. Health certification. This is required for transport/trade purposes, and could be part of a surveillance program or government control programs.
3. Screening of broodstock, sperm and eggs to prevent egg-transmission (vertical transmission) of aquatic animal viruses.
4. Veterinary health investigations or disease surveillance at the state/provincial or national level; for example epidemiological studies to determine prevalence and distribution of viral infections including new, emerging and reemerging aquatic animal viral diseases (see also Chapter 6: Determinants of Emergence of Viral Diseases in Aquaculture).
5. Clinical management of individual cases or infected farms (biosecurity, vaccination, treatment) may require specific viral diagnosis; particularly for diseases of multiple etiology (eg, salmon gill disease).
6. When clinical signs and/or necropsy findings are nonspecific, that is, clinical signs and lesions can be caused by a number of infectious agents; for example, assistance in ruling out a differential diagnosis, in subclinical infections, and in acute disease outbreaks, to establish viral etiology of a disease not previously encountered in a geographical area or in cases of vaccine failure, to check if there is emergence of a new serotype or new variants of preexisting viruses, etc.

4.2 PRINCIPLES OF LABORATORY BIOSAFETY AND BIOCONTAINMENT AS APPLY TO AQUATIC ANIMAL VIRUSES

Laboratory biosafety and biocontainment standards in virology diagnostic laboratories are regulated nationally by federal agencies. The *CDC Biosafety in Microbiological and Biomedical Laboratories* document (CDC, 2009) outlines best practices for the safe conduct of work in biomedical and clinical laboratories, which are consistent with international standards (WHO, 2006; OIE, 2015c). In Canada, the minimum acceptable physical and operational requirements for facilities working with aquatic animal pathogens have been described in *Containment Standards for Facilities Handling Aquatic Animal Pathogens* (CFIA, 2013). These standards recognize four different levels of physical containment, Aquatic Containment Level 1 (AQC1), AQC2, AQC3 and AQC4. The AQC2 for in vitro facilities is analogous to Biosafety Level 2 (BSL-2) laboratories working with human and terrestrial animal pathogens (CDC, 2009).

4.3 QUALITY ASSURANCE

Diagnostic laboratories need to have a Quality Assurance Program in place. The OIE requires that veterinary laboratories be managed under a quality assurance system (OIE, 2015d) preferably accredited to an international standard such as ISO/IEC 17025 (ISO, 2005). The quality standards require that diagnostic tests used in the laboratory should be validated as fit for purpose, and laboratories should participate in proficiency programs (EURL-Fish, 2015a) to ensure that they meet acceptable standards. The international standard for validation of diagnostic tests is established by the OIE (OIE, 2015e). The American Association of Veterinary Laboratory Diagnosticians (AAVLD), assisting the laboratories to meet or exceed the OIE standards (AAVLD, 2014), may also accredit public veterinary laboratories in North America. Those laboratories that perform regulatory testing are periodically required to certify their procedures by the US Department of Agriculture, Animal and Plant Health Inspection Service (APHIS). Similar requirements are in place by federal/national regulatory agencies in other countries.

4.4 PRINCIPLES OF PROPER COLLECTION, HANDLING, STORAGE AND SUBMISSION OF LABORATORY SPECIMENS FOR VIRAL DIAGNOSIS

The chance of detection of a virus depends critically on the attention given by the attending veterinarian or diagnostician to the collection of specimens. Clearly, such specimens must be taken from the right tissue, from the most appropriate animal and at the right time. The right time for virus detection is as soon as possible after the animal first develops clinical signs, because maximal amount (titers) of virus are usually present at the onset signs and then often decrease rapidly during the ensuing days (MacLachlan and Dubovi, 2011). Specimens must be collected according to a sound knowledge of the epidemiology and pathogenesis of the disease under investigation or the disease syndrome to be diagnosed. This will lead to the sampling of tissues or fluids most likely to contain the infectious agent or evidence of the infection. Considerations will include the tissue predilection(s) or target organ, the duration and site of infection in each tissue type and the duration and route of shedding or the time frame in which evidence of past infection, such as an antibody response, can be detected reliably by the tests to be deployed (OIE, 2015f). Generally, whole animals or tissues should be submitted directly to the diagnostic laboratory in leak-proof insulated containers with wet ice or cold packs or shipped under refrigeration by overnight courier service. Many diagnostic laboratories provide viral transport media that help maintain the viability of viruses during shipment to the laboratory. Specific information about the sampling, collection, storage and submission of specimens for specific aquatic animal viral pathogens is described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2015a), the American Fisheries Society, Fish Health Section Blue Book (AFS-FHS, 2014), the European Union (EU) Diagnostic Manual (EURL-Fish, 2015b), the National Aquatic Animal Health Technical Working Group (NAAH-TWG)—Advisory document (Handlinger, 2008) and Policy document (Cameron, 2004), as well as by Midtlyng et al. (2000).

4.5 NECROPSY, GROSS AND HISTOPATHOLOGY EVALUATION OF FISH

4.5.1 In Vivo Examination

Any aberrant behavior should be noted when the fish swim freely in the water. Blood samples should be taken from live fish during anesthesia or immediately after stunning (Midtlyng et al., 2000).

4.5.1.1 Blood Sampling

Blood samples are preferably taken from the caudal vein using a closed system with ready-to-use blood collection tubes or an aspiration syringe. Depending on the intended use of the blood sample, anticoagulants like heparin, EDTA or tubes without anticoagulants should be used (Midtlyng et al., 2000; Stoskopf, 1993). Blood collected for obtaining serum to test for antibodies should be collected in sterile tubes without anticoagulants.

4.5.2 Necropsy

Necropsy or postmortem examination is an important tool for diagnosing fish diseases. Before embarking on the necropsy, the pathologist should obtain a detailed history and make a clinical assessment (Reimchuessel, 1993). The necropsy is achieved through a systematic approach and observation of external and internal structure of organs and tissues, assisted by the collection of samples for further analysis (Bruno et al., 2013). The number of specimens examined will be determined by the purpose of the procedure, but the principle is that samples must represent the population or the group under study.

For instance, in farmed populations suspected of a pathological condition, typically between 5 and 10 animals displaying the observed problem (clinically affected fish) are examined, alongside a smaller number of nonclinically affected individuals. For pathogen screening, the number of fish is statistically calculated considering the expected or threshold prevalence of the pathogen, the level of confidence desired on the results and the performance of the method or test system applied (sensitivity and specificity) (Noguera et al., 2015).

4.5.2.1 External Examination

A skin and gill biopsy must be performed immediately after the subject's death (Noga, 2011). An external examination is essentially a detailed visual inspection of the entire body to evaluate its general condition, including palpation of integument and fin surfaces and sampling for complementary analyses, when relevant (Noguera et al., 2015). A thorough check of the entire body external surface should follow noting the integrity of the skin and fins, changes of normal pigmentation, excessive mucus, raised or lost scales, erosion, ulcer, hemorrhage, exophthalmia, grossly visible parasites, evidence of the skeletal deformity or muscle atrophy.

4.5.2.2 Internal Examination

To access the internal organs the body cavity is opened. The exposed abdomen with all internal organs should be inspected with regards to color, texture and pathological signs like hemorrhages, edema, granulomas and neoplasms (Bruno et al., 2013; Midtlyng et al., 2000). During necropsy, tissues may be collected and stored for microbiology, parasitology, biochemistry and histopathology, as well as for detection of proteins or nucleic acids (OIE, 2015a).

4.5.2.3 Histopathology

The histopathology evaluation of tissues constitutes an invaluable tool in diagnosis of fish diseases. It yields detailed information on the normal or abnormal constitution and appearance of organ structures and cell types, findings that may be pathognomonic or may indicate the nature of the causative agent. Furthermore, histological samples may represent a unique and durable specimen library and valuable documentations of the case. Histological preparations are often used in research, allowing confirmation or reassessment of diagnoses and screening of historical specimens years or decades later, during which time scientific progress may have opened new investigational opportunities (Midtlyng et al., 2000). Today the histopathological study should be complemented by the digital pathology platform which provides a tool to share cases, get second opinion or consultations between two or more experts using the internet, allowing accurate diagnostics.

The necropsy, gross and histopathology evaluation of bivalve mollusks and crustaceans are detailed in a manual by Howard et al. (2004).

4.6 LABORATORY METHODS USED IN THE DETECTION, ISOLATION, IDENTIFICATION AND QUANTIFICATION OF AQUATIC ANIMAL VIRUSES

Historically, laboratory diagnosis of aquatic animal diseases has relied on histological evaluation of tissues, direct visualization of virus particles by EM and/or virus isolation. Early detection of infection, which is important in preventing disease spread and in limiting losses, has relied on development of rapid and sensitive diagnostic methods. Currently, molecular detection by polymerase chain reaction/reverse transcription polymerase chain reaction (PCR/RT-PCR) is favored over other viral diagnostic methods due to advantages in cost, speed and ease of use. There is now a need for assays that can allow unbiased analysis of viruses in a sample, since differential diagnosis is difficult in early infection before the appearance of clinical signs.

Table 4.2 summarizes the methods used for isolation and propagation in cell culture and/or molecular detection by PCR/RT-PCR (virus-specific primers) of important/common aquatic animal viruses. For various reasons, the traditional virologic, microscopic and serologic methods remain important despite the increasing emphasis on molecular detection assays.

4.6.1 Direct Demonstration of Virions, Antigens or Nucleic Acids (the Rapid Methods)

4.6.1.1 Electron Microscopy

EM with negative staining is the method of choice for rapid identification of virus particles in clinical specimens because of its simplicity, rapidity and high resolution. The American Fisheries Society, Fish Health Section Blue Book has general EM techniques for identification of suspected viruses (Lovy and Wadowska, 2014).

TABLE 4.2 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses

Virus Family	Virus Genus	Virus Species or Isolate[a] in Host			Virus Isolation and Propagation		Conventional PCR/RT-PCR
		Fish	Crustaceans	Mollusks	Cell Lines[b]	Incubation Temperature[c]	Virus-Specific Primers and/or Reference
Circoviridae	Unassigned	BaCV1 & 2			–	–	MTL 5′-GTGATTACGCCAACGTGATG-3′
							MTR 5′-GCCCTTTGTGCAGTATTCGT-3′ (Lőrincz et al., 2011)
	Unassigned	CfCV			–	–	HT-F 5′-CAGACCATGCTTCCGGTACT-3′
							HT-R 5′-GGGCTTCCTCGAAGGTTATC-3′ (Lőrincz et al., 2012)
Parvoviridae	*Penstyldensovirus*		IHHNV		–	–	77012F 5′-ATCGGTGCACTACTCGGA-3′
							77353R 5′-TCGTACTGGCTGTTCATC-3′ (Nunan et al., 2000)
	Hepandensovirus		HPV		–	–	HPV600F 5′-TGTCTAAGCGCCAGTAACCAA-3′
							HPV600R 5′-TATACAGTTACTTGGCATGAC-3′ (La Fauce et al., 2007)
Hepadnaviridae	Unassigned	WSHBV					(Hahn et al., 2015)
Adenoviridae	*Ichtadenovirus*	WSAdV			WSS-2	15–30°C	AdVE2B-F 5′-TCMAAYGCHYTVTAYGGBTCDTTTGC-3′
							AdVE2B-R 5′-CCAYTCHSWSAYRAADGCBCKVGTCCA-3′ (Benkő et al., 2002)
Alloherpesviridae	*Cyprinivirus*	CyHV-3			KF-1; CCB	25–26°C	FP 5′-GGGTTACCTGTACGAG-3′
							RP 5′-CACCCAGTAGATTATGC-3′ (Bercovier et al., 2005)
	Ictalurivirus	CCV			CCO, BB	25–30°C	FP 5′-TCATCCGAATCCGACAACTGA-3′
							RP 5′-CCAAGATCGCGGAGAAAC-3′ (OIE, 2009)
	Salmonivirus	OMHV			RTG-2; CHSE-214	10–15°C	FP (F10) 5′-GTACCGAAACTCCCGAGTC-3′
							RP (R5) 5′-AACTTGAACTACTCCGGGG-3′ (Aso et al., 2001)

Malacoher-pesviridae	*Ostreavirus*			OsHV-1	–	–	A3: 5′-GCCAACCGTTGGAACCATAACAAGCG-3′
							A4: 5′-GGGAATGAGGTGAACGAAACTATAGACC-3′ (Renault et al., 2000)
	Aurivirus			AbHV	–	–	AbHV-16 5′-GGCTCGTTCGGTCGTAGAATG-3′
							AbHV-17 5′-TCAGCGTGTACAGATCCATGTC-3′ (OIE, 2015g)
Unclassified			PaV1		–	–	45aF 5′-TTCCAGCCCAGGTACGTATC-3′
							543aR 5′-AACAGATTTTCCAGCAGCGT-3′ (Montgomery-Fullerton et al., 2007)
Iridoviridae	*Ranavirus*	EHNV			BF-2; EPC; FHM; CHSE-214	15–24°C	FP 5′-CGCAGTCAAGGCCTTGATGT-3′
							RP 5′-AAAGACCCGTTTTGCAGCAAAC-3′ (Hyatt et al., 2000)
	Megalocytivirus	RSIV			GF	25°C	1-F 5′-CTCAAACACTCTGGCTCATC-3′
							1-R 5′-GCACCAACACATCTCCTATC-3′ (Kurita et al., 1998)
	Lymphocystivirus	LCDV			SAF-1[d]	20°C	F 5′-TTTGAATGGGAGGATCAC-3′
							R 5′-TCCGTAAATGCTGTTAGC-3′ (Kvitt et al., 2008)
Poxviridae	Unassigned	CEV					(Oyamatsu et al., 1997)
		SPGV					(Gjessing et al., 2015)
Nudiviridae	Unassigned		PmNV		–	–	261F 5′-AATCCTAGGCGATCTTACCA-3′
							261R 5′-CGTTCGTTGATGAACATCTC-3′ (Surachetpong et al., 2005)
Nimaviridae	*Whispovirus*		WSSV		–	–	146F1 5′-ACTACTAACTTCAGCCTATCTAG-3′
							146R1 5′-TAATGCGGGTGTAATGTTCTTACGA-3′ (Lo et al., 1996)
Picornaviridae	*Piscevirus*	EPV-1			EK-1	26°C	
		BGPV			BF-2	15–25°C	FP 5′-CTCGATAGTGTATGACTCGGACC-3′
							RP 5′-CATGGGGTTCAACACTCACA-3′ (Barbknecht et al., 2014)
		FHMPV			EPC, FHM, CHSE-214, BF-2, RTG-2	15–22°C	
	Limnipivirus	CPV-1			FHM		

(*Continued*)

TABLE 4.2 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses (Continued)

Virus Family	Virus Genus	Virus Species or Isolate[a] in Host			Virus Isolation and Propagation		Conventional PCR/RT-PCR
		Fish	Crustaceans	Mollusks	Cell Lines[b]	Incubation Temperature[c]	Virus-Specific Primers and/or Reference
Dicistroviridae	*Aparavirus*		TSV		–	–	9992F 5′-AAGTGAACAGCCGCGCTT-3′
							9195R 5′-TCAATGAGAGCTTGGTCC-3′ (Nunan et al., 1998)
Caliciviridae	*Salovirus*	ASCV			GF-1	15°C	(Mikalsen et al., 2014)
Hepeviridae	*Piscihepevirus*	CTV			CHSE-214	15°C	(+) primer 5′-ACTGTTACACCTCATGTAGC-3′
							(–) primer 5′-GACTTTACTAGCAGTGTGGAT-3′ (Batts et al., 2011)
Togaviridae	*Alphavirus*	SAV			CHSE-214, BF-2, FHM, SHK-1, EPC, CHH-1	15°C	E2F: 5′-CCG–TTG–CGG–CCA–CAC–TGG–ATG-3′
							E2R: 5′-CCT–CAT–AGG–TGA–TCG–ACG–GCA–G-3′ (Fringuelli et al., 2008)
Roniviridae	*Okavirus*			YHV	–	–	YC-F1ab pool: 5′-ATCGTCGTCAGCTACCGCAATACTGC-3′
				GAV			5′-ATCGTCGTCAGYTAYCGTAACACCGC-3′
							YC-R1ab pool: 5′-TCTTCRCGTGTGAACACYTTCTTRGC-3′
							5′-TCTGCGTGGGTGAACACCTTCTTGGC-3′
							(Wijegoonawardane et al., 2008)
Coronaviridae	*Bafinivirus*	FHMNV			FHM	15°C	Sense primer 5′-TTTTGTTGAATTTATAGCTCTT-3′
							Antisense primer 5′-TGGCCATATCCTTAAGGG-3′ (Batts et al., 2012)
Retroviridae	*Epsilonretrovirus*	WDSV			–	–	Primer 3: 5′-TGAAGCAGGAATACCTACCT-3′
							Primer 4: 5′-CTGTAAGTCCGTTCTCTTGT-3′ (Poulet et al., 1996)
Paramyxoviridae	*Aquarespirovirus*	PSPV			CHSE-214	20°C	Sense primer 5′-GCAGAGATATTTTCCTTCTT-3′
							Antisense primer 5′-TGTTTGATTTCCTTCTCCTT-3′ (Batts et al., 2008)

Rhabdoviridae	*Novirhabdovirus*	VHSV			BF-2, EPC, FHM	15°C	VN Forward 5′-ATGGAAGGAGGAATTCGTGAAGCG-3′
							VN reverse 5′-GCGGTGAAGTGCTGCAGTTCCC-3′ (Snow et al., 2004)
		IHNV			EPC, FHM	15°C	Upstream Primer 5′-AGAGATCCCTACACCAGAGAC-3′
							Downstream Primer 5′-GGTGGTGTTGTTTCCGTGCAA-3′ (Emmenegger et al., 2000)
		SHRV			EPC, ZF4	28°C	Sense primer 5′-ATTTATCCGCTGGAGAGGGATTGG-3′
							Antisense primer 5′-GTTGAGCCCATAGGCCTTGAAGTA-3′ (Phelan et al., 2005)
		HIRRV			BF-2, EPC, FHM, RTG	15°C	oPVP278 5′-ACTACAATCAACAAATCGCA-3′
							oPVP279 5′-GTTGGCGAGTGGGATGTTG-3′ (Borzym et al., 2014)
	Perhabdovirus	PerRV			BF-2, EPC	16°C	oPVP116 5′-ACWTGTGAYTWCMGWTGGTATGG-3′
							oPVP118 5′-CTGTTAGCTGTTTTTTTCATA-3′;
							oPVP126 5′-GATATGAAAAAAACTGCAACAG-3′
							Rha-G-seqR2 5′-GAGGAGTCCTCTATGTTGGTC-3′ (Talbi et al., 2011; Gadd et al., 2013)
	Sprivivirus	SVCV			EPC, FHM	20°C	F1 5′-TCTTGGAGCCAAATAGCTCARRTC-3′
							R2 5′-AGATGGTATGGACCCCAATACATHACNCAY-3′ (Stone et al., 2003)
Bunyaviridae	*Phlebovirus*[e]			MoV	–	–	MoV24F 5′-GGGATGGTGTTGCCATACAAAGG-3′
							MoV25R 5′-GTCATTAGCTGGTCTTAGTTTTCAC-3′ (Cowley et al., 2005)
Orthomyxoviridae	*Isavirus*	ISAV			SHK-1, ASK	15°C	HPR Fwd 5′-GCCCAGACATTGACTGGAGTAG-3′
							HPR Rev 5′-AGACAGGTTCGATGGTGGAA-3′ (Kibenge et al., 2009)
	Unassigned	TiLV			E-11	25°C	Nested ext-1 5′-TATGCAGTACTTTCCCTGCC-3′
							Nested ext-2 5′-TTGCTCTGAGCAAGAGTACC-3′
							ME1 5′GTTGGGCACAAGGCATCCTA-3′ CLONE 7450/150R/ME2 5′-TATCACGTGCGTACTCGTTCAGT-3′ (Eyngor et al., 2014)

(*Continued*)

TABLE 4.2 Virus Isolation and Propagation and/or Molecular Detection by PCR/RT-PCR of Important/Common Aquatic Animal Viruses (Continued)

Virus Family	Virus Genus	Virus Species or Isolate[a] in Host			Virus Isolation and Propagation		Conventional PCR/RT-PCR
		Fish	Crustaceans	Mollusks	Cell Lines[b]	Incubation Temperature[c]	Virus-Specific Primers and/or Reference
Reoviridae	*Aquareovirus*	"All species"			CHSE-214, EPC	22°C	GARV-F 5′-TAAAGCTTGCGACGCCTCCATCAC-3′
							GARV-R 5′-TGCTCGGTGGAGGTGACAGT-3′ (Zainathan et al., 2015)
	Orthoreovirus	PRV			GF-1	15°C	PRV-S1-F1 5′- GATAAAGACTTCTGTACGTGAAAC-3′
							PRV-S1-R1 5′- GATGAATAAGACCTCCTTCC-3′ (Kibenge et al., 2013)
	Cardoreovirus			BCRV	–	–	BCRVFor 5′-TCAGTGTCTTCAGCTTTAGGTTG-3′
							BCRVRev 5′- TCTCTTGAGGCCTAGATTCG-3′ (Bowers et al., 2010)
Birnaviridae	*Aquabirnavirus*	IPNV			CHSE-214	15°C	P1 5′-GGTCAACAACCAACTAGTGACC-3′
							P2 5′-GTTGGGATTGACTGCGTAAAC-3′ (Lopez-Lastra et al., 1994)
				TV-1	BF-2		(Nobiron et al., 2008)
Nodaviridae	*Betanodavirus*	SJNNV			SSN-1, E-11, GF-1	20–25°C	VNNV1 5′-ACACTGGAGTTTGAAATTCA-3′
			*Mr*NV				VNNV2 5′- GTCTTGTTGAAGTTGTCCCA-3′ (Dalla Valle et al., 2000)
Totiviridae	*Giardiavirus*	PMCV					(Haugland et al., 2011)
			IMNV		–	–	4587F 5′-CGACGCTGCTAACCATACAA-3′
							4914R 5′-ACTCGGCTGTTCGATCAAGT-3′ (Poulos and Lightner, 2006)

[a]*Virus abbreviations: BaCV1 & 2, Barbel circovirus 1 and 2; CfCV, European catfish circovirus; IHHNV, Infectious hypodermal and hematopoietic necrosis virus; HPV, Hepatopancreatic parvovirus; WSHBV, White sucker hepatitis B virus; WSAdV, White sturgeon adenovirus; CyHV-3, Cyprinid herpesvirus 3 (Koi herpesvirus); CCV, Channel catfish virus (Ictalurivirus herpesvirus 1); OMHV, Oncorhynchus masou herpesvirus; OsHV-1, Ostreid herpesvirus 1 (Oyster herpesvirus); AbHV, Abalone herpesvirus (Haliotid herpesvirus 1); PaV1, Panulirus argus virus 1; EHNV, Epizootic hematopoietic necrosis virus; RSIV, Red sea bream iridovirus; LCDV, Lymphocystis disease virus; CEV, Carp edema virus; SGPV, Salmon gill poxvirus; WSSV, White spot syndrome virus; EPV-1, Eel picornavirus 1; BGPV, Blue gill picornavirus; FHMPV, Fathead minnow picornavirus; CPV-1, Carp picornavirus 1; TSV, Taura syndrome virus; ASCV, Atlantic salmon calicivirus; CTV, Cutthroat trout virus; SAV, Salmonid alphavirus; YHV, Yellow head disease virus; GAV, Gill-associated virus; WDSV, Walleye dermal sarcoma virus; ASPV, Atlantic salmon paramyxovirus; PSPV, Pacific salmon paramyxovirus; FHMNV, Fathead minnow nidovirus; VHSV, Viral hemorrhagic septicemia virus; IHNV, Infectious hematopoietic necrosis virus; SHRV, Snakehead rhabdovirus; HIRRV, Hirame rhabdovirus; PerRV, Perch rhabdovirus; SVCV, Spring viraemia of carp virus; MoV, Mourilyan virus; ISAV, Infectious salmon anemia virus; TiLV, Tilapia lake virus; PRV, Piscine orthoreovirus; BCRV, Blue crab reovirus; IPNV, Infectious pancreatic necrosis virus; TV-1, Tellina virus 1; BSNV, Blotched snakehead virus; SJNNV, Striped jack nervous necrosis virus; MrNV, Macrobrachium rosenbergii nodavirus; PMCV, Piscine myocarditis virus; IMNV, Infectious myonecrosis virus.*

[b]*Cell line abbreviations: WSS-2, White sturgeon spleen; KF-1, Koi fin; CCB, Common carp brain; CCO, Channel catfish ovary; BB, Brown bullhead; RTG-2, Rainbow trout gonads; CHSE-214, Chinook salmon embryo; BF-2, Blue gill fry; EPC, Epithelioma papulosum cyprini; FHM, Fathead minnow; GF, Grunt fin; SAF-1, Gilthead seabream fibroblasts; EK-1, eel embryonic kidney; GF-1, Grouper fin; SHK-1, Salmon head kidney; CHH-1, Chum salmon heart; ZF4, zebrafish embryo fibroblast; ASK, Atlantic salmon kidney; E-11, cloned from SSN-1 cell line; SSN-1, striped snakehead.*

[c]*Fish viruses are typically isolated on cultured fish cell lines at 15–25°C.*

[d]*LCDV grows in SAF-1 cell line; CPE consisted of the rounding and enlargement of infected cells and the formation of cytoplasmic inclusions. The yields of isolated viruses ranged from 1×10^4 $TCID_{50}$/mL to $>1 \times 10^9$ $TCID_{50}$/mL (Garcia-Rosado, E., Castro, D., Cano, I., Alonso, M.C., Pérez-Prieto, S. L., Borrego, J. J., 2004. Protein and glycoprotein content of Lymphocystis disease virus (LCDV). Int. Microbiol. 7, 121–126).*

[e]*Phlebovirus is the tentative genus for Mourilyan virus (Cowley, J.A., McCulloch, R.J., Rajendran, K.V., Cadogan, L.C., Spann, K.M., Walker, P.J., 2005. RT-nested PCR detection of Mourilyan virus in Australian Penaeus monodon and its tissue distribution in healthy and moribund prawns. Dis. Aquatic Organisms 66, 91–104).*

Negative staining of virus particles for EM is not true staining, but instead, involves the deposition of an electron-dense material, such as potassium phosphotungstate, around the surface and is also able to penetrate between small surface projections of the viral structure so as to provide contrast. Use is made of transmission electron microscopy, and only electrons that pass through the specimen are involved in the formation of the final image. The technique was first applied in virology by Brenner and Horne (1959). Briefly, the virus suspension is placed on an electron microscope grid (coated with Formvar, Collodion, Butvar or Pioloform film stabilized with carbon coat). For staining, the grid is placed upside down on a drop of 1–2% aqueous heavy metal salt solution, such as sodium or potassium salt of phosphotungstic acid (at pH 6–7). The mixture forms a thin film. The specimen is dried and irradiated by ultraviolet light to inactivate the virus. The grid is then ready to be examined. When viewed, the virus appears as light (electron lucent) particles with dark (electron dense) background. The salt has the ability to enter even the finest crevices on the surface of the virus particle, and the surface features stand out. Unlike in histological sections for light microscopy, here the virus particles themselves are not stained, rather the increased contrast is provided by the darkening of the background, hence the term "negative staining". Only electrons that pass through the specimen are involved in the formation of the final image. The resulting image is a light virus particle against a dark background. The inner part of a virus can be dark if the envelope of an enveloped virus is damaged/broken or in the case of naked viruses if the virus particle contains no genome (ie, empty particle), allowing for leakage of the salt inside the particle.

EM is a good rapid diagnostic method, particularly for specimens containing a large number of virus particles, such as vesicular fluid, fecal extracts and serum (Goldsmith and Miller, 2009). In such samples, one can obtain a rapid diagnosis within an hour (or 2 hours in case of samples with low virus yield (eg, respiratory mucus) requiring other methods of virus concentration). Using specific antiserum to aggregate virions enhances the sensitivity and accuracy of diagnosis by EM. This technique is referred to as *immunoelectron microscopy (IEM)*. Other methods routinely used for enhancing virus visualization in clinical specimens are ultracentrifugation and agar gel diffusion (Fong, 1989). Thin sectioning with positive staining can be used for cells and tissues. The main limitation of virus diagnosis by thin sectioning is that if the infection is focal, the sampling might miss the area containing viruses (Goldsmith and Miller, 2009).

The morphology of a virus particle is the primary criterion for virus classification (see Chapter 2: Classification and Identification of Aquatic Animal Viruses). Virus morphology includes the size and shape of the virion, number of capsomers, symmetry of capsid and presence or absence of envelope, all of which can be determined using EM. The morphology of most viruses is sufficiently characteristic to assign an "unknown" virus to the correct family (Anonymous 1, 1995), and from there to identify it specifically. For example a viral isolate from the kidney tissue of a fish, identified by negative staining electron microscopy as a birnavirus, might be submitted immediately for serological identification and would be identifiable serologically as infectious pancreatic necrosis virus (IPNV) serotype 1–12 or may be a new aquabirnavirus of fish. However, viral diagnosis by EM has lost its importance due to more rapid methods, such as PCR-based tests. Some of the advantages and disadvantages of EM as a method of virus detection (Goldsmith and Miller, 2009) are listed in Table 4.3.

TABLE 4.3 Electron Microscopy for Virus Detection

Advantages	Disadvantages
1. Rapid, simple and fairly accurate identification of "unknown" virus based on morphology—allowing classification into virus family; will identify isolates even with unusual biological or antigenic properties, and new viruses whose antigenic properties are presently unknown	1. Cannot differentiate between viruses of the same family; eg, fish rhabdoviruses VHSV, IHNV, SVCV, PerRV, HIRRV
2. Can work in mixed infections	2. Has limited sensitivity. Needs 10^6 particles/mL to see virus in 15 minutes. Therefore, a negative EM result is inconclusive
3. Detects both infectious and noninfectious virus particles	3. Too time consuming for screening several samples, and is expensive
4. Can provide direct evidence of presence of virus in target tissues, allowing understanding of viral pathogenesis	4. Needs specialized equipment and trained personnel
	5. Needs good quality specimens. This is true for all diagnostic tests

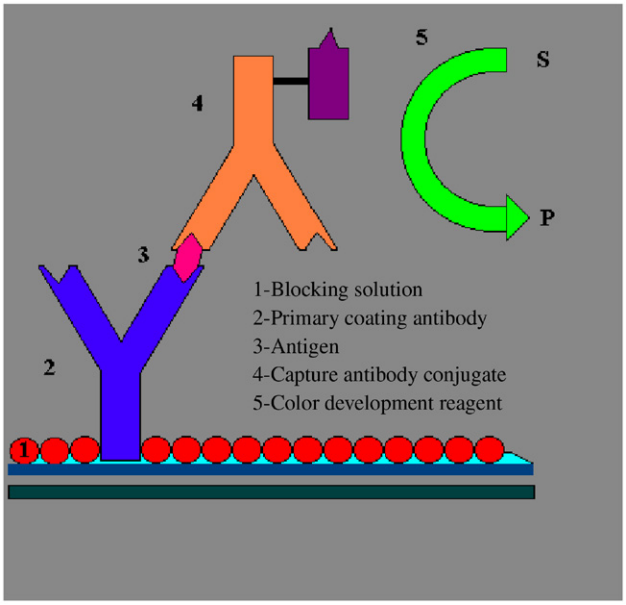

FIGURE 4.2 Basic format for a sandwich ELISA for detection of virus antigen.

4.6.1.2 Serological Procedures

Enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID), fluorescent antibody testing (FAT) and indirect FAT (IFAT), immunochromatography and hemagglutination/hemagglutination-inhibition (HA/HI) tests are rapid serological assays that can be used to detect either virus antigen or virus-specific antibodies (Fig. 4.1). Virus neutralization (VN) or serum neutralization (SN) specifically detects virus-specific antibody and is discussed separately in Section 4.7 on serology.

4.6.1.2.1 ELISA for the Detection of Virus Antigen or Virus Antibodies

This technique is based on the principle that an antibody molecule can be covalently linked with an enzyme to form a conjugate which retains both immunologic and enzymatic function. This assay most often uses alkaline phosphatase (AP), horseradish peroxidase (HRP) or β-galactosidase conjugated to an antibody that when supplied with the proper colorless chromogenic substrate (p-nitrophenyl phosphate for alkaline phosphatase, o-phenylenediamine for peroxidase and lactose for β-galactosidase) results in a strongly colored compound after degradation, and thus a very sensitive detection system. The antibody–enzyme conjugate can be bound to specific virus antigen and specific virus antibody in a clinical specimen by a variety of steps (Fig. 4.2) and allowed to react with a substrate to produce a color that can be assessed visually (as in kits for on-site detection of viral infections) or with greater sensitivity by measuring optical density (OD) using an ELISA reader machine. The ELISA for antigen detection is referred to as *direct ELISA* or if the solid phase is first coated with antibody to immobilize the antigen in the test sample as *sandwich ELISA* whereas the one for antibody detection is called *indirect ELISA*. The intensity of the color reaction is usually proportional to the amount of virus antigen (when testing for antigen) or to the amount of antibody (when testing for antibody) except for the *prozone effect*. Prozone effect is when a negative result is obtained in antibody excess (ie, in concentrated serum) due to the presence of blocking antibody. A positive result is obtained in diluted serum. The prozone effect error can be avoided by using serial dilutions of serum. Some of the advantages and disadvantages of ELISA as a method of virus antigen detection are listed in Table 4.4.

In the indirect ELISA (for the detection of virus-specific antibody), the viral antigen is first adsorbed to a solid phase. Serum samples are then tested for presence of virus-specific antibody, the binding of which is detected with enzyme-conjugated anti-species IgG followed by addition of enzyme substrate (Kibenge et al., 2002). A variation of this is *competitive ELISA*, where the enzyme-conjugated anti-species IgG is replaced by enzyme-conjugated antiviral IgG such that the color development after addition of enzyme substrate is inversely proportional to the level of antibody present in the serum sample.

There are several ELISA kits available commercially for diagnosis of viral infections; most are essentially for the small animal veterinarian but some for aquatic animal pathogens are also beginning to appear on the market. These kits can be for detection of either viral antigen or viral antibody (Table 4.1).

TABLE 4.4 Antigen ELISA for Virus Detection

Advantages	Disadvantages
1. Rapid	1. Narrow specificity—ie, only a particular viral antigen is identified, and viruses which share this antigen or that were used to raise antiserum
2. Very sensitive. 10^2 virus particles may be detectable. Therefore, a positive ELISA result is conclusive for that particular antigen	2. Cannot be used if antigenic properties are unusual or nonclassical
3. Good for screening several samples	3. Does not provide virus for culture/further study
4. Cheap	4. Kits must be used within a specified time frame before they expire
5. Can be used even in vaccinated animals (with inactivated vaccines)	5. Not available for all viral diseases
6. Can be performed by the clinician (therefore convenient)	
7. Can be semiquantitative	

4.6.1.2.2 AGID for the Detection of Virus Antigen or Virus Antibodies

The basis for the immunodiffusion test is concurrent migration of antigen and antibody towards each other through an agar or agarose gel. As the antigen and antibody come in contact, they combine to form a precipitate that is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentrations of antigen and antibody are optimum. An extreme variation in the concentration of antigen or antibody will alter the location of the precipitin line or cause it to be dissolved. Electrolyte concentration, buffer, pH and temperature also affect precipitate formation. An increase in the incubation temperature allows for more complete reactions. However, sharper and more distinct precipitin lines occur at lower temperatures. Temperature changes during migration may lead to artifacts.

Immunodiffusion tests are simple, economical and rapid tests (with results in 24–72 hours). The disadvantage is that they are relatively insensitive and are best for the detection of either viral antigens or antibodies in persistent viral diseases in which viral antigens constantly are present. Interpretation of immunodiffusion tests is often hindered by nonspecific reactions and lack of quantitation. Diagnosis is based on the result of a single sample (no seroconversion can be detected by this method because of the lack of quantification). It is recommended that all samples that have positive reactions be retested to confirm the validity of the test. All weak positive samples should be retested before reporting the results. A weak positive reaction may occur if there has been seepage of positive control serum under the agar layer into a negative test serum well. A retest should identify this error. This test is more commonly used in terrestrial animal viral diseases such as equine infectious anemia virus (EIAV) with the Coggins test (Pearson et al., 1971), bovine leukemia virus (BLV) and ovine progressive pneumonia (Maedi/Visna), all lifelong infections caused by retroviruses.

4.6.1.2.3 Immunochromatography

Immunochromatography also known as *lateral flow immunoassay* or *strip testing* is a variation of ELISA in which the antibody or antigen reagent flows laterally through a membrane (typically a hydrophobic nitrocellulose or cellulose acetate membrane) and localizes at a site where the corresponding antigen or antibody is bound on the membrane. The technology was made famous for its application in the home pregnancy test (Leuvering et al., 1980) and is now widely used in diagnostic kits including those for detection of aquatic animal viral infections both on-site in the field (point-of-care) and in the laboratory either directly on submitted specimens or to confirm isolated virus (Table 4.1). The research and application of the lateral flow assay technology including its advantages and pitfalls and future developments have been widely reviewed (Ngom et al., 2010; Sajid et al., 2015).

4.6.1.2.4 FAT and IFAT

FAT detects viral antigens in cytological preparations (blood smears, tissue imprints, scrapings, cell cultures) and tissue sections with specific viral antibody labeled with a fluorescent dye (fluorescein isothiocyanate or FITC). This is also referred to as direct FA (DFA). It is one of the most widely used viral antigen/antibody methods for routine viral diagnosis (Wise et al., 2005); the other one is ELISA. If the antibody is conjugated to an enzyme (horseradish peroxidase or alkaline phosphatase) instead of FITC, the procedure then becomes immunohistochemistry (IHC) (refer to Section 4.6.1.3).

An indirect FAT uses two antibodies in a two-step procedure in which the specimen is first reacted with an unlabeled antibody specific for the viral antigen; after incubation, the antibody is washed off and the FITC-labeled antibody is applied. This second antibody that is labeled is directed against the IgG of the animal species in which the unlabeled virus-specific antibody was prepared. Thus one labeled second antibody can be used to detect binding of different unlabeled antibodies (commonly monoclonal antibodies) specific for different viral antigens in different preparations. The existence of a large number of constantly evolving viral serotypes can render IFAT nearly impossible for certain viruses.

4.6.1.2.4.1 ***Immunofluorescence Assay*** The FAT used to detect and measure virus-specific antibody is called the *immunofluorescence assay*. It is formatted like the IFAT except that it uses cell cultures infected with the known virus as the source of viral antigens. Serum samples are then tested for the presence of virus-specific antibodies, the binding of which is detected with the FITC-conjugated anti-species IgG.

4.6.1.2.5 Hemagglutination/Hemagglutination-Inhibition

HA involves the agglutination of erythrocytes by binding to virus particles or viral proteins. Many animal viruses have the ability to bind to the surface structures on red blood cells of different animal species resulting in agglutination. The classical examples of hemagglutinating viruses are the members of the virus family *Orthomyxoviridae* such as the avian and mammalian influenza viruses and infectious salmon anemia virus (ISAV) with the hemagglutinin protein as part of the viral envelope. The HA assay is carried out in a 96-well microtiter plate with round-bottom wells, and is very useful for assaying noncytopathic virus isolates in cell culture and also for detecting viruses directly in clinical specimens. The HA test is quantitative; 1 hemagglutinating unit (HAU) equals the highest dilution of the viral sample giving complete HA. InDevR markets an automated hemagglutination analyzer, Cypher One (Anonymous 2, 2015).

In HI, antibodies to the virus in the serum sample bind to the virus and block the binding sites on the viral envelope or capsid thereby preventing the virus from agglutinating the red blood cells. In an antibody positive serum, the red blood cells settle out in "button," whereas in a serum sample without the specific viral antibody, the red blood cells form a "mat." A standard number of HAU (4–8 HAU), depending on the virus to be detected, is used in the HI test.

If erythrocytes are first coated with a soluble viral antigen, the coated red blood cells can be used in a *passive hemagglutination* test either for the measurement of the viral antigen (through competition with the antigen coated on the red blood cells) or for antibody to the soluble antigen.

4.6.1.3 Immunohistochemistry/Immunoperoxidase Staining

IHC involves the detection of a viral antigen in tissue by a specific antibody (typically monoclonal) conjugated to an enzyme (horseradish peroxidase or alkaline phosphatase) that reacts with a substrate to produce a color similarly to ELISA. Proper reading of IHC requires that the test include a negative control of the same tissue because background tends to differ among different tissue types (Nuovo, 2006) due to the presence of endogenous peroxidase in the cells of many tissues. Similarly to FAT, localization of antigen in specific cells adds to surety of diagnosis.

4.6.1.4 Nucleic Acid-Based Tests

All viruses contain either DNA or RNA, and are thus amenable to nucleic acid-based detection. However, viruses lack correlates of the universally conserved 16S ribosomal ribonucleic acid (rRNA) for prokaryotes (bacteria) and 18S rRNA for eukaryotes (fungi) that facilitate unbiased detection and phylogenetic characterization through PCR amplification (Kapoor and Lipkin, 2001; Edwards and Rohwer, 2005). Moreover, virus genomes easily generate mutants, strains and variants (refer to Chapter 6: Determinants of Emergence of Viral Diseases in Aquaculture, eg, of mutations in aquatic animal viruses). Virus variants differing in sequence, even by a single nucleotide, can vary in host range, transmissibility and pathogenicity (Guillot et al., 1994), making it difficult to have a universal pan-viral nucleic acid-based assay to precisely diagnose infections. Therefore, a staged strategy for pathogen detection has been advocated (Quan et al., 2008): singleplex PCR/RT-PCR testing is good in a characteristic disease outbreak or in situations suggestive of infection with one known pathogen but is not ideal in the absence of clinical signs; multiplex PCR testing alleviates the problem by reducing bias and allowing simultaneous detection of a wide range of candidate viral and bacterial pathogens that may act alone or in concert and is good where the differential diagnosis list contains fewer than 30 agents and/or clinical signs are not pathognomonic of infection; microarrays are good where multiplex PCR testing has failed or cannot be set up for some reason and they are well suited to pathogen detection in animal populations likely to have large numbers of samples and pathogens; and NGS and viral metagenomics are useful where other methods have failed—they are completely unbiased and therefore ideal for surveillance and for pathogen discovery (Fig. 4.3).

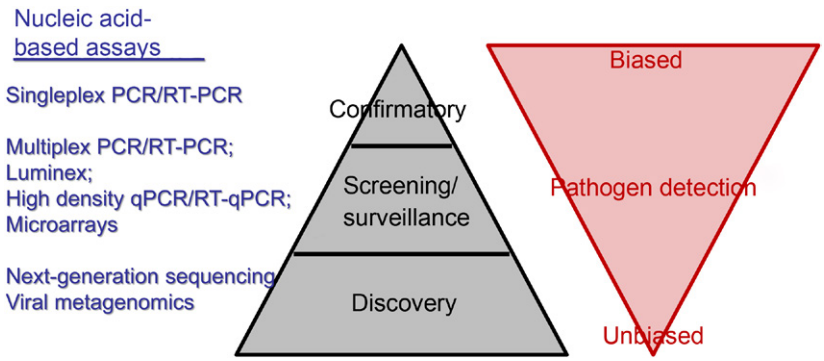

FIGURE 4.3 Nature of nucleic acid-based laboratory diagnostic tests. Singleplex PCR/RT-PCR is not ideal in the absence of clinical signs. It is based on a known sequence; multiplex PCR/RT-PCR alleviates the problem by reducing bias and allowing simultaneous detection of multiple pathogens and/or subtypes. Next-generation sequencing (NGS) allows unbiased detection; it is therefore ideal for pathogen discovery. *Modified from* Kibenge, F.S.B., Godoy, M.G., Kibenge, M.J.T., 2012. Diagnostic methods for aquatic animal diseases: global issues and trends. In: Proceedings of the OIE Global Conference on Aquatic Animal Health Aquatic Animal Health Programmes: their benefits for global food security 28–30 June 2011, Panama City (Panama), pp. 59–67. Accessible at: http://www.oie.int/doc/ged/d12238.pdf.

4.6.1.4.1 PCR and RT-PCR

Pathogen detection using nucleic acid-based assays is a cornerstone of any modern animal disease (veterinary) diagnostic laboratory. PCR is a simple and powerful method for enzymatic amplification of DNA in vitro using a thermocycler. The technique consists of repetitive cycling of three simple reactions (denaturation, annealing and extension) in presence of thermostable DNA polymerase and a pair of specific oligonucleotide primers for selective amplification of small amounts of target dsDNA present in a complex sample, providing the most sensitive method for detecting nucleic acids. For detection of pathogen transcripts or of viruses with an RNA genome (RNA viruses), amplification by PCR requires that the RNA first be copied by reverse transcriptase enzyme to cDNA and then used in PCR; this technique is referred to as reverse transcription-PCR (RT-PCR). Multiplex PCR assays are more difficult to establish than singleplex PCR/RT-PCR because primer sets may differ in optimal reaction conditions, and complex primer mixtures are more likely to result in primer-primer interactions that reduce assay sensitivity and/or specificity (Lipkin and Briese, 2014). Greene SCPrimer software was developed to design multiplex PCR primer panels for detection and differentiation of viral pathogens (Jabado et al., 2006).

There is now a growing and necessary trend among veterinary diagnostic laboratories to complement and in some cases, replace the classical PCR assays with real-time or quantitative (q) PCR and RT-qPCR because of the very high sensitivity and rapid turnaround time of the test. Moreover, because qPCR assays use a close-tube format, there is reduced risk of cross-contamination. These nucleic acid platforms rely only on in vitro amplification of the pathogen transcripts (ie, are culture-independent) and can be performed in readily available BSL-2 facilities, even for highly pathogenic agents. Such technologies offer great potential for improved specificity, sensitivity and speed of pathogen detection and, therefore, great effort should be focused on optimizing these technologies for routine use (Kibenge et al., 2012). qPCR/RT-qPCR is a mature technology that is ready for prime time in aquatic animal diagnostic virology, and most aquatic animal diagnostic laboratories worldwide now use this technology.

4.6.1.4.1.1 ***Principle of Real-Time PCR and RT-PCR*** Although PCR is usually considered a repetitive process where three reactions (denaturation, annealing, and extension) occur at three different temperatures for three times during each cycle, the temperatures do not change instantaneously but occur as smooth transitions; that is, it is kinetic. Thus, in real-time PCR, there is no need to hold denaturation and annealing temperatures (times often reduced to zero); temperature is always changing; denaturation, annealing and extension occur at different rates depending on temperature; and multiple reactions may occur simultaneously. The real-time PCR/RT-PCR (qPCR/RT-qPCR) technique uses fluorescent reporter dyes (fluorophores) to combine DNA amplification and detection steps in a single tube or well format. In qPCR/RT-qPCR, the accumulation of PCR product (amplicon) is monitored continuously (ie, in real time) by measuring the fluorescence signal of a reporter using various chemistries (Kubista et al., 2006) during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. This allows the assay to reliably quantify the DNA or RNA content in a given sample. The increase in fluorescent signal recorded during the assay is proportional to the amount of DNA synthesized during each amplification cycle. Individual reactions are characterized by the cycle fraction at which fluorescence first rises above a defined background fluorescence, a parameter known as the *threshold cycle* (C_t)

or *crossing point* (C_p). The lower the C_t, the more abundant the initial target. The technique permits accurate quantification of target molecules over a wide dynamic range, while retaining sensitivity and specificity of conventional endpoint PCR assays. Use of such a highly sensitive method for detecting pathogen nucleic acids allows detection of pathogens before appearance of clinical signs and in the absence of clinical disease.

The TaqMan assay, which uses a probe usually 25–35 nucleotides long containing a fluorescent reporter dye (eg, 6-FAM) at the 5′-end and a quencher dye (eg, TAMRA) at the 3′-end, is the most commonly used qPCR/RT-qPCR technique. Probes shorter than 20 nucleotides long are designed as Minor Groove Binder (MGB) probes. A further step in the evolution of TaqMan chemistry is the use of probes that consist of locked nucleic acid (LNA) oligonucleotides, 7–15 mers in length, to provide superior hybridization characteristics and enhanced biostability compared with conventional DNA oligonucleotides (Ugozzooli et al., 2004). Moreover, some commonly used quenchers such as DABCYL and TAMRA suffer from a number of drawbacks, including poor spectral overlap between the fluorescent dye and quencher molecule (DABCYL) or inherent fluorescence of the quencher (TAMRA), resulting in a relatively poor signal-to-noise ratio. Black hole quencher (BHQ) molecules have been developed to overcome these drawbacks. TaqMan assays are specific, highly reliable and applicable to a wide range of diagnostic technology, including multiplex PCR and array technology that allow development of assays to detect multiple pathogens or pathogen subtypes in a single sample (by allowing for amplification of various targets in the same real-time PCR machine); TaqMan assays are highly amenable to quality assurance/quality control and are highly repeatable and reproducible, key requirements for diagnostic laboratories. Minimum information for publication of quantitative real-time PCR experiments (MIQE) and MIQE précis standards (Bustin et al., 2010) have been described for "best practice" of fluorescence-based qPCR, which facilitate the design of de novo assays and assure reproducibility of the results (Huggett and Bustin, 2011).

With the application of both robotic technology and miniaturized technology to routine molecular biology techniques, future trends indicate further development into pathogen (genomic pattern) high-density qPCR arrays (Jungkind, 2001) such as the OpenArray platform (Brenan and Morrison, 2005), and nucleic acid microarrays (Boonham et al., 2003) and microfluidics digital PCR (dPCR) arrays (Sanders et al., 2011). The OpenArray real-time qPCR, which combines the parallelism of microarrays with the quantification capabilities, sensitivity and specificity of qPCR, can run 3000 PCR tests on one sample or as few as 64 PCR tests against 48 samples. It is therefore possible to test for multiple pathogens simultaneously, making the array techniques ideal for aquatic animal disease diagnosis and surveillance. In dPCR, the sample is diluted to achieve approximately 0 or ≥1 copy of target nucleic acid per reaction chamber prior to PCR (Vogelstein and Kinzler, 1999). This not only dilutes out background signal, increasing the signal-to-noise ratio of low-abundance targets, but also provides accurate absolute quantitation of the target nucleic acid present at low levels (Sanders et al., 2011) without depending on the number of amplification cycles, and the technique is routinely used for clonal amplification of samples for deep sequencing (White et al., 2009). dPCR can be performed in standard 96- or 384-well plates, but this is labor-intensive and prone to pipetting errors. Alternative approaches to the multiwell plate method include the microfluidic dPCR array chip. The unit uses a microfluidics sample handling system to split one sample into hundreds of individual reaction chambers that reside on an "integrated fluidic circuit" or chip, which allows performance of close to 10,000 PCRs per chip (Sanders et al., 2011).

4.6.1.4.2 Non-PCR-based Nucleic Acid Amplification

Non-PCR-based nucleic acid amplification is also referred to as *isothermal amplification*. This technique does not require the temperature cycling characteristic of PCR, eliminating the need for a thermocycler. This makes these techniques extremely attractive in poorly resourced laboratories and in point-of-care settings (Table 4.1). Two DNA amplification strategies are: (1) loop-mediated isothermal amplification (LAMP), which uses 4–6 primers and autocycling strand displacement DNA synthesis with Bst DNA polymerase; the enzyme initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification. The 4–6 primers recognize a total of 6–8 distinct sequences on the target DNA, making the amplification highly specific (Notomi et al., 2000). LAMP provides high sensitivity (fg levels or <10 copies of target), and reactions can be performed in as little as 5–10 min. LAMP can also be used to detect RNA either by adding a reverse transcriptase (RT) to the LAMP reaction or by using a DNA polymerase with RT activity, for example, *Bst* 3.0 DNA polymerase (New England BioLabs). Amplified DNA can be visualized: (a) with naked eye (due to precipitation of Mg pyrophosphate); (b) real-time with SYBR green; or (c) by gel electrophoresis. (2) Strand displacement amplification (SDA) (Walker et al., 1992), which also uses four primers, two of which have *Hinc* II recognition sites; DNA synthesis is by Klenow fragment in presence of dATPαS at 37°C, which allows enzyme to create a single-stranded nick in the primer region and the nicked strands are displaced by newly synthesized DNA strands. SDA products are detected by denaturing gel electrophoresis using 5′-32P-labeled SDA primers and autoradiography. The most common RNA amplification strategy is called *nucleic acid sequence-based amplification (NASBA)* (Compton, 1991); detection of the RNA generated can be by nucleic acid hybridization or real-time with a molecular beacon and Förster resonance energy transfer (FRET) (Polstra et al., 2002).

4.6.1.4.3 Bio-Plex Assays

Bio-Plex (or Luminex) assays are based on xMAP technology (multianalyte profiling beads produced by Luminex) that enables the detection and quantification of multiple RNA or protein targets simultaneously. The Luminex xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), lasers and digital signal processing to effectively allow multiplexing of up to 150 different nucleic acid sequence targets within a single sample (Li et al., 2007; Mahony et al., 2007). Thus multiple PCR amplification products bound to matching oligonucleotides that are attached to differently colored fluorescent beads are detected by flow cytometry (Brunstein and Thomas, 2006).

4.6.1.4.4 Microarrays (Nucleic Acid Arrays)

Microarrays are miniaturized multiplexed detection platforms consisting of arrays (or clusters) of thousands of short, single-stranded probes (25–70 mer oligonucleotides, PCR products or cDNA) bound at defined locations, either by spotting or direct synthesis to solid substrates. The probes typically target conserved sequences at different levels of the taxonomy (family, genus and species), which allows detection of pathogens that share homology with known, previously characterized viruses (Mikhailovich et al., 2008; Chiu, 2013). For use, target nucleic acid in the clinical specimen (ie, RNA or DNA) is labeled with fluorescent reporter group (Cy3 or Cy5) or nonisotopic label (eg, biotin; detected with streptavidin–phycoerythrin conjugate) or radioisotope. This is a reversal of conventional hybridization assays in that the target nucleic acid is the one that is labeled in microarrays (Boonham et al., 2003). The hybridization signals are collected by scanners (eg, DNAsope), and analyzed using clustering algorithms in computer programs. Several pan-microbial DNA microarrays have been developed (Chiu, 2013), including the ViroChip (Chen et al., 2011) with the capacity to detect all known viruses as well as novel variants on the basis of conserved sequence homology and a turnaround time of about 24 hours (Wang et al., 2002). These platforms rely on random PCR strategies to amplify and label target nucleic acids for detection. However, poor reproducibility (Draghici et al., 2006) and low sensitivity have hindered microarray-based technologies from becoming standard for clinical diagnostics (Rasmussen, 2015).

4.6.1.4.5 Next-Generation Sequencing

The term *next-generation sequencing (NGS)* is used here as an umbrella term to describe a collection of DNA sequencing methods also referred to as *second-generation high-throughput sequencing (SGS)* (Illumina/Solexa, Roche/454, SOLiD, Ion PGM/Proton) and *third-generation sequencing (TGS)* (Pacific Biosciences), which were preceded by the automated Sanger method that is considered as a first-generation technology (Metzker, 2010). The NGS methods are known as *high-throughput sequencing (HST)* or *deep sequencing*. They each use different biochemical approaches and instruments to produce data in vastly larger amounts, at greatly lower costs, in shorter time, and with less manual intervention than the Sanger method (Goldberg et al., 2015). These HST-based methods when coupled with fast computers, cloud computing and bioinformatics tools are providing robust methods for discovering new pathogens in clinical specimens (Radford et al., 2012; Chiu, 2013; Rasmussen, 2015). Unlike PCR or array methods in which investigators are limited by known sequence information, NGS requires no a priori knowledge of the viruses in the sample (ie, is sequence-independent) and therefore provides a random and unbiased screening method that is as sensitive as PCR for universal amplification of any virus. It is thus excellent for identification of emerging viruses with unique genetic features (Chiu, 2013), particularly as the NGS instruments and methods are increasingly becoming accessible (Loman et al., 2012). The raw sequence reads obtained are filtered for quality and redundance before assembly into contiguous sequence streams or contigs. These contigs and any reads that cannot be assembled are identified using BLAST programs available via the National Center for Biotechnology Information (Altschul et al., 1990) against the nonredundant (NR) Genbank database. The bioinformatic analysis allows the virus sequences to be characterized with regard to taxonomy to the level of virus subtype (taxonomic assignment) and even the presence of genes associated with virulence, etc. The biggest challenge to successfully applying NGS as standard diagnostic assays is the presence of large amounts of irrelevant nucleic acids (eg, host DNA, rRNA, etc.) or very low amounts of viral nucleic acids in clinical specimens. Therefore, a lot of effort is expended in depletion techniques to remove irrelevant nucleic acids (through subtraction of host nucleic acid via nuclease digestion and rRNA depletion) or enrichment procedures such as "pathogen capture" (Bent et al., 2013) to increase viral sequence yield. Hall et al. (2014) found a three-step method of centrifugation, filtration and nuclease-treatment to show the greatest increase in the proportion of viral sequences.

4.6.1.4.5.1 ***Viral Metagenomics*** The term *metagenomics* refers to the comprehensive sequence analyses of all genomic DNA (metatranscriptomics for all genes/mRNA) recovered directly from an environmental sample, without separating the genomes or culturing the organisms (Chen and Pachter, 2005; Goldberg et al., 2015). Recent advances in NGS (metagenomic NGS) have significantly enhanced its application. Viral metagenomics (Edwards and Rohwer, 2005; Mokili et al., 2012)

therefore provides a powerful culture-independent and sequence-independent (ie, completely unbiased) approach to analysis of the virome in a wide range of samples; that is, by sampling viruses in natural environments and identifying the genomic composition of a sample (Soueidan et al., 2015). For example, metagenomic shortgun sequencing (MSS) is used to characterize viral populations in microbial communities. A variation of MSS with increased sensitivity is ViroCap, designed to enrich nucleic acid from DNA and RNA viruses from 34 families that infect vertebrate hosts (Wylie et al., 2015). Another example with increased sensitivity of sequence-based virus detection and characterization is the virome capture sequencing platform for vertebrate viruses (VirCapSeq-VERT), with a limit of detection comparable to that of targeted qPCR/RT-qPCR (Briese et al., 2015). VirCapSeq-VERT allows both the simultaneous identification and the comprehensive genetic characterization of all known vertebrate viruses, their genetic variants and novel viruses (Briese et al., 2015), even in high-background specimens with low-abundance viruses (Rasmussen, 2015).

Breitbart et al. (2002) conducted the first viral metagenomic study applied to marine viruses and found a high diversity of marine viruses, as well as a high number of sequences (65%) with no homologs in the Genbank database (referred to as the "unknown"; Mokili et al., 2012). Alavandi and Poornima (2012) reviewed the use of viral metagenomics as a tool for virus discovery and diversity in aquaculture. The application of this virus detection method to establish causation in infectious disease (ie, fulfillment of Koch's Postulates; Koch, 1890; Fredericks and Relman, 1996) has also been reviewed (Lipkin, 2013), and Mokili et al. (2012) proposed a new model of Koch's postulates, which they named the "Metagenomic Koch's Postulates." Most recently, Hall et al. (2015) proposed actions to address seven major issues identified when considering use of viral metagenomics as a diagnostic tool.

4.6.1.4.6 In Situ Hybridization

Nucleic acid hybridization is the formation of a stable duplex between two complementary strands of nucleic acid by means of hydrogen bonding between base pairs. The ability of complementary nucleic acid sequences to form double stranded hybrids with high efficiency and specificity in the presence of mixture of noncomplementary sequences was first reported by Hall and Spiegelman (1961). In situ hybridization (ISH) refers to the method of detection of specific nucleic acid sequences in cytological preparations and tissue sections by hybridization to specifically labeled nucleic acid probes. The concept is analogous to detection of antigens by IHC (Nuovo, 2006), and a combination of both techniques provides a very powerful experimental tool. Unlike for IHC, protease digestion is invariably needed in any ISH reaction that uses paraffin-embedded, formalin-fixed tissue to partially digest the cross-links between DNA/RNA and proteins; otherwise, the probe cannot access the target and hybridize to it (Nuovo, 2006). The primary objective of ISH is to reflect accurately inter and intracellular distribution of target molecules in a sample (Fig. 4.4). As a virus detection method, ISH is primarily applied to pathogenesis or cell biology rather than diagnostics. However, it is ideally suited for use in mollusk diseases where diagnosis relies heavily on histological evaluation of tissues.

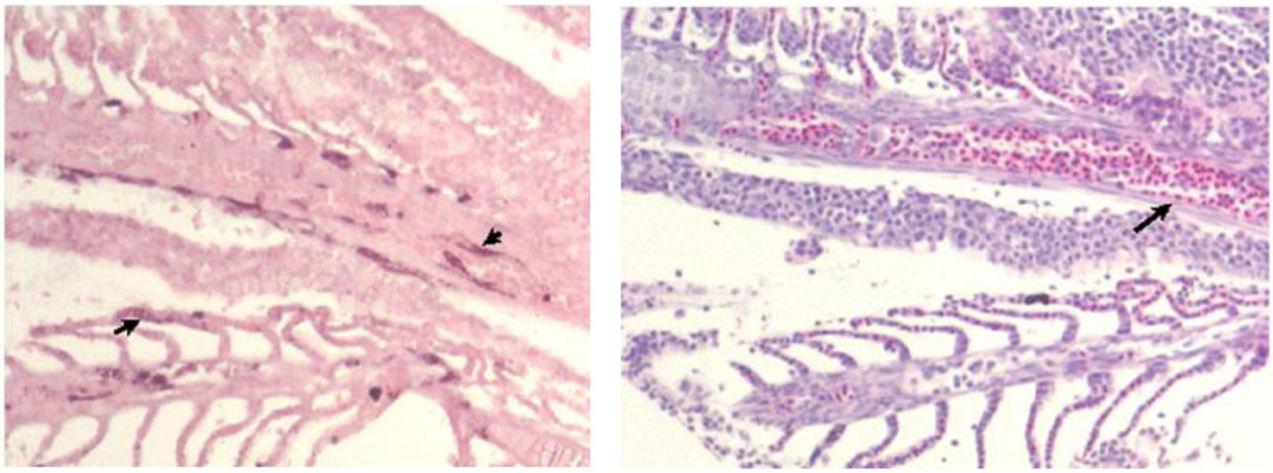

FIGURE 4.4 In situ hybridization using a digoxigenin riboprobe. Gill tissue of Atlantic salmon infected with infectious salmon anemia virus (ISAV): (A) ISH with digoxigenin riboprobe. Digoxigenin-11-dUTP is detected with antidigoxigenin antibodies (Roche). (B) Hematoxylin and Eosin (H&E) staining.

4.6.2 Virus Isolation

Virus isolation is the most sensitive method available for fish viral diseases (Crane and Williams, 2008) if properly collected material is used, although the sensitivity of this method may now be surpassed by qPCR/RT-qPCR techniques. Virus isolation followed by a confirmatory assay such as EM, FAT, PCR/RT-PCR is the "gold standard" method for most regulatory inspections to support interstate or international commerce in live fish (Goodwin et al., 2010). Virus culture has several advantages; for example, it provides material for further study, and it enables insights into pathogenesis and immunity. However, it has the distinct disadvantage of being able to select for the virus that is more fit to replicate, which may or may not be the cause of the disease, allowing it to outgrow and obscure detection of the causative virus that is more abundant in vivo. No one cell line can support the growth of all fish viruses. Thus diagnostic laboratories have to maintain several different cell lines (Lakra et al., 2011), and the use of at least two cell lines, primary and complimentary, for each sample is advocated (Peters, 2004). Moreover, many viral pathogens are completely refractory to virus culture despite implication in disease, and in the case of some fish viruses and all crustacean and molluskan viruses, there is a lack of permissive cell lines for use; hence, the importance of advances in metagenomics (refer to Section 4.6.1.4.5.1). Some viruses may require use of RNA interference (RNAi) to suppress innate immune responses or must be inoculated in young live animals. Other viruses such as the nonpathogenic infectious salmon anemia virus (ISAV-HPR0) that may be carried by apparently normal fish are inherently refractory to in vitro culture (Kibenge et al., 2009) and to being propagated in live fish.

The virus isolation result by itself is not automatically confirmatory of a viral cause of disease. It is common to have subclinical viral infections unrelated to the disease in question (especially if disease is immunosuppressive), and some of the viruses are not proven pathogens. The isolated virus may be a primary or secondary agent or immunosuppressive agent or nonpathogenic. Koch's Postulates are applicable here. For proper interpretation of the result, consider the following:

1. The site from which virus was isolated: sterile versus unsterile sites (eg, blood vs gastrointestinal sample)
2. In the case of unsterile site, the same isolation if made from several cases of the same clinical presentation during an epizootic is significant
3. Knowledge that the virus and the disease in question are causally associated.

Recovery of more than one virus presents a problem, especially if both viruses are known pathogens. Confirmation may require other tests such as serology on paired sera.

4.6.2.1 Fish Cell Lines

Virus isolation is carried out on cultured cells in vitro. There are three basic types of cell cultures: (1) primary cells, (2) diploid cell lines and (3) heteroploid or continuous cell lines. Primary cells are produced by the dissociation of organized tissue fragments into single cell suspensions using proteolytic enzymes such as trypsin (Versene or ethylenediamine tetraacetic acid, EDTA, a chelating agent which ties up divalent cations necessary for cell attachment and prevents cell clumping may be included in the trypsin) and denote the first cultivation of cells from an organ. These cells represent a heterogenous population of different cell types that closely resemble cells from the parental organ. This makes primary cell cultures sensitive to a wide range of viruses and therefore useful in the isolation of virus from clinical specimens. In the absence of crustacean and molluskan cell lines, primary cell cultures may be useful in virus isolation attempts of crustacean and molluskan viruses. Diploid cell lines originate from primary cells and represent cells with normal growth properties (not continuous and therefore do not induce tumors in test animals) and hence find usage in viral vaccine production. The term diploid denotes a cell line in which, arbitrarily, at least 75% of the cells have the same karyotype as the normal cells of the species from which the cells were originally obtained. Diploid cells have a finite life span of usually about 50 subcultivations or passages. Heteroploid cell lines are capable of indefinite serial propagation in vitro and possess nuclei containing chromosome numbers other than diploid. The term heteroploid cell line means that less than 75% of the cells have the diploid chromosome number. Most cell lines are actually aneuploid and often have a chromosome number between the diploid and tetraploid value (Levin and Müntzing, 1963). The advantage of diploid and heteroploid cell lines is that they are easy to propagate and can be stored in the frozen state for use when desired without having to resort to the preparation of primary cell cultures. Cell lines also provide a homogenous population of cells, unlike primary cell cultures.

To date there are more than 280 fish cell lines established from a broad range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain and skin (Fryer and Lannan, 1994; Lakra et al., 2011; Zheng et al., 2015). The methodology for culture of fish cells is similar to those for mammalian and avian cell cultures except that fish cell lines have a wider temperature range for incubation (Lakra et al., 2011). Fish cells can be maintained with little care for long periods of time because of lower metabolic rates than eurythermal cells and fish cell lines, in contrast to mammalian and avian cell lines, are

easier to maintain and manipulate (Lakra et al., 2011). Most virology laboratories freeze several early passages of the cell lines in liquid nitrogen tanks (Freshney, 2010) to safeguard against changes or contamination in higher passages (Hughes et al., 2007).

4.6.2.2 Standard Virus Isolation Procedure Using Cell Culture Monolayers

Several publications have general recommendations for sampling, shipping and processing of fish tissues for viral assays (La Patra, 2014) and detailed procedures for the isolation of viruses using fish cell lines (Peters, 2004; Crane and Williams, 2008; AFS-FHS, 2014; OIE, 2015a). Tissue samples for virus isolation are first homogenized to a 10% homogenate in medium with 10× antibiotics, clarified, diluted and then used to infect monolayers of fish cell lines following the standard protocols in the OIE Aquatic Manual (OIE, 2015a) and/or in the Blue Book (AFS-FHS, 2014). Cell culture plates are normally sealed with plate film or placed in an airtight container and incubated at the appropriate temperature (Peters, 2004; Crane and Williams 2008). Evidence of virus replication is by presence of cytopathic effect (CPE) in the inoculated cell monolayer. CPE is the macroscopic and microscopic appearance of the cell damage produced by cytocidal (or cytopathic or cytolytic) viruses. It may consist of cell rounding and detachment from the substrate, cell lysis, syncytia formation (cell fusion or giant cell formation as revealed by presence of more than one nucleus) or inclusion body formation (demonstrated using special stains). The CPE may be characteristic of a particular virus family. Virus replication is monitored daily using an inverted light microscope until CPE is evident (Fig. 4.5) or up to 21 days and the plates or flasks are frozen at −80°C (Crane and Williams, 2008). The presence of virus replication is also monitored by RT-PCR in cell lysates because this may occur without apparent CPE. Noncytocidal viruses (viruses that do not cause CPE) can be assayed by FAT. The cultures negative by CPE and RT-PCR or FAT are passaged on fresh cell monolayers. A sample is considered negative if no CPE or RT-PCR amplification or FAT is observed after three blind passages.

Cultures with CPE are subcultured and analyzed by other tests such as EM or FAT to corroborate and identify the isolated virus and subtyped with monoclonal antibodies or by DNA sequencing. Normally, the identification of virus isolates of regulatory or reporting concern is performed in international (OIE or EU) or national reference laboratories.

4.6.2.3 Quantitative and Quantal Assays of Viral Infectivity

The most important property of a virus is its infectivity (the ability to invade a cell and parasitize that cell to replicate itself). To measure infectivity, one could look at any virus-cell interaction indicative of virus replication and develop an assay to obtain a titer for a given virus stock. A *virus titer* is defined as a given number of infectious virus units per unit volume; an *infectious unit* is the smallest amount of virus that produces some recognizable effect in the host system employed. Viruses are propagated in the laboratory in one of three host systems (Fig. 4.1): (1) cell culture: evidence of virus replication is CPE; noncytocidal viruses (viruses that do not cause CPE) can be assayed by hemadsorption or by FAT; (2) laboratory animals (normally newborn) and (3) natural host (young): monitored for clinical signs or mortality and then confirmed for the presence of virus.

There are two basic types of infectivity assays. The first is the quantitative assay, which involves the actual enumeration of plaques on a cell monolayer (*plaque assay*). Dulbecco (1952) introduced the plaque assay as a means of quantitating lytic animal viruses. In this assay, serial dilutions of a known viral suspension are added to a susceptible cell monolayer, and the inoculum is allowed to adsorb. The unadsorbed virus is washed off and an overlay medium is added. The overlay medium contains a solid gel that ensures that the spread of progeny particles is restricted to the immediate vicinity of the originally infected cell. Thus, each infective viral particle gives rise to a localized focus or plaque of infected cells. The plaque is recognized when a stain overlay is added. This stain overlay contains a vital dye (stains only living cells) such as neutral red. The viable cells are stained with the dye and the plaques are clear zones in the cell monolayer. One virus plaque, therefore, corresponds to one infectious unit. The virus titer is expressed as the number of plaque forming units (PFU) per volume of viral inoculum (Burke and Mulcahy, 1980).

An important application of the plaque assay is the *plaque reduction bioassay*. In this case dilutions of serum to be tested for viral neutralizing antibodies are added to confluent cell monolayers that are then infected with a dose of virus adjusted to yield a countable number of plaques. This can also be useful in assessing inhibitory titers of antiviral products. Another application of the plaque assay is *plaque purification*. For this procedure, a clone of the virus is picked from an isolated plaque by a Pasteur pipette (only from cultures stained with neutral red) and propagated, and the procedure is repeated at least three times.

The second type of infectivity assay measures an all-or-nothing response (ie, whether there are infectious viral particles present at all in the viral suspension). The endpoint is an arbitrary selection of a given effect of the virus on the indicator system; is there CPE or not (for $TCID_{50}$)? Is the animal dead or alive (for LD_{50})? This assay is not quantitative, but rather quantal and is not nearly as precise as a quantitative assay.

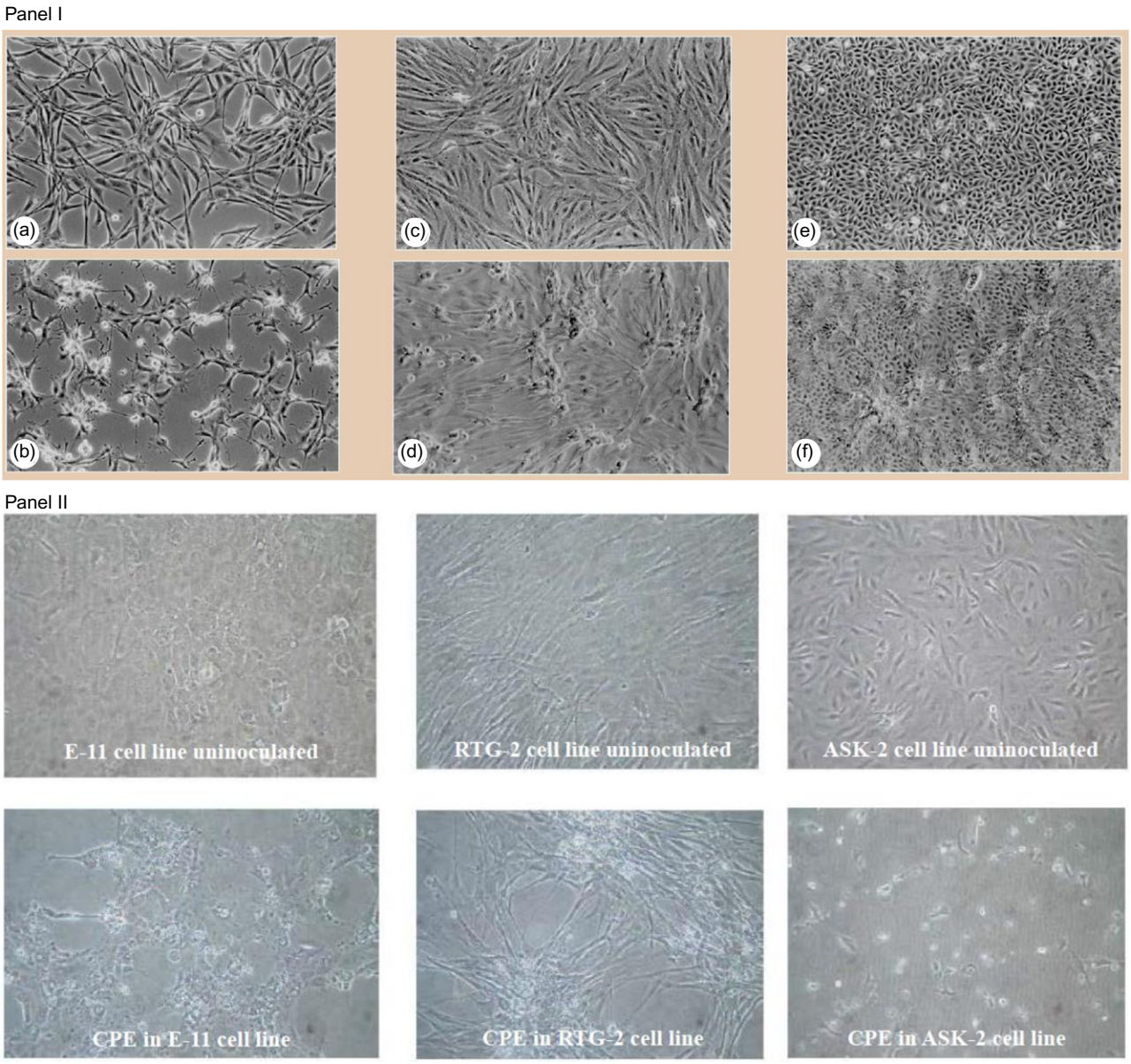

FIGURE 4.5 CPE induced by two different viruses in various fish cell lines. Panel I: (a), (c) and (e) are 24-hour-old uninfected TO, SHK-1 and CHSE-214 cell monolayers, respectively; (b) TO, (d) SHK-1 and (f) CHSE-214 cells showing CPE caused by infectious salmon anemia virus (ISAV) strain NBISA01 at 4, 6 and 12 days postinoculation, respectively (Joseph et al., 2004). Panel II: top row—uninoculated E-11, RTG-2 and ASK-2 cell monolayers, respectively; bottom row—E-11, RTG-2 and ASK-2 cells showing CPE caused by Atlantic salmon bafinivirus (ASBV) at 7 days postinoculation, respectively (Kibenge et al., 2016).

Quantal assays in cell culture are performed in a fashion similar to that of the plaque assay except that liquid medium is used as the overlay and adequate time is allowed for the virus to multiply and spread to destroy the entire cell monolayer. At the lower virus dilutions all the cell monolayers are infected, and at higher virus dilutions the cell monolayers are unaffected. The viral titer is calculated using the intermediate dilutions where only some of the cell monolayers are scored as being infected. The results are calculated on the basis of statistical assays such as the Karber (Minikel, 2015) or Reed and Muench (1938) methods used to determine a median infective dose response; that is, the highest dilution of the tissue culture fluid that produces CPE in 50% of infected cultures. Thus, viral titers are then expressed as a median tissue culture infective dose ($TCID_{50}$). A $TCID_{50}$ of $10^{5.0}$/0.1 mL means that a virus pool diluted 1:100,000 and inoculated onto cell culture in 0.1 mL amounts will destroy 50% of all cultures inoculated. When experimental animals are used, the lethal dose that kills 50% of the inoculated animals is referred to as the LD_{50}.

4.7 LABORATORY METHODS USED FOR DEMONSTRATION OF PRESENCE OF SPECIFIC VIRAL ANTIBODY (SEROLOGY)

There are numerous serological techniques used to diagnose viral infections. Diagnostic virology utilizes the following techniques, either singly or in combination, in order to accurately identify a viral isolate, determine the antigenic relationship of different viral isolates and to identify and quantify viral antibody: ELISA (see Section 4.6.1.2.1), AGID (see Section 4.6.1.2.2), immunochromatography (see Section 4.6.1.2.3), immunofluorescence assay (see Section 4.6.1.2.4.1), hemagglutination-inhibition (HI) (see Section 4.6.1.2.5) and virus neutralization (VN) (see Section 4.7.1) to name a few. Each of these assays has advantages and disadvantages. Properties to be considered before selecting one to use or request include: sensitivity, specificity, simplicity, expense and time required. Crustaceans and mollusks do not produce antibodies and therefore a viral diagnosis cannot be reached by detection of viral antibody in the host (ie, serologic diagnosis) although antibody tests can be used for detection of virus antigens in the host (see Section 4.6.1.2). In the case of fish, detection of viral antibody can be useful in diagnosis of a viral infection, but where vaccination is practiced, antibody detection tests are unable to differentiate between infection and vaccination responses by fish. Paired sera (acute and convalescent sera) tested in a VN test allows for determination of an increase in antibody titer suggesting a recent infection.

4.7.1 Virus Neutralization or Serum Neutralization

In a neutralization test, serum and virus are reacted together in equal volumes and inoculated into a susceptible animal host or cell culture. If antibodies to the virus are present then clinical disease or CPE will not be observed; that is, the virus replication will be inhibited and virus is neutralized. Thus any assay that measures viral infectivity can be used in virus neutralization. Viral neutralization tests are used to either identify an unknown virus using known reference antisera/monoclonal antibody or measure virus neutralizing antibody levels in serum samples against a known infectious virus. Specific antibody levels in an animal serum indicate previous exposure to a particular virus and often may give an indication of the susceptibility to a virus. Diagnosis of a virus infection requires the submission of paired serum samples: acute phase sample taken at first signs of clinical illness, and convalescent phase sample approximately 2–3 weeks later. For accurate interpretation, both acute and convalescent samples should be assayed simultaneously by the same method in the same assay. An increase in antibody titer of fourfold or greater in the convalescent sample is considered diagnostic of an active infection by a particular virus at the time the acute specimen was taken (and is termed *seroconversion*).

The virus neutralization test is sensitive and specific, but also more complex, time consuming and expensive than many other assays. Virus neutralization tests are now done using microtiter systems (as for a quantal assay), which are economical, easier to perform and use smaller amounts of reagents. Plaque reduction tests are performed as for a plaque assay following reaction of virus with antibody (refer to Section 4.6.2.3).

There are two types of neutralization tests—alpha and beta. In the alpha procedure, equal quantities of a constant amount of serum and increasing dilutions of virus are incubated and then inoculated into the indicator system. In the more common beta procedure, virus at a concentration of 100 $TCID_{50}$ is incubated with equal volumes of increasing dilutions of serum.

4.7.2 Western Blot Analysis

In this test, viral proteins of a purified virus preparation are first separated into discrete bands according to their molecular mass (M_r) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins are then transblotted onto nitrocellulose/nylon membranes. Then the test serum is applied to the membrane where antibodies in serum bind to the viral proteins. The bound antibodies are detected by adding a second antibody (which is directed against the IgG of the animal species of the test serum sample) conjugated to an enzyme (horseradish peroxidase or alkaline phosphatase) that reacts with a substrate to produce a color similarly to ELISA or IHC. The test permits demonstration of antibodies to some or all the viral proteins in the virus preparation.

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Chapter 5

Prevention and Control of Viral Diseases in Aquaculture

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5.1 INTRODUCTION

The global aquaculture industry has increased tremendously in the past few decades, which has led to the corresponding increase in the discovery of aquaculture viruses. Given that some viruses have had a devastating impact on the aquaculture industry worldwide, it is imperative that preventive and control measures aimed at reducing their occurrence are devised. In general, prevention describes the measures taken to avert the introduction of a disease in a susceptible population while disease control refers to measures taken to reduce infection to tolerable levels in already infected populations. Thus, the old adage "an ounce of prevention is better than a pound of cure," is highly applicable in aquaculture virology.

Prevention and control of viral diseases is, to a large extent, influenced by the properties, environmental stability, reservoir hosts, host species susceptibility, transmission dynamics and pathogenicity of individual viruses that infect aquatic organisms. In addition, farming in the aquatic environment where farmed and wild aquatic organisms will be in (close) contact and absolute biocontainment is difficult, not to say impossible, using floating net-pen production system, is in strong contrast to the terrestrial environment in animal agriculture (such as commercial poultry farming) where absolute biosecurity is easier to implement. One possible solution to this would be to move current net pens to on-land production structures with sea water, which will be subject to filtration and disinfection prior to being pumped into the system to prevent intake of any pathogens. The cost of production has thus far precluded development of such production structures, but there are test facilities currently operating.

Most of these parameters have not been studied in detail because most aquaculture viruses have only been discovered in recent years. In the absence of such information, it is difficult to design effective preventive and control measures for some of the viral diseases in aquaculture. For example, viruses discovered in the past few years, such as piscine myocarditis virus (PMCV) (Haugland et al., 2011) and piscine orthoreovirus (PRV) (Palacios et al., 2010) have not had their properties fully characterized. Similarly, a novel virus shown to cause high mortality in farmed tilapia in Israel has just been identified (Eyngor et al., 2014) and characterized as an orthomyxo-like virus likely representing a new genus in the family *Orthomyxoviridae* (Bacharach et al., 2016). For such viruses, research is still in its infancy to fully establish their properties that would serve as a guide to develop measures for their prevention and control. Apart from viral properties, several factors influence the prevention and control of viral diseases in aquatic organisms. Current aquaculture farming practices that depend on high-stocking densities to maximize production outputs have the propensity to increase the transmission of viral infections between individuals. The expansion of international trade for eggs and live fish has been linked to the transcontinental spread of fish diseases. Another important factor that influences the distribution of aquatic viral diseases is the differences in ecological factors. For example, cold-water fish species that thrive in low temperatures ($\leq$12°C) are infected by viruses that replicate at $\leq$15°C, while tropical and subtropical fish species are mostly infected by viruses that grow at high temperatures ($\geq$20°C). However, although some viruses infect a wide host range of aquatic organisms, other viruses are host species specific, which limits their spatial distribution. Although thousands of aquatic organisms exist in the world, only a few selected species are utilized in aquaculture. Thus, the current scope of prevention and control of aquatic viruses is limited by host species utilized in aquaculture, which is likely to change as more aquatic organisms are incorporated into aquaculture farming. Nevertheless, this chapter brings into perspective current preventive and control measures, together with the novel strategies being explored to reduce the prevalence of viral infections in aquaculture.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00005-X
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5.2 DISEASE PREVENTION AND CONTROL USING BIOSECURITY MEASURES

Biosecurity refers to measures that prevent the introduction of infectious agents into defined geographical locations or facilities. It also includes biocontainment, which refers to control of diseases already present in a particular area, but aims at preventing novel transmission to other areas. The major concerns in biosecurity are the potential carriers of infectious agents, which include infected hosts, nonhost biological carriers and contaminated inanimate objects (Lotz, 1997). To ensure biosafety in aquaculture, biosecurity measures should be implemented at all stages of the aquaculture production cycle, which include brood-stations, individual farms, national and international organizations responsible for aquaculture exports and imports.

5.2.1 Implementation of Biosecurity on Brood Stations

Brood-stations serve as starting points because of their role in the supply of eggs and live fish for fish farming and seed stock for shrimp farming. Some of the routine biosecurity measures carried out on brood stations for the control and prevention of infectious diseases are discussed next.

5.2.1.1 Routine Screening of Parent-stock and Eggs

To prevent vertical transfer of pathogens, parent stocks should be routinely screened using laboratory tests for the identification and removal of infected fish so that only disease-free fish are used to produce eggs for fish farming. Individual fish from parent-stock should be tagged and screened for viral infections using sperms, ovarian fluid and other materials for virus isolation and identification using cell culture and/or reverse transcription polymerase chain reaction (RT-PCR) (Watanabe et al., 2000). For example, in Japanese flounder screening for viral nervous necrosis virus (VNNV), using enzyme-linked immunosorbent assay (ELISA) is done 3 months before spawning and only negative fish are reared as brood stocks for egg production (Yoshimizu, 2009). Apart from parent stock, eggs should be screened for viral infections using cell culture and RT-PCR. Infected eggs should be removed from the breeding program and only eggs certified free of disease infections should be sold for fish farming. In shrimp, the use of specific pathogen free (SPF) postlarvae from brood-stocks screened and certified free of the major shrimp viral diseases such as white-spot syndrome virus (WSSV), Taura-syndrome virus (TSV), infectious hematopoietic and hemorrhagic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) have contributed to the significant reduction of disease outbreaks on shrimp farms (Moss et al., 2012).

5.2.1.2 Manuals of Standard Operating Procedures for Brood Stations

To ensure consistency in the implementation of biosecurity measures on brood stations, it is important that manuals for standard operating procedures (SOPs) are prepared and that these SOPs include biosecurity control measures as part of routine operations on brood stations. The SOPs should include details on facility design, facility flow for both personnel and stock, showing restricted and nonrestricted areas, equipment and personnel disinfection procedures, inflow water and wastewater management, feeding regimes and other management procedures. In addition, SOPs should provide protocols for sample collection used in routine screening of viral diseases, guidelines for handling disease outbreak and reporting of notifiable diseases to regulatory national veterinary institutions. For shrimp farming, protocols should include guidelines on screening of the seed-stock used for generating SPF postlarvae for the supply to shrimp farmers. It is important that standard data entry forms are prepared and filled in routinely, showing records on the health status, disinfection of equipment and facilities, quantity of fish-eggs or postlarvae shrimp produced per batch, diseases screened per batch, diagnostic tests carried out, immunostimulants used or vaccinations, sources of feed, feed consumption, weight gain and other relevant biosecurity and management practices used on the brood station.

5.2.1.3 Compliance with National Biosecurity Regulations on Brood-Stations

As shown in Fig. 5.1, national biosecurity plays a pivotal role in coordinating all disease control and prevention activities for brood-stations and individual farms by linking them to local and international organizations involved in fish trade. It is vital that implementation of all biosecurity programs on brood-stations complies with national biosecurity guidelines. Therefore, routine screening of postlarvae shrimp, fish-eggs and breeding stock should be carried out in compliance with national disease screening procedures for aquatic organisms. All notifiable diseases when suspected or detected should be reported to relevant authorities and appropriate disease control measures should be implemented in accordance with governing regulations. Only diagnostic tests approved by national veterinary services or when applicable, OIE standards, should be

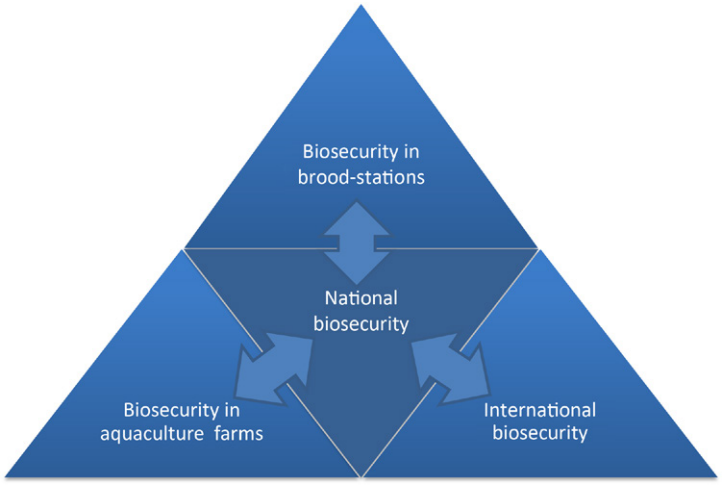

FIGURE 5.1 Integration of brood stations, individual aquaculture farms and national biosecurity with international biosecurity activities. Note that national biosecurity takes a central coordination role as a linker (blue (gray in print versions) arrows) of brood-stations, individual aquaculture farms and international biosecurity.

used for the screening of eggs and parent stocks. For brood-stations exporting postlarvae shrimp, frozen shrimp, fish eggs or live-fish, it is important that international biosecurity regulations are strictly adhered to and that all packaging and shipment are in compliance with biosecurity measures recommended by the importing country and international bodies.

5.2.2 Implementation of Biosecurity on Aquaculture Farms

Apart from brood-stations, implementation of biosecurity measures should be part of the routine farming activities carried out in aquaculture.

5.2.2.1 Management Practices in Aquaculture Farming

Implementation of biosecurity measures should be part of the routine management practices routinely carried out on fish or shrimp farms and can be divided into physical, chemical and biological disease control activities, as shown next.

5.2.2.1.1 Physical Disease Control Measures

This refers to physical activities carried out to prevent infectious agents from coming into contact with reared fish or shrimp. Therefore, it is vital that the design of the farm deters the contact of disease vectors from getting into contact with reared stock. All newly acquired stock should be quarantined before they can be put in the same facility as resident stock. The quarantine facility should be built away/physically separated from the resident stock. The duration of observation in quarantine can be variable, although a period of 4–6 weeks is ideal for viral diseases. There should be no exchange of equipment and personnel between the quarantined and resident stock. All acquired fish should, to the extent possible, be vaccinated before they are put in the mainstream facility with the resident fish. Other biosecurity factors that require physical control include regulation of temperature, which has been shown to play an important role in reducing the occurrence of diseases such as hirame infection rhabdovirus virus (HIRRV) in Japanese flounder (Nishizawa et al., 2011). In addition, studies in fish immunology have shown that temperature is an important regulator of immune response to vaccination and therefore, maintaining optimal temperatures during fish production is a prerequisite to developing protective immunity in vaccinated fish (Morvan et al., 1998). The use of recommended stocking densities is important because high stocking densities increase the transmission of viral diseases between fish and also tend to introduce stress, which exacerbates disease transmission. Filtration and irradiation of inflow water using ultraviolet (UV) light reduce the chances of introducing viral infections to reared fish, as shown that several fish and Table 5.1 show the UV-doses and duration of exposure required for the inactivation of different fish viruses, while Table 5.2 shows the temperature and duration of exposure required to inactivate different fish viruses.

TABLE 5.1 UV Sensitivity Range for Selected Fish Viruses

Virus	Viral Family	UV Dose (mJ/cm^2)	Reference
Infectious pancreatic necrosis virus	*Birnaviridae*	100–337	(Liltved et al., 1995; Liltved et al., 2006; Oye and Rimstad, 2001; Yoshimizu et al., 1986; Yoshimizu et al., 1990; Yoshimizu et al., 2005)
Sea bass neuropathy nodavirus	*Nodaviridae*	211	(Frerichs et al., 2000; Liltved et al., 2006)
Atlantic halibut nodavirus	*Nodaviridae*	105	(Yoshimizu et al., 2005)
Japanese flounder lymphocytic virus	*Iridoviridae*	100	(Yoshimizu et al., 2005)
Infectious salmon anemia virus	*Orthomyxoviridae*	3.3–7.5	(Liltved et al., 2006; Oye and Rimstad, 2001)
Viral hemorrhagic septicemia virus	*Rhabdoviridae*	3.1–7.9	(Oye and Rimstad, 2001)
Hiramine rhabdovirus	*Rhabdoviridae*	1.0	(Yoshimizu et al., 2005)
Infectious hematopoietic necrosis virus	*Rhabdoviridae*	1.0–10.0	(Yoshimizu, 2009; Yoshimizu et al., 1991; Yoshimizu et al., 1986; Yoshimizu et al., 1990; Yoshimizu et al., 2005)
Spring viremia of carp virus	*Rhabdoviridae*	1.0	(Yoshimizu et al., 2005)
Channel catfish herpesvirus	*Herpesviridae*	1.0–3.0	(Yoshimizu et al., 1986; Yoshimizu et al., 1990)
Koi herpes virus	*Herpesviridae*	4.0	(Kasai et al., 2005)

5.2.2.1.2 Chemical Disease Control Measures

This involves the use of chemical substances for the inactivation of viral pathogens on aquaculture farms. Some of the basic guidelines used for the selection of effective disinfectants include inactivation efficacy, environmental impact, human safety and impact of the disinfectant on the reared aquatic organisms. Table 5.3 shows the concentration and duration of exposure required to inactivate different fish viruses for the common disinfectants used in aquaculture. It is important that all equipment, personnel and all facilities are disinfected routinely. In additions, all fish eggs brought on the fish farm should be disinfected and the common disinfectants used for fish eggs include iodophor used at 25 ppm for 20 min or 50 ppm for 15 min and 4–6% sodium hypochlorite per liter for 5 min. Other biosecurity measures that require chemical control include the checking of the chemical composition of water used for rearing fish to determine the oxygen, ammonia, nitrate and pH levels. High levels of ammonia, nitrate and low pH induce stress on fish and these chemicals have the potential to cause tissue damage, making fish more vulnerable to infection. In addition, it is important to ensure that there is adequate aeration to avoid introducing hypoxia, which can cause death in fish eggs.

5.2.2.1.3 Biological Control Measures

This involves the use of biological processes to protect reared aquatic organisms from viral infections, which include vaccination to prevent infection in immunized fish, purchase of certified disease free eggs or live fish as well as the use of disease-resistant strains of aquatic organisms used for fish farming. Kent and Kiesler (2003) showed that the policy of "eggs only" combined with the policy of only using certified disease-free eggs for fish farming eliminated the introduction of disease infections in Atlantic salmon on fish farms in British Columbia, Canada, suggesting that the transfer of live fish from brood-stations to fish farms had a higher risk of disease transmission than the eggs. In the case of shrimp, protective biological processes include the use of SPF postlarvae and disease resistant strains of shrimp. Given that shrimp do not develop adaptive immune responses, stimulants such as lipopolysaccharides, β-1-3 glucan and peptidoglycans are used to induce innate immune responses that are protective against viral infections in shrimp.

5.2.2.2 Compliance to National Biosecurity Regulations

It is important that all aquaculture farmers comply with national guidelines for the implementation of biosafety measures. Routine disease inspection should be carried out and when a notifiable disease is detected, it should be reported to competent authorities immediately. Depending on the severity and nature of the disease, high-risk diseases (emerging infections) might require stamping-out of all stock, followed by disinfection and fallowing during which period the farm is completely dried-off of all stock. Thereafter, the farms can be restocked with certified disease-free stock.

TABLE 5.2 Disinfectant Inactivation Concentrations for Selected Fish Viruses

Chemical Compound	Application	Abbreviation	Concentration; Duration	References
Chlorine	Equipment, pumps, vehicles, buckets, nets, foot baths and other equipment	IHNV	0.1–1.0 mg/L; 30 s	(Wedemeyer et al., 1978)
		VHSV	25–54 mg/L; 5 min	(Ahne, 1982)
			98 mg/L; 2 min	(Ahne, 1982)
		SVCV	7.6 mg/L; 20 min	(Ahne, 1982)
			36–55 mg/L; 2 min	(Ahne, 1982)
		ISAV	50–100 mg/L; 15–30 min	(Torgersen, 1998)
		EHNV	200 ppm; 2 h	(Langdon, 1989)
		VNNV	25–100 ppm; 5–30 min	(Arimoto et al., 1996; Frerichs et al., 2000)
		IPNV	4–40 mg/L; 2–30 min	(Ahne, 1982; Desautels and Mackelvie, 1975; Elliott and Amend, 1978; Inouye et al., 1990)
Formalin	Fumigation of enclosed facilities	VHSV	3%; 5 min	(Ahne, 1982)
		ISAV	pH 3.5–4.0	(Torgersen, 1998)
		VNNV	2%; 6 h	(Arimoto et al., 1996)
		IPNV	2–3%; 5 min	(Ahne, 1982; Vestergaard-Jørgensen, 1973)
Iodophor	Disinfection of eggs, equipments, boots, nets, vehicles	IHNV	12–25 ppm; 15–30 s	(Amend and Pietsch, 1972)
		VHSV	100 ppm; 4 min	(Ahne and Held, 1980)
		VNNV	25–100 ppm; 10–30 min	(Arimoto et al., 1996; Frerichs et al., 2000)
		IPNV	2.5–35 ppm; 5–60 min	(Amend and Pietsch, 1972; Desautels and Mackelvie, 1975; Elliott and Amend, 1978; Inouye et al., 1990)
Quaternary ammonia	Disinfection of aquaculture ponds, hand sanitation and disinfection of plastic surfaces	VHSV	1 mL/L; 5–30 min	(Kurita et al., 2002)
		SVCV	100 ppm; 4 min	(Ahne and Held, 1980)
		VNNV	50 ppm; 10 min	(Arimoto et al., 1996)
Ozone	Sterilization of waterborne pathogens, disinfection of surfaces and equipment	IHNV	0.01–70 mg/L;10 s to 10 min	(Wedemeyer et al., 1978)
		VNNV	0.1–5.3 ppm; 30 s to 2.5 min	(Arimoto et al., 1996)
		IPNV	70–90 mg/L; 10 min	(Wedemeyer et al., 1978)
Phenol	Disinfection of surfaces and equipment	IPNV	5%; 5 min	(Inouye et al., 1990)
		VHSV	2.5%; 5 min	(Kurita et al., 2002)
Cresol	Disinfection of surfaces and equipment	IPNV	5%; 5 min	(Inouye et al., 1990)
		VHSV	0.1–0.25%; 5–25 min	(Kurita et al., 2002)
Methanol	Hands and working surfaces	IPNV	80%; 30 min	(Inouye et al., 1990)
Ethanol	Hands and working surfaces	EHNV	70%; 2 h	(Langdon, 1989)
Isopropanol	Hands and working surfaces	VHSV	20–30%; 30 s to 2 min	(Kurita et al., 2002)
Formic acid	Fumigation of facilities	ISAV	pH 3.5–4.0; 8–24 h	(Torgersen, 1998)
Sodium hypochlorite	Disinfection of nets, clothing and boots	VHSV	50–100 ppm; 1–5 min	(Kurita et al., 2002)

TABLE 5.3 Inactivation Temperatures and pH for Selected Fish Viruses

Method	Virus	Abbreviation	Virus Family	Temperature/ pH	Duration	Reference
Heat	Infectious hematopoietic necrosis virus	IHNV	*Rhabdoviridae*	55°C	30 s	(Whipple and Rohovec, 1994)
				45°C	10 min	(Whipple and Rohovec, 1994)
	Epizootic hematopoietic necrosis virus	EHNV	*Rhabdoviridae*	40°C	24 h	(Langdon, 1989)
				60°C	15 min	(Langdon, 1989)
	Viral hemorrhagic septicemia virus	VHSV	*Rhabdoviridae*	70°C	1 min	(Vestergaard-Jørgensen, 1974)
				50°C	10 min	(Vestergaard-Jørgensen, 1974)
	Spring viremia of carp virus	SVCV	*Rhabdoviridae*	45°C	20 min	(Ahne, 1976)
				50°C	5 min	(Ahne, 1976)
	Infectious salmon anemia virus	ISAV	*Orthomyxoviridae*	50°C	2 min	(Torgersen, 1998)
				55°C	1–10 min	(Torgersen, 1998)
	Infectious pancreatic necrosis virus	IPNV	*Birnaviridae*	70°C	2 h	(Whipple and Rohovec, 1994)
				80°C	10 min	(Whipple and Rohovec, 1994)
	Nervous necrosis virus	NNV	*Nodaviridae*	37°C	4 days	(Frerichs et al., 2000)
				60°C	1 h	(Frerichs et al., 2000)
				70°C	10 min	(Maltese and Bovo, 2001)
Acid (pH)	Infectious hematopoietic necrosis virus	IHNV	*Rhabdoviridae*	pH 3.8–4.3	30 s	(Whipple and Rohovec, 1994)
	Epizootic hematopoietic necrosis virus	EHNV	*Rhabdoviridae*	pH 4.0	1 h	(Langdon, 1989)
				pH 12.0	1 h	(Langdon, 1989)
	Viral hemorrhagic septicemia virus	VHSV	*Rhabdoviridae*	pH 3.0	3 h	(Ahne, 1982)
				pH 12.2	5–10 min	(Ahne, 1982)
	Spring viremia of carp virus	SVCV	*Rhabdoviridae*	pH 3.0	3 h	(Ahne, 1976)
				pH 12.0	5–10 min	(Ahne, 1982)
	Infectious pancreatic necrosis virus	IPNV	*Birnaviridae*	pH 11.9	5 min	(Ahne, 1982)
				pH 12.2	10 min	(Amend and Pietsch, 1972)
	Nervous necrosis virus	NNV	*Nodaviridae*	pH 2.0	42 days	(Frerichs et al., 2000)

5.2.2.3 Manuals of SOPs on Aquaculture Farms

Similar to brood-stations, manuals of SOPs should be prepared for use as part of routine operations on aquaculture farms. The SOPs should include designs of farm facilities, facility flow of personnel and equipment, movement/admission restrictions, disinfection procedures, inflow water treatment and wastewater management, sample collection procedures for disease screening and guidelines for handling disease outbreaks. Data entry should include the health status of reared aquatic organisms, vaccination status, stocking densities, feeding regimes, source of feeds, relevant environmental parameters and several other relevant data on health management of aquatic organisms reared on the farm.

5.2.3 National Biosecurity

National biosecurity programs integrate disease-control activities carried out on individual farms and brood-stations with the international organization (Fig. 5.1). National biosecurity guidelines should include details on handling disease outbreaks, compliance with eradication programs, movement restriction of fish-eggs, live-fish, postlarvae shrimp, frozen shrimp and other aquatic organisms. Further, national authorities should carry out routine surveillance programs and provide feedback on the disease status in the country and layout programs to equip farmers with effective disease-control strategies in high-risk areas. Where possible, national authorities should provide diagnostic services and supervise eradication programs. In addition, they should regulate the import and export of aquaculture products. Thus, by coordinating all biosecurity activities in the country, national authorities have an obligation to improve the health status of farmed aquatic organisms in their countries, as well as safeguarding their local aquaculture industry from exotic viral diseases.

5.2.4 International Biosecurity

Although the profile of aquatic viral diseases found in different countries might differ, the distribution of some of the diseases cuts across different countries, which demands common strategies for their control. To coordinate these activities, different international organizations engaged in animal health activities have been established, which include the Food and Agriculture Organization (FAO) of the United Nations and the World Organization for Animal Health (Office International des Epizooties, OIE). The FAO provides guidelines on the control and prevention of diseases for food animals while the OIE deals with all animal health issues including diseases of aquatic organisms. Since its inception in 1924, the OIE has been involved in developing science-based disease control strategies and standards to guide countries in protecting their animals from major diseases (Hastein et al., 2008). Through the OIE Aquatic Animal Health Code (Aquatic Code) and Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual), the OIE provides frameworks and guidelines for countries to develop biosecurity programs for use at national, brood-stations and individual farm levels so that the requirements for health certification in connection with trade in aquatic animals and aquatic animal products can be met. The OIE Aquatic Code and Aquatic Manual rank all diseases into high- to low-risk categories and then provide guidelines on prevention and control based on their ranking. In addition, they provide guidelines for determining the prevalence of these diseases in defined epidemiological units, diagnosis, disinfection, surveillance, prevention and eradication and restocking procedures. In principle, the FAO and OIE are not only responsible for the control of trans-boundary animal diseases, but they also provide guidelines for countries to develop their own biosecurity control measures.

5.3 SELECTIVE BREEDING FOR DISEASE RESISTANCE

Disease resistance has been defined as the ability of the host to limit infection by reducing or preventing pathogen replication following exposure to the pathogen (Doeschl-Wilson et al., 2012; Raberg et al., 2007). Currently, there are two approaches used for generating disease-resistant fish in aquaculture and these include selective breeding of naturally resistant fish strains to infection and the use of molecular biology tools to identify the markers for resistance in fish.

5.3.1 Natural Selection of Disease-Resistant Strains

The breeding of disease-resistant farmed aquatic organisms based on selecting survivors of lethal challenge to infections or epidemics dates as far back as the 1950s when Wolf (1954) tested brown trout and brook trout for resistance against furunculosis and ulcer disease. However, studies on breeding disease-resistant fish against viral infections has only gained impetus in the past two decades, mainly because the majority of fish viral diseases have only been discovered during this time. In Norway, disease resistance has been a target trait for the Norwegian salmon breeding program for bacterial and viral diseases since 1993 (Gjoen and Bentsen, 1997), which led to identification of Atlantic salmon strains that are highly susceptible to infectious pancreatic necrosis virus (IPNV) infection. This trait paved the way to optimization of IPNV challenge models in which highly susceptible fish to IPNV infection gave a wide discriminatory capacity between the vaccinated and nonvaccinated fish, making it easy to distinguish between the highly and less-protective vaccines in vaccine efficacy trials (Munang'andu et al., 2012). Therefore, selective breeding has a dual benefit of identifying fish strains resistant to disease infections, as well as identifying highly susceptible fish suitable for use in optimizing challenge models for vaccine efficacy trials.

In shrimp, selective breeding for resistance against TSV started in the mid-1990s after an epizootic that devastated the *Litopenaeus vannamei* with cumulative mortality reaching up to 80–90% (Lightner et al., 2009; Moss et al., 2012). Argue

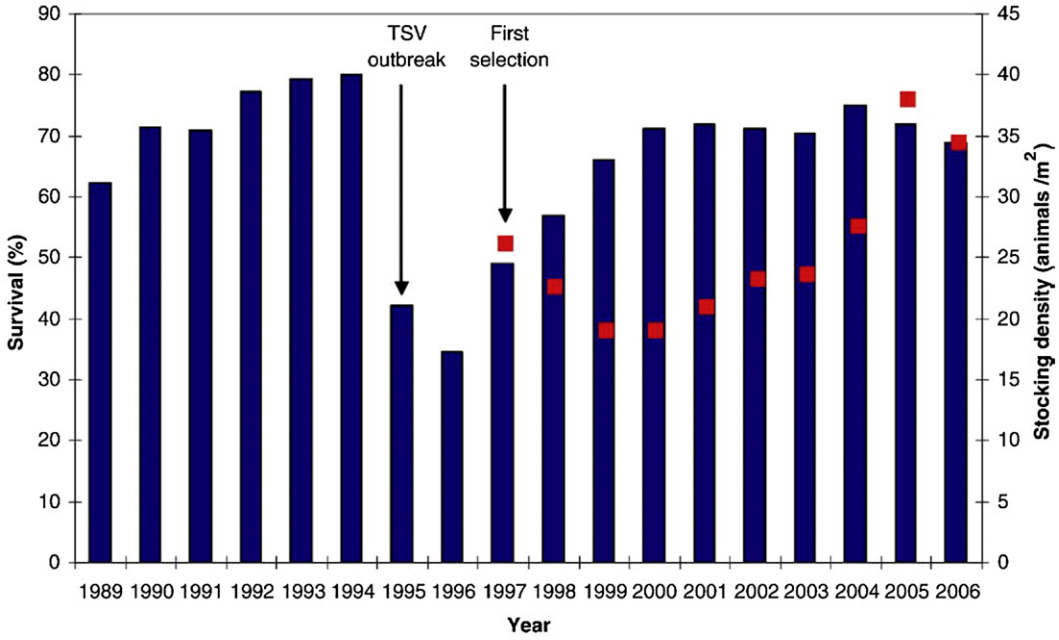

FIGURE 5.2 Survival of shrimp following TSV infection in commercial ponds in the Atlantic Coast of Colombia. Red (gray in print versions) squares denote to stocking density while the blue (dark gray in print versions) bars denote the percentage of survivors. The virus first appeared in 1995, which led to a significant decrease in the percentage of survivors with the lowest amount of survivors being in 1996 on the Atlantic Coast of Colombia. Note that the percentage of survivors increased after introducing the selection for TSV-resistant shrimps in 1997 as source of breeding stock in shrimp production. *Adapted from Cock, J., Gitterle, T., Salazar, M., Rye, M., 2009. Breeding for disease resistance of Penaeid shrimps. Aquaculture 286, 1–11.*

et al. (2002) showed that selection of *L. vannamei* resistant families to TSV increased the survival rate by 18.4% after one generation compared with unselected families while White et al. (2015) reported a survival increase from 65% to 100% by selecting resistant families after 3 years of selective breeding. Selective breeding for TSV resistance carried out at the Oceanic Institute (OI, Waimanalo, HI) for 15 years has shown that several families of *L. vannamei* that exhibit 100% survival after TSV exposure have been identified (Moss et al., 2011). As shown in Fig. 5.2, selecting for disease resistance has led to the increase in shrimp production after the mid-1990s epidemic in South America (Cock et al., 2009). Other viral diseases for which traits for disease resistance have been explored in shrimp include WSSV, although it has been observed that heritability (h^2) traits for WSSV are lower than TSV (Gitterle et al., 2006a,b, 2005). However, it is important to point out that the conventional approach of selecting disease-resistant fish or shrimp based on postchallenge survivors does not underpin the genetic markers of disease resistance on host chromosomes and, thus, the use of molecular biology-based tools to identify the genetic markers of disease resistance has taken precedent in recent years.

5.3.2 Genetic Selection of Disease-Resistance Strains Based on Quantitative Loci Analysis

The use of high throughput genome mining techniques has led to identification of genetic markers linked to disease resistance in farmed aquatic organisms. The most common approach used to identify the genomic markers of disease resistance in fish is the quantitative trait loci (QTL) analysis (Goddard and Hayes, 2009), which links phenotypic traits to specific regions on fish chromosomes. In principle, QTL analysis is a statistical method that links phenotypic measurable traits to genotypic data in an attempt to explain the genetic basis of variability (Falconer and Mackay, 1996; Kearsey, 1998; Lynch and Walsh, 1998). To determine the QTLs for disease resistance, two parameters are essential: (1) phenotypic traits expressing disease susceptibility and resistance traits, and (2) genetic markers located on specific loci of host chromosomes that account for differences in the phenotypic traits. Fish expressing different phenotypic traits are crossed and thereafter challenged using a pathogenic virus strain to produce heterozygous individuals that respond differently to the challenge virus. QTLs are then identified using high throughput genome mining techniques such as RNA-seq to identify individuals whose QTL correlates with postchallenge mortality. The ultimate goal is to generate homozygous individuals whose QTL codes for susceptibility or resistance to virus infections.

TABLE 5.4 Fish Species and Viruses Where Genetic Markers of Resistance and Immunity Have Been Determined

Trait	Virus Family	Fish Species	References
A: Disease Resistance QTLs/Heritability			
IPNV resistance	*Birnaviridae*	Atlantic salmon	(Houston et al., 2010; Houston et al., 2008)
IPNV resistance	*Birnaviridae*	Rainbow trout	(Ozaki et al., 2001)
ISAV resistance	*Orthomyxoviridae*	Atlantic salmon	(Moen et al., 2007; Odegard et al., 2007a; Odegard et al., 2007b)
SPDV resistance	*Togaviridae*	Atlantic salmon	(Norris et al., 2008)
VHSV resistance	*Rhabdoviridae*	Rainbow trout	(Verrier et al., 2013a; Verrier et al., 2013b)
VHSV resistance	*Rhabdoviridae*	Turbot	(Rodriguez-Ramilo et al., 2014)
IHNV resistance	*Rhabdoviridae*	Rainbow trout	(Palti et al., 1999)
LCD resistance	*Iridoviridae*	Japanese flounder	(Fuji et al., 2006)
Koi herpesvirus	*Herpesviridae*	Carp (*Cyprinus carpio*) strains	(Odegard et al., 2010a)
NNV resistance	*Nodavirus*	Atlantic cod (*Gadus morhua* L.)	(Bangera et al., 2014; Bangera et al., 2013; Odegard et al., 2010b)
TSV resistance	*Dicistroviridae*	Pacific white shrimp	(Odegard et al., 2011)
WSSV resistance	*Nimaviridae*	Indian black tiger shrimp	(Robinson et al., 2014)
B: Immune-Related QTL/Markers			
VHSV innate immune QTL	*Rhabdoviridae*	Rainbow trout	(Verrier et al., 2013a)
IHNV-MHC-loci	*Rhabdoviridae*	Rainbow trout	(Palti et al., 1999)
IHNV-MHC-loci	*Rhabdoviridae*	Atlantic salmon	(Miller et al., 2004)
ISA-MHC-I and II loci	*Orthomyxovirus*	Atlantic salmon	(Grimholt et al., 2003)
Natural killer cell activity	*Birnaviridae*	Rainbow trout	(Zimmerman et al., 2004)
QTL innate immunity		Tilapia	(Cnaani et al., 2004)

Postchallenge survival data as a marker for resistance or susceptibility has been widely used as a phenotypic trait for identifying QTLs in fish. For example, Moen et al. (2009) used postchallenge survival data as a measurable trait to identify QTLs for susceptibility and resistance to IPNV infection in Atlantic salmon. Similarly, Houston et al. (2010) and Gheyas et al. (2010a,b) have shown that the susceptibility of Atlantic salmon fry to IPN is largely explained by QTL analysis and these QTLs account for susceptibility during the freshwater and seawater stages of the Atlantic salmon production cycle, suggesting that these QTLs can be used for identification of IPNV-resistant fish at an early stage of the Atlantic salmon production. In terms of genetic markers, the most commonly used QTLs are single nucleotide polymorphism (SNPs) (Houston et al., 2014) and microsatellites (Ozaki et al., 2001), although other genetic markers are being sought (Palti et al., 1999; Verrier et al., 2013). For example, Ozaki et al. (2001) used microsatellites to identify QTLs linked to IPN-resistance (YN-RT201) and susceptibility (YK-RT101) on chromosomes A (IPN R S-1) and C (IPN R/S-2), respectively. Similarly Moen et al. (2009), used microsatellite markers to identify QTLs linked to IPNV mortality in Atlantic salmon, showing that microsatellites can be used to identify genetic markers for IPNV susceptibility and resistance in both rainbow trout and Atlantic salmon. On the other hand, Houston et al. (2012) used genome-wide restriction site-associated DNA (RAD) markers to identify a SNP that correlates with IPNV-induced mortality in Atlantic salmon. Overall, these studies show that QTLs generated by microsatellites and SNPs have great potential for use in marker-assisted selection (MAS) for IPN resistance in salmonids.

Table 5.4 shows that salmonids are the leading fish species that have attracted a lot of research into identification of QTLs for disease resistance (Houston et al., 2008). Identified QTLs for IPNV are being incorporated into MAS schemes for the genetic improvement of disease resistance in Atlantic salmon in Norway and Scotland (Houston et al., 2010; Moen et al., 2009). For salmon alphavirus type 3 (SAV3), differences in susceptibility have been observed in Norway and research

is ongoing to establish the QTLs for SAV3 resistance in Atlantic salmon. Similarly, in Iceland efforts are ongoing to establish a brood-stock resistant to SAV1 infection of which QTL selection is in the early stages (Mcloughlin and Graham, 2007). For infectious salmon anemia (ISA), two QTLs for resistance have been detected in Atlantic salmon (Moen et al., 2004; Moen et al., 2007) while in rainbow trout, major diseases for which genetic markers for resistance have been identified this far include infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV) and IPNV (Table 5.4). As shown in Table 5.4, studies on genetic selection for disease resistance in shrimp have also started (Robinson et al., 2014). In general, these studies have paved the way to selective breeding of disease-resistant farmed aquatic organisms, which is likely to reduce the prevalence of some of the infectious disease currently ravaging the aquaculture industry. Given that selective breeding has only gained application in recent years, existing data are insufficient to draw conclusions. Thus, the efficacy of this disease control strategy is yet to be determined, although preliminary observations suggest that it is positively contributing to the reduction of IPNV outbreaks in Norway.

5.4 DISEASE CONTROL BY ERADICATION

The central goal of eradication is to eliminate the disease from a defined geographical area (Cockburn, 1961). In principle, eradication ensures that the prevalence of a disease is reduced to zero with or without the use of intervention measures and that there is total cessation of disease transmission in the defined geographical area. Although eradication has rarely been used for the elimination of viral diseases in aquaculture, lessons learnt from the eradication of VHSV in Denmark (Jensen et al., 2014) and spring viremia of carp virus (SVCV) in the United Kingdom (Taylor et al., 2013) suggest that fish viral diseases can be eradicated from different countries in the world. For a viral pathogen to continue reproducing itself in a population, it must be transmitted from one susceptible individual to another. Therefore, the reproductive index (R) of the pathogen increases as transmission increases ($R > 1$), which in most cases culminates into an outbreak or epidemic depending on the number of new cases. On the contrary, a decrease in transmission results in a reduced reproductive index ($R < 1$), of which continued reduction can culminate in total elimination of the pathogen ($R = 0$). Therefore, in disease eradication, the target is to design intervention strategies aimed at eliminating the pathogen from a susceptible population ($R = 0$). As shown in Fig. 5.3, disease eradication is a gradual process that starts with high disease prevalence to total elimination of the disease in a defined geographical area based on the steps described next.

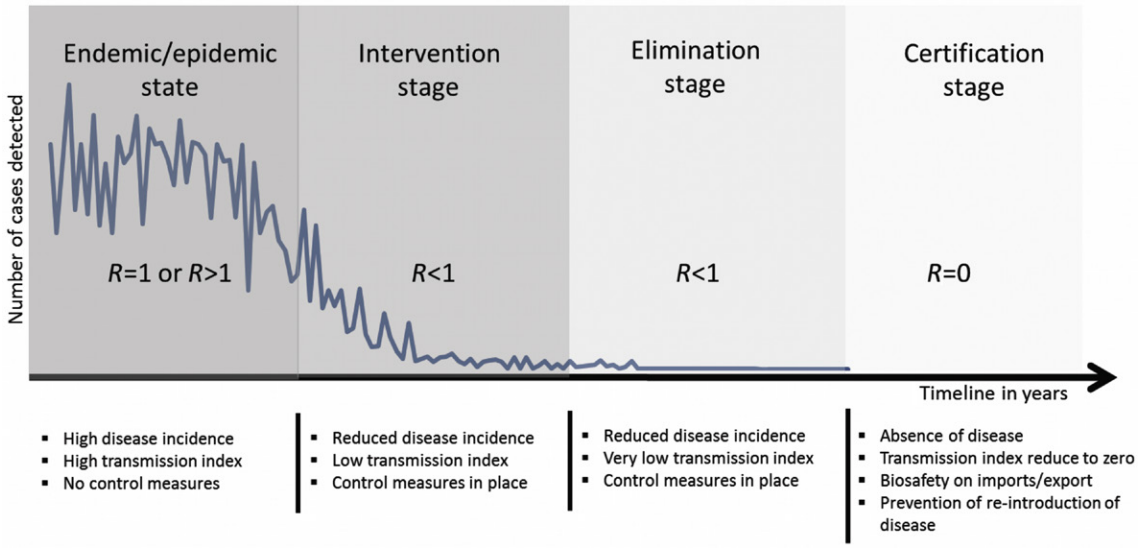

FIGURE 5.3 Timeline decline of disease eradication programs from an endemic or epidemic state to elimination of the pathogen in a defined geographical area. $R \geq 1$ shows that the reproduction index is high during the endemic/epidemic period when transmission and disease incidence is high in the susceptible population due to lack of control measures. During the intervention period the reproductive index of the pathogen is low, which is characterized by the decline in the number of cases ($R < 1$) due to introduction of disease control measures. The elimination stage shows insignificantly low cases ($R < 1$). Certification is only achieved after a period of continuous absence of the disease ($R = 0$) for a number of years. Decrease in color shading denotes the progressive decline of the disease prevalence from an endemic or epidemic state to total elimination of the disease.

5.4.1 Endemic/Epidemic State

An endemic state can be defined as the constant presence of the disease in a population or area where a stable state exists between the host and pathogen ($R \approx 1$), while an epidemic state refers to a sporadic increase in the number of disease cases in a defined geographical area ($R > 1$). To design an effective disease eradication program, it is important to carry out extensive surveys during the endemic or epidemic period in order to identify the intervention points in the fish production cycle when the disease can be eliminated. Data obtained should include the prevalence of the disease, distribution, high-risk areas, reservoir hosts, factors that enhance disease transmission and the timing when transmission can be reduced ($R < 1$) during the fish production cycle. For example, in the case of VHSV in Denmark, the disease was endemic in the 1960s, when more than 50% of the farms were infected (Kahns et al., 2012) and surveillance studies during this period showed that transmission of the virus occurred via river water from upstream to downstream farms following the river flow (Olesen and Korsholm, 1989). Surveillance data also showed that outbreaks occurred between spring and early summer, when higher temperatures shortened the duration of clinical infection and virus shedding, which set the timing that all stamping out activities were to be carried out when temperatures were high to minimize the occurrence of persistently infected wild fish (Jensen et al., 2014; Olesen and Korsholm, 1989). Thus, surveillance data obtained during the endemic period was useful in designing an intervention strategy that was used to reduce the occurrence of VHSV in Denmark in subsequent years.

5.4.2 Intervention Stage

Intervention in disease eradication is mainly focused on reducing disease transmission in a susceptible population ($R > 1$). The most effective intervention strategy used for fish viral diseases is the stamping out of all fish from infected farms, followed by disinfection to ensure total elimination of the infectious agent from all fomites, vehicles, equipment, fish-rearing facilities and environment followed by fallowing. Only fish certified free of the eliminated disease should be used for restocking and the infectious agent must have no reservoir hosts in the environment during eradication. Extensive surveillance should be carried out using diagnostic tests with high sensitivity and specificity to evaluate the efficacy of the intervention strategy.

In Denmark, eradication of VHSV was carried out using a stepwise approach in which fish on upstream farms were eliminated first, followed by those in the downstream farms. All stamping out activities were carried out in spring to early summer when temperatures were high. All fish on infected farms were culled and the farms were completely emptied, all ponds were drained-off, facilities were completely dried off and disinfected using calcium hydroxide. This was followed by fallowing for a minimum period of 6 weeks, after which farms were restocked with fish certified free of VHSV. All eradication activities were carried out with voluntary payment of eradication costs by the fish farmers. Consequently, this led to the significant decline on the number of infected farms, such that by 1990 more than 90% of the fish farms were free of VHSV (Fig. 5.4). However, total eradication could not be implemented based on farmers' voluntary participation. Thus, a more stringent approach was implemented, which demanded compulsory removal of all fish from the remaining infected farms and was supported by compensation from the Danish government to the farmers. The Danish Veterinary Services then prepared a legislature to eradicate VHSV from all infected farms, which paved the way to total elimination of the disease (Kahns et al., 2012). In the case of SVCV in the United Kingdom, more than 108 cases were reported by 1997, which declined drastically by 2005 after introducing disease-control measures that led to the elimination of the disease from United Kingdom (Taylor et al., 2013).

5.4.3 Elimination Stage

Elimination aims at exterminating the disease from the infected areas in order to reduce transmission to zero ($R = 0$). This is carried in the final stages of the intervention programs by stamping-out of all fish from the remaining infected farms, followed by extensive surveillance to ensure total absence of the disease for at least two fish production cycles. Continuous absence of the disease after extensive surveillance for at least two fish productions cycles entails that the area can be certified free of the eradicated disease. In Denmark, the final eradication programs for VHSV was started in 2009 when it was mandatory to cull all fish on infected farms and the government compensated the fish farmers for all losses (Jensen et al., 2014). Thereafter, intensive surveillance was carried out each spring-to-summer for 2 years to ensure that VHSV was not detected on any fish farms in Denmark.

The United Kingdom officially entered into the program of eliminating SVCV, which was implemented by the Fish Health Inspectorate in 2005 (Taylor et al., 2013). To maximize the likelihood of detecting the virus during eradication, surveillance was carried out during the high-risk period when water temperatures were between 10°C and 17°C, which is ideal

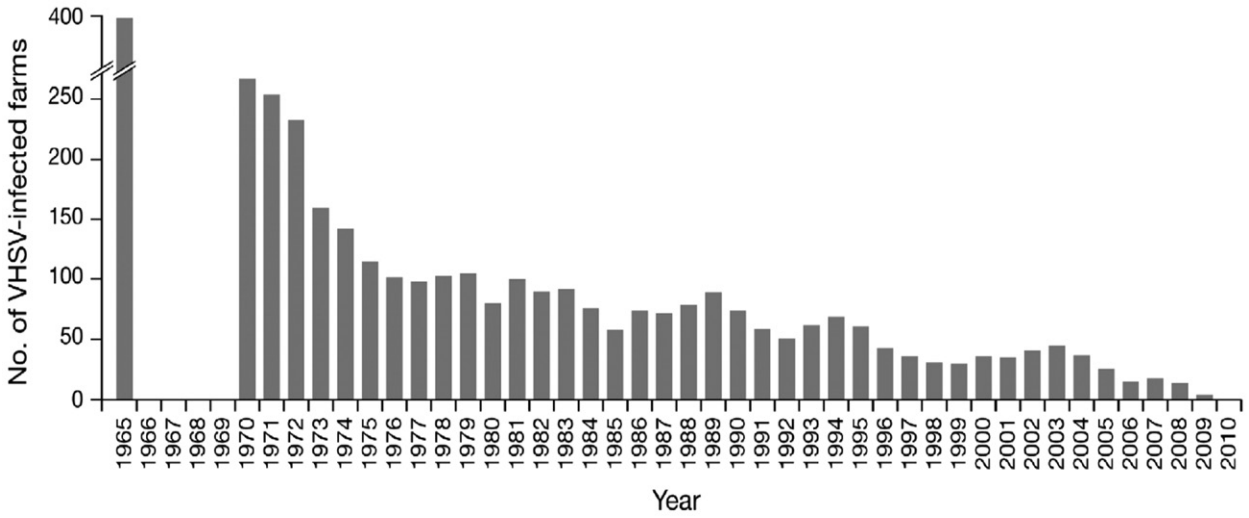

FIGURE 5.4 The progressive decline of viral hemorrhagic septicemia virus (VHSV) cases during the eradication program implemented in Denmark for the period from 1965 to 2010. Note that the last cases of VHSV were reported in 2009 and no cases were reported in 2010. *Adapted from Jensen, B.B., Ersboll, A.K., Korsholm, H., Skall, H.F., Olesen, N.J., 2014. Spatio-temporal risk factors for viral haemorrhagic septicaemia (VHS) in Danish aquaculture. Dis Aquat Organ 109, 87–97.*

for the virus to replicate in the host when transmission between fish is high (Ahne et al., 2002). All testing was carried out using cell culture for virus isolation and confirmation by RT-PCR (Stone et al., 1997), as recommended and validated by the Office International des Epizooties (2009). In line with the EU guidelines, 30 samples, each comprising pooled tissues from five individual fish, were used for laboratory diagnosis, which provided a 95% chance of detecting the pathogen in a susceptible population (Taylor et al., 2013). Sites confirmed to be positive for SVCV had all fish culled, followed by implementation of biosecurity measures. All sites that had SVCV were monitored until three consecutive negative annual tests showed absence of the disease, and by 2007 no further cases were detected in the United Kingdom (Taylor et al., 2013), which enabled application of an SVCV disease-free status for the European Union.

5.4.4 Certification Stage

Certification entails recognition of the absence of the eradicated disease by international organizations, such as the OIE and European Union, that regulate the trade of farmed aquatic organisms and their products. This promotes free trade and eliminates movement restrictions imposed on countries previously infected with high-risk diseases. After the eradication of VHSV, Denmark applied to the European Union for the certification of being free of VHSV and it was granted the VHSV free status in 2013. Similarly, the United Kingdom was declared free of SVCV in 2010 by the European Union (EC Decision 2010/221/EC) (Taylor et al., 2013). It is important, however, to point out that restriction of introduction of the disease from outside sources should be carried out by ensuring that imported fish products from outside sources are free of the eradicated disease.

5.5 DISEASE PREVENTION BY VACCINATION

Vaccination in aquaculture has emerged as one of the most effective disease preventive strategies, evidenced by the fact that vaccinated fish are less at risk of contracting viral infections compared with unvaccinated fish. However, a successful vaccine in the modern era requires antigen identification on the surface of the pathogen, which should be recognized by the immune system in order to induce protective immune responses (Gudding et al., 1999; Munang'andu et al., 2013). As a result, the choice of antigen delivery system in vaccine design is becoming wide because vaccines can be made from inactivated whole virus (IWV) or attenuated live virus, expressing immunogenic epitopes in their native form or the use of molecular biology-based tools to clone the immunogenic proteins in heterologous vectors (Munang'andu et al., 2012). The choice of antigen delivery system is dependent on several factors that include safety, production costs, efficacy and type of immune response induced (Tafalla et al., 2014).

5.5.1 Types of Vaccine

Vaccines used in aquaculture for fish viral diseases can be classified into two broad categories, namely replicative and nonreplicative vaccines.

5.5.1.1 Nonreplicative Vaccines

Nonreplicative vaccines include IWV, immunogenic component of the native virus cloned in heterologous vectors, as subunit vaccines or synthetic peptides linked to carrier proteins. The main advantage for nonreplicative vaccines is their safety because inactivated virus pathogens do not cause disease (Munang'andu et al., 2014).

5.5.1.1.1 IWV Vaccines

Inactivated or "killed" vaccines account for the largest proportion of vaccines currently used in aquaculture. They are based on whole virus inactivation, using chemical compounds that alter the intracellular replication capacity of the virus by irreversible modification of the viral genome with moderate alterations of the outer capsid morphology and the immunogenic protein(s) (Tafalla et al., 2014). The major limitation for these vaccines is that they do not induce cellular immune responses, or do so to a very limited extent, while humoral immune response induced by these vaccines is short lived, unless formulated for antigen retention at the injection site. Thus, they require the use of adjuvants to form a depot at the injection site for the slow release of the antigen to prolong the duration of the immune response.

5.5.1.1.2 Subunit Vaccines

The basic principle of subunit vaccine is that the gene encoding the immunogenic protein is isolated form the native virus and transferred into a heterologous vector for replication. Genes encoding antigenic proteins for viral antigens are structural proteins that form the outer capsids, while the choice of heterologous vectors used to express these proteins is highly governed by their efficiency in expressing the conformational structure of the antigenic proteins. For example, nodavirus structural proteins expressed in *Escherichia coli* spontaneous self-assemble into a nonenveloped $T = 3$ quasi symmetrical capsid resembling the native virus structural protein (Lin et al., 2001; Tang et al., 2002), while the VP2 structural proteins of IPNV expressed in yeast cells have also been shown to spontaneously self-assemble into virus-like particles (VLPs) that form the outer capsid resembling the capsid of the native virus (Allnutt et al., 2007; Shivappa et al., 2005). Given the similarity of these protein structures to their native capsid, these subunit vaccines create a good basis for displaying the immunogenic epitopes encoded in the virus genome. Structural proteins for different fish viruses used for the production of subunit vaccines and the heterologous vectors used to express the different immunogenic proteins have recently been reviewed by different scientists (Gomez-Casado et al., 2011; Munang'andu et al., 2014). In general subunit vaccines do not induce cellular immune responses (in general) and are confined to humoral immune responses because they are nonreplicative and are delivered by the exogenous route to the antigen presenting cell.

5.5.1.1.3 Peptide Vaccines

Peptide vaccines use short fragments of amino acid sequences encoding the immunogenic component of the native virus and, because of their limited size, they are not potent enough to require coupling to carrier molecules/haptens. This mode of vaccine production is not widely used in the production of fish vaccines apart from one study (Emmenegger and Kurath, 2008) that made a synthetic peptide vaccine for IHNV by coupling the immunogenic protein to bovine serum albumin. To enhance its immunogenicity, the vaccine was delivered in Freud's complete adjuvant intraperitoneally.

5.5.2 Replicative Vaccines

Replicative vaccines are live viruses or recombinant antigens encoded by live virus vectors with the capacity to replicate in host cells with altered pathogenicity, lacking the ability to cause disease. Replicative vaccines can be classified into live vaccines derived from variant strains of the pathogenic native virus or DNA vaccines encoding the immunogenic protein of the native virus.

5.5.2.1 DNA Vaccines

This mode of vaccination is based on the principle that the plasmid DNA, which encodes the immunogenic protein, is injected into host tissues where it enters the cells and is transcribed in vivo to direct the synthesis of its polyprotein antigen

from the plasmid vector. In general, the transcribed polyprotein self-assembles into a capsid structure that resembles the native virus protein and evokes immune responses comparable to the native live virus infection. Thus, this mode of vaccination evokes both cellular and humoral immune responses. Fish DNA vaccines have been reviewed by different scientists (Evensen and Leong, 2013; Gillund et al., 2008; Lorenzen and LaPatra, 2005; Salgado-Miranda et al., 2013). However, only the DNA vaccine against IHNV has been licensed for use in aquaculture (Novartis, Canada). The major drawback to licensure of fish DNA vaccines is their inability to induce protective immunity that surpasses the IWV vaccines, except for the rhabdoviruses. Generally, DNA vaccines are inferior to IWV vaccines in fish vaccinology. It is not known whether this is due to failure of host cells to express immunogenic proteins in vivo or the inefficiency of the promoter genes used. Therefore detailed studies to elucidate the factors linked to poor performance of DNA vaccines in aquaculture are necessary.

5.5.2.2 Live Virus Vaccines

Live virus vaccines can be made from antigenic components encoded in live recombinant vectors or derived from attenuated pathogenic virus with altered pathogenicity that replicate in host cells without causing disease. These viruses replicate inside host cells and induce cellular and humoral immune responses, immune responses comparable to the native virus. The viruses induce a longer immune response than the inactivated viral vaccines because they are replicative and, thus, they do not need the support of adjuvants. Several methods for generating live vaccines have been explored and these include the selection of naturally occurring avirulent strains (Roberti et al., 1998), attenuation by serial passages (Fryer et al., 1976; Tebbit, 1976) and attenuation of virulent viral strains by reverse genetics to generate avirulent strains that can be used as vaccine strains (Munang'andu et al., 2013; Santi et al., 2004). However, only a few live viral vaccines have been licensed in aquaculture thus far. A live vaccine against VHSV was licensed in Germany, but this vaccine is no longer available on the market. In Israel, a live vaccine obtained by natural selection of an avirulent strain for Koi herpesvirus has been licensed and is currently available on the market (Enzmann et al., 1998). The safety profile of the vaccine is uncertain. In general, only a few live vaccines have been licensed for use in aquaculture.

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Chapter 6

Determinants of Emergence of Viral Diseases in Aquaculture

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6.1 INTRODUCTION

The intensive water rearing of fish, mollusks and crustaceans remains the world's fastest growing sector producing food of animal origin. In fact, it is the only animal food-producing sector growing faster than the human population, and provides an acceptable supplement to and substitute for wild fish. Fish, crustaceans and mollusks represent the most economically important global aquaculture industry sectors, at 60 million tonnes in 2010 with an estimated total farmgate value of US$119.4 billion (FAO, 2012) that is expected to reach US$202.96 billion by 2020 (Grand View Research Inc., 2014). For the first time, in 2014, aquaculture contributed more to aquatic animal food destined for human consumption than fisheries. This share of aquaculture is projected to rise to 62% by 2030, as catches from wild capture fisheries level off (FAO, 2014). However, the intensification of aquaculture and increased densities involve substantial animal stress, which facilitates diseases. Aquaculture presents unique challenges unlike all other intensive animal production systems because the aquatic farmed and wild animals occur in the same water column, and the aquatic environmental parameters cannot be very closely controlled (as, eg, in the poultry and swine industries). Consequently both farmed and wild aquatic animals in the same water column experience the same pathogen challenges (Kurath and Winton, 2011; Saksida et al., 2014; Jones et al., 2015), and absolute bio-containment cannot be attained (Uglem et al., 2009; Arechavala-Lopez et al., 2013).

Generally, viruses in nature (aquatic or terrestrial) coevolve with their hosts within their natural range to ensure their long-term survival. Viral diseases occur in both farmed and wild aquatic animals; normally, the aquatic viruses have their natural reservoir in wild aquatic animals (Hill, 2005; Raynard et al., 2007; Snow et al., 2010; Johansen et al., 2011; Garver et al., 2013; Taranger et al., 2015) where they are often not sufficient to sustain the natural transmission cycle density (Ward and Lafferty, 2004), which is readily facilitated by aquaculture (Murray, 2009; Rimstad, 2011). The high-density confinement within the aquaculture environment and chronic stress (Snieszko, 1974; Wedemeyer, 1996; Weyts et al., 1999; Yada and Nakanishi, 2002) provide opportunities for the emergence of diseases caused by pathogens that may be harmless under natural conditions (Kurath and Winton, 2011). In addition, the burgeoning international aquaculture expansion and expanding global trade in live aquatic animals and their products facilitates long-distance geographical redistribution of aquatic animal species and their viruses. The most knowledge is regarding viral diseases of farmed aquatic animals (Kurath and Winton, 2011) because of the advances in health management in aquaculture, with viral diseases in wild aquatic animals mainly studied in an opportunistic manner, for example, during episodes of large-scale die-offs of wild aquatic animal species (Stephens et al., 1980; Hyatt et al., 1997; Hedrick et al., 2000; Whittington et al., 2008; Gaughan et al., 2000; Skall et al., 2005; Garver et al., 2010; Moreno et al., 2014). The wild aquatic animals that overcome the disease become asymptomatic carriers, exhibiting light viral loads and constantly excreting viruses (Gozlan et al., 2006). Increased aquaculture production will be accompanied by increased disease risk, thereby ensuring that conditions for exposure to and the spread of aquatic viruses will persist. The viral genetic features that account for the emergence, virulence and persistence of aquatic viruses are not well studied. Improved methods for laboratory diagnosis and pathogen surveillance, and more extensive molecular analyses of viruses from farmed and wild aquatic organisms, will improve our understanding of the many complex factors that underlie viral disease emergences in aquaculture and fisheries.

Rosenthal et al. (2015) reviewed the evolution of the term emerging infectious disease (EID) in the literature as applied to human hosts, and by examining the pathways through which diseases emerge, proposed a new framework for classifying pathogens and diseases as "emerging" that distinguishes EIDs from emerging pathogens and novel potential pathogens. The current definition of an EID is that which has "recently increased in incidence, impact or geographic range. Specifically, it

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00006-1
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is caused by a pathogen that has recently evolved or entered the human population for the first time, or which has occurred previously, but is increasing in incidence or expanding into an area in which it has not previously been reported, or which has significantly changed its pathological or clinical presentation" (Jones et al., 2008, p. 38). The timeframe for emerging pathogens in human populations is "usually over the last 20 years" (Taylor et al., 2001). Most recently, Engering et al. (2013) categorized EIDs into three main groups, with emergence of: (1) a pathogen in a novel host; (2) a pathogen with novel traits within the same host and (3) a disease complex moving into a novel geographical area; all three categories are driven by human actions that modulate the interplay between pathogens, hosts and the environment. However, viral pathogens can also appear to emerge or reemerge simply because of increased awareness, improved diagnosis and the discovery of previously unrecognized etiological agents of existing diseases (Woolhouse and Dye, 2001). The World Organisation for Animal Health (OIE) Aquatic Animal Health Code (2015) (Aquatic Code) defines an emerging disease as "a disease, other than listed diseases, which has a significant impact on aquatic animal or public health resulting from: (a) a change of known pathogenic agent or its spread to a new geographic area or species; or (b) a newly recognized or suspected pathogenic agent." The Aquatic Code mandates Member Countries to report emerging diseases in aquatic animals (World Organisation for Animal Health (OIE), 2015), as does the European Fish Health Legislation (EC, 2006) for EU member states. Walker and Winton (2010) reviewed the major emerging viral diseases of fish and shrimp that cause significant losses in aquaculture and are expanding in host or geographic range.

In this chapter, emerging aquatic animal viruses are grouped into eight categories: (1) a virus introduced in a new host and new geographic area; (2) a virus infecting the same host but introduced in a new geographic area; (3) enzootic virus with increased pathogenicity in same host (manifesting as increased incidence or more severe disease); (4) enzootic virus with increased host range; (5) enzootic virus with expanded geographic range; (6) increased awareness; (7) improved diagnosis and surveillance and (8) newly discovered viral cause of existing disease. Categories (1) and (2) would originate from an existing viral disease conveniently in wild aquatic animals, with the virus able to jump to a new host species ("species jump" or "host jump") and/or transported over long distances (geographic jump) whereas categories (3)–(5), in addition, require mutations in the infecting virus (virulence jump). A successful "species jump" by a virus and/or "virulence jump" may be accompanied by a "geographic expansion" associated with natural movement of the infected hosts. Categories (6)–(8) constitute "emergence of knowledge" (Gessain and García-Arenal, 2015). Thus, as observed for human EIDs (Rosenthal et al., 2015), an emerging aquatic animal viral disease may fit in several of the eight categories, depending on the sequence of events involved and geographical area concerned (Table 6.1). For example: (a) the emergence of infectious hematopoietic necrosis virus (IHNV) in rainbow trout aquaculture in Europe, Japan and Korea as a result of shipments of IHNV-contaminated rainbow trout eggs or fry (Bootland and Leong, 1999) (ie, category (2)) was accompanied by mutations in the virus related to host specificity and virulence (ie, categories (3) and (5)) (Penaranda et al., 2009); (b) the emergence koi herpesvirus (KHV; *Cyprinid herpesvirus 3*, CyHV3) as a result of movement of latently-infected (carrier) koi carp via the extensive trade in cultured ornamental fish (Haenen et al., 2004); (c) the emergence of viral hemorrhagic septicemia virus (VHSV) in marine fish as a result of both improved surveillance efforts (Mortensen et al., 1999; Gagne et al., 2007) and development of new marine-farmed aquaculture species (Isshiki et al., 2001); (d) the emergence of VHSV IVb in the Great Lakes Basin of North America, likely a result of the natural movement of infected fish up the Saint Lawrence River from the near-shore areas of the Atlantic coast of Canada (Groocock et al., 2007; Walker and Winton, 2010) and via baitfish (Bowser, 2009) or through introduction by ballast water from ships (Bain et al., 2010) (ie, categories (4) and (5)); (e) the emergence of infectious salmon anemia virus (ISAV) in Atlantic salmon aquaculture in Chile, likely a result of shipments of ISAV-contaminated Atlantic salmon eggs (Vike et al., 2009) (ie, category (2)) was accompanied by mutations in the virus related to virulence (ie, categories (3) and (5)) (Kibenge et al., 2009) that are still continuing (Godoy et al., 2013, 2014); (f) the emergence of white spot syndrome virus (WSSV) throughout Asia and the Americas (Lightner et al., 1997; Walker and Mohan, 2009), the rapid spread of Taura syndrome virus (TSV) in the Americas and then to Asia, emergence of infectious myonecrosis virus (IMNV) in Indonesia, Thailand and China (Senapin et al., 2007) are all attributed to a prolific international trade in live shrimp and other crustacean seed and broodstock (Walker and Winton, 2010) and an expanded host range and (g) the emergence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Americas through importation of infected *Penaeus monodon* from the Philippines, resulting in an expanded host range providing opportunities for pathogenicity and a vastly expanded geographic distribution (Tang and Lightner, 2002) (ie, categories (1)–(5)).

The drivers (attributes or causal factors) responsible for the emergence of viral diseases in aquaculture can be categorized into virus drivers, animal host drivers, environment drivers and anthropogenic drivers (Fig. 6.1 and Table 6.2). The interplay of the virus–host–ecosystem then manifests into new or previously unrecognized viral diseases or reemerging viral diseases in aquaculture.

TABLE 6.1 Categories of Emerging[a] Aquatic Animal Viruses With Examples

Aquatic Animal Group	Emerging Aquatic Animal Viruses							
	Newly Introduced		Enzootic			Increased Awareness	Improved Diagnosis and Surveillance	Newly Discovered Cause of Existing Disease
	To New Host, New Geographic Area	To New Geographic Area, Same Host	Increased Pathogenicity in Same Host (Increased Incidence or More Severe Disease)	Increased Host Range	Expanded Geographic Range			
Fish							SGPV (Gjessing et al., 2015)	
	EHNV in Australia (Hedrick and McDowell, 1995; Whittington et al., 2010)			EHNV (Hyatt et al., 2000; Whittington et al., 2010)				
	MCIV in Australia (Lancaster et al., 2003; Whittington and Chong, 2007)	*Megalocytivirus* in Japan, south China Sea and southeast Asian countries (Sudthongkong et al., 2002)	RSIV in Japan (Matsuoka et al., 1996; Miyata et al., 1997)	RSIV (Kawakami and Nakajima, 2002)				ISKNV (Lu et al., 2005)
								Herpesvirus-induced mass mortality in the pilchard *Sardinops sagax neopilchardus* in Australia and New Zealand (Jones et al., 1997; Hyatt et al., 1997; Whittington et al., 2008)
		CyHV-2 in Australia (Stephens et al., 2004; Whittington and Chong, 2007); CyVH-3 global epidemic (Hedrick et al., 2000; Gilad et al., 2003; Haenen et al., 2004; Uchii et al., 2009)						
		IHNV in Europe, Japan and Korea (Enzmann et al., 2005; Kim et al., 2007; Nishizawa et al., 2006)						

(*Continued*)

TABLE 6.1 Categories of Emerging[a] Aquatic Animal Viruses With Examples (Continued)

Aquatic Animal Group	Emerging Aquatic Animal Viruses							
	Newly Introduced		Enzootic			Increased Awareness	Improved Diagnosis and Surveillance	Newly Discovered Cause of Existing Disease
	To New Host, New Geographic Area	To New Geographic Area, Same Host	Increased Pathogenicity in Same Host (Increased Incidence or More Severe Disease)	Increased Host Range	Expanded Geographic Range			
	VHSV IVb in Great Lakes (Elsayed et al., 2006; Groocock et al., 2007; Lumsden et al., 2007)	VHSV III in farmed rainbow trout in Norway in 2007 (Dale et al., 2009)	VHSV III in farmed rainbow trout in Norway in 2007 (Snow and Cunningham, 2000; Dale et al., 2009)	VHSV IVa in Asia (Isshiki et al., 2001; Kim et al., 2009); VHSV IVa in Atlantic salmon in BC-Canada (Skall et al., 2005)	VHSV (Isshiki et al., 2001; Hedrick et al., 2003; Kim et al., 2009); VHSV IVb in Great Lakes (Elsayed et al., 2006; Groocock et al., 2007; Lumsden et al., 2007)		VHSV (Meyers and Winton, 1995; Mortensen et al., 1999; Skall et al., 2005; Gagne et al., 2007)	
				HIRRV in Japan (Kimura and Yoshimizu, 1991)				
		SVCV global epidemic (Ahne et al., 2002; Miller et al., 2007)					SVCV (Walker and Winton, 2010)	
		ISAV in New Brunswick-Canada, Maine, United States, United Kingdom, Faroes Islands (Kibenge et al., 2004); ISAV in Chile (Godoy et al., 2008, 2013, 2014; Kibenge et al., 2009; Vike et al., 2009)	ISAV in Chile (Godoy et al., 2014)	ISAV in Norway (Plarre et al., 2005)	ISAV in Nova Scotia and Newfoundland-Canada (CFIA, 2012)			
							TiLV in Israel and Ecuador (Bacharach et al., 2016)	
		SD in United Kingdom (Graham et al., 2007); SAV2 in Norway (Hjortaas et al., 2013)						

				PRV-HSMI like in rainbow trout (Olsen et al., 2015)		PRV in Canada and Chile (Kibenge et al., 2013)	PRV-HSMI (Palacios et al., 2010)	PRV-HSMI (Palacios et al., 2010)
							PMCV-CSM (Løvoll et al., 2010; Haugland et al., 2011)	PMCV-CSM (Løvoll et al., 2010; Haugland et al., 2011)
	Betanodavirus global epidemic (Munday et al., 2002)							
Crustaceans	WSSV in China in 1992 (Zhan et al., 1998)	WSSV in Americas in 2001 (Walker and Mohan, 2009)						
	IHHNV[b] in Hawaii in 1981 (Lightner et al., 1983); in Gulf of Mexico in 1987 (Morales-Covarrubias et al., 1999)							
	YHV in Thailand in 1990 (Chantanachookin et al., 1993); GAV in Australia in 1996 (Spann et al., 1997)	YHV in Asia (Walker et al., 2001); YHV in Mexico (De la Rosa-Velez et al., 2006)						
	TSV in Ecuador in 1992 (Hasson et al., 1995)	TSV in Asia (Tu et al., 1999; Nielsen et al., 2005)						
	IMNV in Brazil in 2002 (Lightner et al., 2004)	IMNV in Indonesia, Thailand and China (Senapin et al., 2007; Andrade et al., 2008)						
	MrNV in Caribbean (Arcier et al., 1999)				MrNV in China, Taiwan, Thailand and India (Tung et al., 1999; Qian et al., 2003; Yoganandhan et al., 2006)		MrNV in Australia (Owens et al., 2009); PvNV in Belize (Tang et al., 2007)	
Mollusks				AbHV (Hooper et al., 2007; OIE, 2015b)				

Virus name abbreviations: SGPV, salmon gill poxvirus; EHNV, epizootic haematopoietic necrosis virus; RSIV, red sea bream iridovirus; ISKNV, infectious spleen and kidney necrosis virus; MCIV, Murray cod iridovirus; CHV-3, Cyprinid herpesvirus 3; CHV-2, Cyprinid herpesvirus 2; IHNV, infectious hematopoietic necrosis virus; VHSV, viral hemorrhagic septicemia virus; SVCV, spring viremia of carp virus; ISAV, infectious salmon anemia virus; TiLV, Tilapia lake virus; SD, sleeping disease; SAV, salmon alphavirus; WSSV, white spot syndrome virus; IHHNV, infectious hypodermal and hematopoietic necrosis virus; YHV, yellow head virus; GAV, gill-associated virus; TSV, Taura syndrome virus; IMNV, infectious myonecrosis virus; MrNV, Macrobrachium rosenbergii nodavirus; AbHV, Abalone herpesvirus.

[a]*The term "emerging" is used here within the context of the timeframe for emerging pathogens in human populations, which is usually over the past 20 years (Taylor et al., 2001); in this case, since 1995. The list of examples is not exhaustive.*

[b]*IHHNV, infectious hypodermal and hematopoietic virus of prawns and shrimp (new name is Decapod penstyldensovirus 1).*

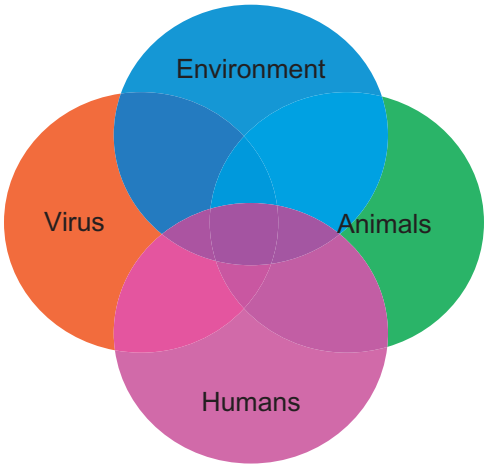

FIGURE 6.1 A venn diagram depicting the drivers of emerging viruses in aquaculture and their relationships.

TABLE 6.2 Drivers of Emergence of Viral Diseases in Aquaculture and Fisheries[a]

Drivers	Examples		
Virus drivers	Genetic variation in RNA viruses	Elevated rates of spontaneous mutation; very short replication times; generate very high virus yields	Viral RNA polymerase lacks proofreading-repair mechanisms; generate viral *quasispecies* clouds
		Genetic recombination mutation	Insertions; deletions
		Genome segment reassortment	
	Broad host range		
Animal host drivers	Host susceptibility	Species, age, nutritional status, stress, hormonal conditions, coinfections, genetic determinants	
	Natural animal movements	Migratory infected wild fish (asymptomatic carriers)	
		Scavenging wild fish	
		Scavenging wild birds	
		Aquatic turtles	
		Sea lice	
Environment drivers	Environmental factors	Oxygen saturation, salinity, temperature, pH, water flow	Aquatic viruses adapted to survive in water
	Climate change	Increases in temperature	
Anthropogenic drivers	Aquaculture (including intensive aquaculture in an enzootic area and global expansion of aquaculture)	Introduction of nonnative species	
		Expanding range of emerging farmed aquatic animal species such as Atlantic halibut, Arctic char, Sablefish, Atlantic cod, etc.	
		New production approaches such as integrated multitrophic aquaculture	
		High-density rearing of either genetically homogeneous single-species or mixed species aquatic animal hosts, of short lifespans	
		Use of wild broodstock that may be healthy carriers of viral pathogens	

(*Continued*)

TABLE 6.2 Drivers of Emergence of Viral Diseases in Aquaculture and Fisheries[a] (Continued)

Drivers	Examples		
	Global trade in live aquatic animals	Translocation of successfully farmed live aquatic animals to new destinations (eg, transfer of live nonindigenous fish for farming), particularly animals with low prevalence of clinical disease, subclinical infections or asymptomatic carrier hosts	
		Trade in ornamental aquatic animals	
		Shipment of infected/contaminated fish eggs	
	Global trade in aquatic animal products	Use of raw (unpasteurized) marine fish as feed	
		Importation of infected/contaminated fish carcasses	
	Anthropogenic drivers unrelated to aquaculture	Ballast water in ships	
		Movement of bait anglers	
		Climate change	
		Artificial fish aggregating devises (wharfs, seawalls) attract wild fish	

[a]*Refer to text for details.*

While the emphasis is on aquaculture, disease emergence is also a concern in wild fisheries due to environment drivers and anthropogenic drivers (Naish et al., 2008; Miller et al., 2014). An illustration of aquatic animal virus transmission pathways in wild and farmed populations is depicted in Fig. 6.2 by Arechavala-Lopez et al. (2013).

Finally, a separate distinct category needs to be recognized for those viral diseases whose incidence is decreasing as a result of anthropogenic means or through natural means because this is the ultimate goal of disease control and prevention. This category, for example, includes infectious salmon anemia (ISA), which has been eliminated in Scotland (Murray, 2013) and Faroe Islands (Christiansen et al., 2011).

6.2 VIRUS ATTRIBUTES THAT CONTRIBUTE MOST TO EMERGING VIRAL DISEASES IN AQUACULTURE

The pathogenicity of a virus (ie, the capacity of a virus to cause disease in a host) depends on both viral factors and host factors (eg, viral attachment proteins and matching cellular receptors, viral enhancers, etc.), and is defined in terms of virus dose (ie, amount of virus entering the host), route of entry of virus into host (skin, respiratory tract, gastrointestinal tract, genitourinary tract), age of host (young animals are usually more susceptible), immune status of host (immune system, cytokines, etc.) and host range (the animal species that support a productive infection). These properties are important determinants of whether a disease will emerge or remain sporadic (Rimstad, 2011).

The categories of emerging aquatic animal viruses that involve a "species jump" and/or "virulence jump" are facilitated by viruses with strong potential for genetic variation (giving rise to "host range mutants" and "virulent variants") and with a broad host range (Woolhouse et al., 2005). These features are especially common in RNA viruses (Table 6.2).

In general, RNA viruses (and DNA viruses that have RNA as replicative intermediate, eg, the DNA family *Hepadnaviridae*), have elevated rates of spontaneous mutation (typically on the order of 10^{-3} to 10^{-5} errors per nucleotide per round of replication for a 10-kb genome) (Drake et al., 1998; Drake and Holland, 1999; Chao et al., 2002; Domingo, 2010). The reason for this high mutation rate in RNA viruses is the viral RNA-dependent RNA polymerases (RdRp) and RNA-dependent DNA polymerases (RdDp) (or reverse transcriptases) have a high misincorporation frequency and lack 3′–5′-exonuclease activity and therefore have no proofreading-repair mechanisms (Ferrer-Orta et al., 2006; Menéndez-Arias, 2002; Steinhauer et al., 1992; Sanjuan et al., 2010). Moreover, the cellular post-replicative-repair mechanisms that act to correct mismatches in double-stranded (ds) DNA are not effective in repairing either dsRNA or RNA–DNA hybrids (Friedberg et al., 2006) and cannot operate on single-stranded (ss) RNA progeny genomes. In contrast, DNA viruses, particularly those with large genomes (poxviruses, iridoviruses, herpesviruses, adenoviruses) encode DNA polymerases with

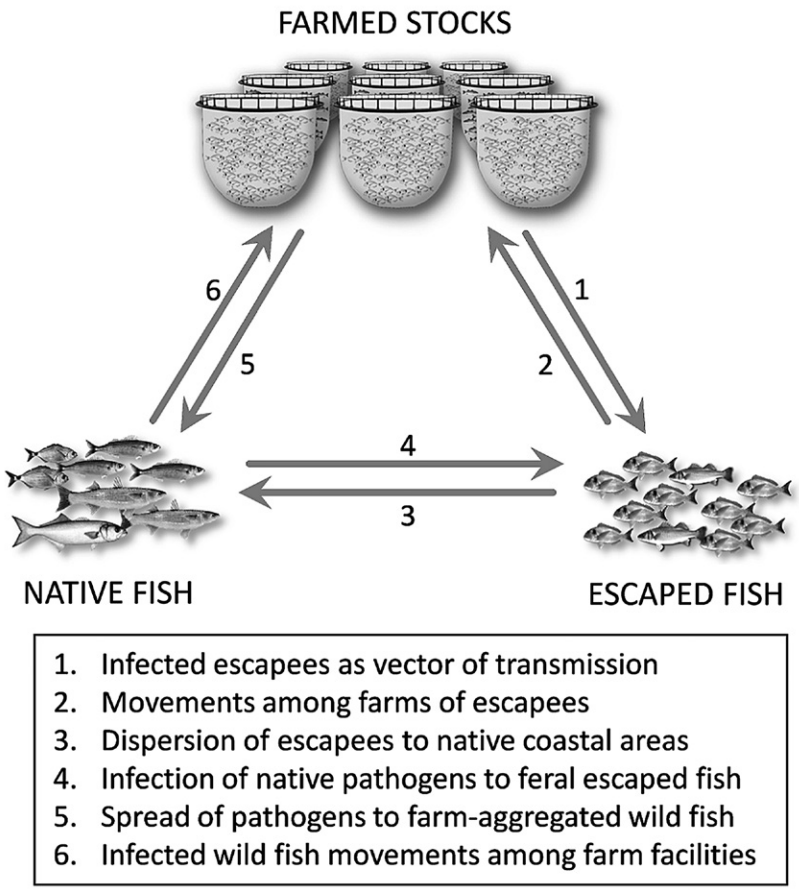

FIGURE 6.2 Generalized flow chart of potential pathogen transmission through movements of wild fish and escapees. *From Arechavala-Lopez, P., Sanchez-Jerez, P., Bayle-Sempere, J.T., Uglem, I., Mladineo, I., 2013. Reared fish, farmed escapees and wild fish stocks—a triangle of pathogen transmission of concern to Mediterranean aquaculture management. Aquac. Environ. Interact. 3, 153–161. Figure 1.1 Inter-Research 2013.* www.int-res.com*, with permission* http://creativecommons.org/licenses/by/3.0/legalcode *no changes were made.*

a proofreading 3′–5′ exonuclease activity similar to those in cellular DNA-dependent DNA polymerases; are expected to have lower mutation rates (Domingo, 2010), and are more often associated with virus–host cospeciation (Holmes, 2004; Shackelton and Holmes, 2004). Consequently, during their replication, RNA viruses exist as groups of closely related, viral genomes in sequence space (*viral quasispecies* cloud) (Holland et al., 1992; Eigen, 1993, 1996; Lauring and Andino, 2010; Domingo, 2006). The mutant clouds may include phenotypic variants, such as cell tropism and host-range mutants that can respond to selective constraints (Vignuzzi et al., 2006). During transmission, the "wild type" virus goes through a population bottleneck (only some virions succeed in infection of a new host), and those virions expand to a new quasispecies cloud in the new host. Mutations that are introduced during replication are tolerated because they are neutral for virus fitness in a particular environment, rapidly lost because they reduce fitness, or expanded because they are advantageous (Lauring and Andino, 2010). Thus, the multiphenotypic potential inherent to viral quasispecies represents a major driving force for viral disease emergence because of the higher capacity to adapt to new hosts. In fact, it has been shown with terrestrial animal viruses that directly-transmitted ssRNA viruses are the pathogens most likely to jump between host species (Woolhouse et al., 2005). In addition, RNA viruses have very short replication times and generate very high virus yields (Domingo and Holland, 1997; Moya et al., 2004), two characteristics that strongly accelerate intra-host genetic variation.

Virus recombination has also been reported to drive the increasing genomic diversity of some virus families, such as *Orthomyxoviridae* (ISAV), *Rhabdoviridae* (VHSV), *Roniviridae* (YHV), etc. Within shrimp aquaculture, homologous genetic recombination is considered to have contributed to the increasing genetic diversity of yellow-head-complex viruses detected in *P. monodon* from across the Asia-Pacific region recombinants (Wijegoonawardane et al., 2009; Walker and Winton, 2010).

Genome segment reassortment is another major evolutionary driver for viruses with segmented genomes, such as members of *Orthomyxoviridae* and *Reoviridae*. For example, the two genotypes of ISAV (North American and European) have been reported to occur in Atlantic salmon in Atlantic Canada (Kibenge et al., 2001), signifying the potential for classical genome segment reassortment; although none has been reported to date, probably because there is no selective advantage since any new antigenic types would be recognized by the neutralizing antibodies present in the farmed Atlantic salmon population. On the other hand, genetic reassortment and recombination mutations in ISAV isolates have been indicated by molecular and phylogenetic sequence analyses of the virus envelope genes (Cottet et al., 2010; Devold et al., 2006; Markussen et al., 2008; Kibenge et al., 2009; Godoy et al., 2014). The fusion (F) protein of some ISAV strains contains insertions near the putative proteolytic cleavage site, acquired through nonhomologous recombination between the F and RNA polymerase PB1 (Kibenge et al., 2009), RNA polymerase PB2 (Godoy et al., 2014) or nucleoprotein (NP) (Devold et al., 2006; Markussen et al., 2008) genes. The virulent strains of ISAV have deletions in a highly polymorphic region (HPR) in the stem of the hemagglutinin-esterase (HE) protein (designated ISAV-HPRΔ). The low pathogenic or nonvirulent strains of ISAV (designated ISAV-HPR0) have a full-length sequence (35 amino acids) of HPR (Cunningham et al., 2002) and are considered to have an ancestral relationship with ISAV-HPRΔ (EFSA, 2012). The deletions in HPR probably occur through homologous recombination (copy-choice recombination), presumably because of the viral RNA polymerase's strand-switching (Castro-Nallar et al., 2011) during negative RNA strand synthesis from one nucleic acid template of one virus to another.

The emergence and reemergence of ISAV in the salmon aquaculture industry in Chile (Kibenge et al., 2009; Godoy et al., 2013) provides an interesting example in a fish host with mutations involving both viral envelope genes and viral polymerase genes. The first involvement of ISAV in a disease outbreak in Chile was in Atlantic salmon in mid-June 2007 (Godoy et al., 2008) and then the virus spread throughout the salmon industry; 85% of the outbreak cases in which ISAV isolates had their RNA segment 6 (HE gene) sequenced had ISAV-HPR7b virus, similar to isolates from Norway, but had acquired a mutation consisting of an 11-amino acid insert in the F gene (Kibenge et al., 2009). However, beginning in 2010, the nonpathogenic ISAV-HPR0 virus (without deletions in RNA segment 6 and no mutation or small sequence insertions in RNA segment 5) appeared and by 2012, it had completely replaced the virulent ISAV-HPRΔ strain responsible for the 2007–10 ISA outbreaks as the dominant ISAV variant in marine-farmed Atlantic salmon in Chile (Godoy et al., 2013). Subsequent ISA outbreaks in Chile have been linked directly to the presence of ISAV-HPR0, supporting the notion that under appropriate conditions, ISAV-HPR0 can mutate to virulent ISAV-HPRΔ (Godoy et al., 2013, 2014). In 2013–14, six ISA cases were caused by ISAV-HPR7a (with an 18-amino acid insert in F gene with 100% sequence identity with ISAV RNA segment 1 encoding PB2 polymerase) and ISAV-HPR7b (without sequence insertion in segment 5); with ISAV-HPR7a appearing more virulent (Godoy et al., 2014).

6.3 ANIMAL HOST ATTRIBUTES THAT CONTRIBUTE MOST TO EMERGING VIRAL DISEASES IN AQUACULTURE

Animal host factors that contribute to emerging viral diseases in aquaculture are those that affect host susceptibility to virus infection, and the natural animal movements (eg, migratory fish as vectors) (Table 6.2).

Host factors influencing the outcome of virus infections are aspects such as species, age, nutritional status, stress, hormonal conditions, other clinical and subclinical infections and disease, genetic determinants, etc. (Rimstad, 2011). For example, in contrast to terrestrial-farmed animals, the animal strains used in aquaculture usually have been very recently derived from wild strains (Stuart et al., 2007; Duarte et al., 2007; FAO, 2016; Rosenlund and Skretting, 2006; Svasand et al., 2004) and may not have had enough time to adapt to high-density confinement within the aquaculture environment (Rodríguez-Ramilo et al., 2011).

Most of the viral diseases that currently cause problems in fish farms are likely enzootic, originating from wild fish (Kurath and Winton, 2011). This implies that these infections occur or occurred in the past at some "background" level in wild stocks (Taranger et al., 2015), and are unlikely to cause disease outbreaks in wild populations. For example, although ISA occurs naturally only in farmed Atlantic salmon, ISAV can replicate in several other species, which can serve as carriers of the virus and may possibly function as reservoirs (Kibenge et al., 2004). Often, virus infections have been detected in wild aquatic animals but not clinical disease; for example, ISAV in wild salmonids, but not ISA (Nylund et al., 2003; Kibenge et al., 2004); salmon alphavirus (SAV) in wild salmonids, but no pancreas disease (Nylund, 2007; Snow et al., 2010; Biering et al., 2013); piscine orthoreovirus in wild fish but no HSMI (Garseth et al., 2013). Whereas farmed fish that may survive a lethal virus infection are eventually harvested and removed from the water column, the wild fish that overcome the disease become asymptomatic carriers, exhibiting light viral loads and excreting viruses constantly (Gozlan

et al., 2006). This continual circulation of virus strains in a population provides stock for infection of other fish species in contact with the carrier fish species or contamination of the environment (Arechavala-Lopez et al., 2013). The presence of asymptomatic hosts allows the virus to be shed across a wider temporal and spatial scale than by symptomatic individuals, whose movement may be limited by the infection. Wild aquatic animals in contact with farmed aquatic animals, particularly if the latter are nonnative species, could be the source of emerging viral diseases in aquaculture.

Wild fish can transport viral pathogens over hundreds of kilometers, either through large-scale movement of individual fish (Tucker et al., 1999; Dopazo and Bandín, 2006; Wallace et al., 2008; Matejusova et al., 2010) similar to migratory wild birds for avian influenza viruses (Webster et al., 1992) or by transmission through intervening populations (Murray et al., 2003; Uglem et al., 2009; Arechavala-Lopez et al., 2013). It is speculated that VHSV IVb virus (Elsayed et al., 2006) was introduced to the Great Lakes via migrating infected native fish up the Saint Lawrence River from the near-shore areas of the Atlantic coast of Canada (Groocock et al., 2007; Walker and Winton, 2010); the VHSV IVb virus is similar to VHSV IVc isolates from stickleback *Gasterosteus a. aculeatus*, mummichog *Fundulus heteroclitus* (Olivier, 2002), striped bass *Morone saxatilis* and wild brown trout *Salmo trutta* on the Atlantic coast of North America (Gagne et al., 2007). The European eel *Anguilla anguilla* travels long diseases to its spawning grounds in the Sargasso Sea, south of Bermuda off the coast of the Bahamas and the larvae float on oceanic currents back to Europe (Aarestrup et al., 2009). A survey of wild eels found them infected with *Anguillid herpesvirus*, *Aquabirnavirus* and *Betanodavirus* (Bandín et al., 2014). Tuna from the Mediterranean breed in the Gulf of Mexico (Block et al., 2005). Predatory vertebrates, for example, scavenging wild birds can also act as disease vectors (Peters and Neukirch, 1986; McAllister and Owens, 1992; Peeler et al., 2004), and even aquatic turtles that prey upon infected fish (Goodwin and Merry, 2011). Eels also scavenge on dead fishes (Coad, 1995). Farmed fish may be the most important reservoir of IPNV in the aquatic environment because fish develop a lifelong persistent infection (Johansen et al., 2011). However, the worldwide distribution of IPNV is not only a consequence of aquaculture and commercial movements of cultured fish, particularly when wild fish populations are considered (Dopazo and Bandín, 2006). For example, the genetic variation of IPNV isolates in Chile cannot be explained entirely by trade in fish eggs (McAllister and Reyes, 1984; Mutoloki and Evensen, 2011); analysis of more recent Chilean IPNV isolates has identified a cluster of Genogroup 1 isolates (Kibenge et al., 2012) that are most similar to those made in 1999 by Romero-Brey et al. (2009) from 12 different species of wild fish from the Flemish Cap fishery (located in international waters near Newfoundland, on the Northeastern coast of Canada) (Callejas et al., 2012). Sea lice may also serve as vectors of aquatic animal viruses (Petterson et al., 2009; Jacob et al., 2011).

The first reported outbreak of IHNV in marine-farmed Atlantic salmon occurred in British Columbia in 1992 (Armstrong et al., 1993; Traxler et al., 1993). Since then, the virus has occurred at epizootic levels on Atlantic salmon farms from 1992 to 1996 (St-Hilaire, 2000), 2001 to 2002 (Saksida, 2004) and 2012 (Anonymous 1, 2012). It was hypothesized that the source of these IHNV outbreaks was a stock of wild salmon, most likely sockeye, carrying a virulent strain of IHNV; and because of the cyclical nature of the sockeye life history (Groot, 1996), the virulent virus strain would only appear every 4–5 years with the return of this particular stock of sockeye to the rivers to spawn (Saksida, 2004). However, cultured salmonid fish, such as Chinook salmon (St-Hilaire et al., 2001) and the nonsalmonid fish that occur around sea net-pens, such as shiner perch *Cymatogaster aggregata* and tubesnout *Aulorhynchus flavidus*, could also serve as carriers of the virus because they can have high virus titers without showing any clinical signs (Traxler and Richard, 1996).

The crustacean aquatic animal group is dominated by the black tiger shrimp *P. monodon* and the white Pacific shrimp *Penaeus vannamei* (also called *Litopenaeus vannamei*). Since 1981, several viral pathogens have emerged in shrimp aquaculture in Asia and the Americas (Walker and Mohan, 2009). The manifestation of these viral infections (ie, virus–host interaction) differs from that in fish primarily because shrimp are invertebrates (which lack adaptive immune-response mechanisms). Most shrimp viral pathogens cause persistent infections in apparently healthy shrimp populations (Flegel, 2006; Nunan et al., 2001; Fegan et al., 1991), and almost all shrimp pathogens are transmitted vertically (but usually not transovarially) (Lightner, 1996; Walker and Winton, 2010). Disease outbreaks usually either follow environmental or physiological stresses that facilitate massive virus replication (Cowley et al., 2002; Lotz et al., 2005; Liu et al., 2006; Peng et al., 1998; Vidal et al., 2001; Wijegoonawardane et al., 2008) or horizontal transmission accompanied by high viral loads (Walker and Winton, 2010). Consequently, shrimp are commonly infected simultaneously or sequentially with multiple viruses (Flegel et al., 2004; Umesha et al., 2008) or even different strains of the same virus (Hoa et al., 2005). *Penaeus vannamei*, which is native to the west coast of America now accounts for more than 65% of total farmed shrimp production in Asia. This switch that started in 2002 was dictated by the need to use a shrimp species that was resistant to viral diseases and could be obtained as genetically selected, specific pathogen free.

6.4 ECOSYSTEM ATTRIBUTES THAT CONTRIBUTE MOST TO EMERGING VIRAL DISEASES IN AQUACULTURE

Environmental factors, such as oxygen saturation, salinity, temperature, pH, water flow, etc., are not only important for the outcome of an infection in the host (Rimstad, 2011), but also in terms of survival of the virus and its transmission in the environment. Aquatic viruses are transmitted fecal-orally and persist in water, making the aquatic environment ideal for cross-species transmission. Thus, aquaculture farms are "ecotones" (ecological transitional areas where opportunities arise for cross-species transmission—Mendenhall et al., 2015). This can lead to coinfection with different virus strains and opportunities for recombination. Most aquatic viruses are adapted to survive for several weeks in the water environment (Reno, 1999; Graham et al., 2007; Hawley and Garver, 2008; Altuntas and Ogut, 2010) and thus may be carried long distances with currents (Mortensen, 1993; McAllister and Bebak, 1997; Wallace et al., 2005; Raynard et al., 2007; Gustafson et al., 2007; Viljugrein et al., 2009; Johansen et al., 2011; Stene, 2013; Stene et al., 2014; Aldrin et al., 2010; Kristoffersen et al., 2013). An interesting example is the Pilchard herpesvirus (PHV), which when introduced into native populations of pilchard *Sardinops sagax neopilchardus* in South Australia was spread to >5000 km of Australian coastline at 30 km/day (Whittington et al., 1997) or 10,000 km/year (McCallum et al., 2003). The most likely explanation for the relatively rapid spread of aquatic pathogens (compared with terrestrial pathogens) is the lack of barriers to dispersal in some parts of the ocean, and the potential for long-term survival of pathogens outside the host (McCallum et al., 2003) (Table 6.2).

Most viral diseases of fish occur only within certain temperature ranges (Goodwin and Merry, 2011; Leimbach et al., 2014). These ranges are set by interactions between the ability of the virus to replicate and the ability of the host to mount an effective immune response. Disease occurs at temperature conditions under which the pathogen replicates well, but under which the host response is constrained (Snieszko, 1974; Workenhe et al. 2010; Hershberger et al., 2013). In the case of mollusks without adaptive immune response mechanisms, pathogen replication is heavily dependent on environmental factors (Petton et al., 2015). Increases in temperature are expected to lead to the introduction of pathogens to new regions by producing environmental conditions that favor pathogen growth and transmission (Harvell et al., 2002). Leung and Bates (2013) reviewed some of the effects of climate change on EIDs in aquaculture.

6.5 HUMAN ATTRIBUTES THAT CONTRIBUTE MOST TO EMERGING VIRAL DISEASES IN AQUACULTURE

The human attributes are also referred to as anthropogenic factors. Changes of anthropogenic nature are the basis of virtually all EIDs in humans and animals (Engering et al., 2013). This is also true for emerging viral diseases in aquaculture. The three primary anthropogenic drivers of emerging viral diseases in aquaculture are: (1) aquaculture (including intensive aquaculture in an enzootic area and global expansion of aquaculture), (2) prolific global trade in live aquatic animals and (3) global trade in aquatic animal products (Table 6.2).

Aquaculture is highly dynamic and characterized by enormous diversity in both the range of farmed species and in the nature of production systems (Walker and Winton, 2010). Among the three main aquaculture groups (fish, crustaceans, mollusks), there were 354 fish species, 59 crustacean species and 102 mollusks species recorded as cultured by FAO (2012). Aquatic viruses occur in the numerous farmed species in all the three main aquaculture groups (refer to Chapter 2: Classification and Identification of Aquatic Animal Viruses, Table 2.1), which are often globally distributed (Subasinghe and Bondad-Reantaso, 2008; Walker and Winton, 2010). The techniques, systems and facilities used in aquaculture are as diverse as the species currently being raised and also vary by geographical region (Bostock et al., 2010). World aquaculture production takes place at different levels and scales, from little to no contact with wild species for on-land systems through intensive culture of single species (monoculture) and age class in floating-net cages in a lake or river to marine aquaculture in floating-net cages in the sea in the same water column with wild species unabated. Increased risk for new diseases occurs with introduction of nonnative species (Maccrimmon, 1971), the expanding range of emerging farmed aquatic animal species such as Atlantic halibut *Hippoglossus hippoglossus*, Arctic char *Salvelinus alpinus*, Sablefish *Anoplopoma fimbria* and Atlantic cod *Gadus morhua*, and with the new production approaches, such as integrated multitrophic aquaculture (McAlaster, 2011). In all cases, aquaculture with consistently high-density rearing and therefore high contact frequency (Nokes and Anderson, 1988) with either genetically homogeneous single species (Louz et al., 2010) or mixed species aquatic animal hosts (of short lifespans, Mennerat et al., 2010) often with compromised defensive responses, and in water allowing rapid spread (Rimstad, 2011), favors the selection of virulent virus strains (Woolhouse et al., 2002; Kennedy et al., 2016) and/or host-switching (Kurath and Winton, 2011). For example, novel ISAV virulent strains (ISAV-HPRΔ) were

suggested to be produced from nonvirulent strains (ISAV-HPR0) (Cunningham et al., 2002; Nylund et al., 2003) during intensive aquacultural practices (Mjaaland et al., 2002). The short lifespan of aquaculture species as a result of compression of the rearing cycle is predicted to favor increased virulence of viruses that cause chronic, persistent infections with lifelong potential for pathogen transmission (Kennedy et al., 2016), such as KHV *Cyprinid herpesvirus 3* (CyHV3) in koi and carp (Ilouze et al., 2011); infectious pancreatic necrosis virus in salmonids (Yamamoto, 1975) and WSSV in shrimp (Tsai et al., 1999). The viruses adapted to various types of aquaculture ("domestic" viruses) invariably "spillback" to wild aquatic animal hosts (Krkosek et al., 2006; Wallace et al., 2008; Kurath and Winton, 2011; Arechavala-Lopez et al., 2013; Jones et al., 2015) that aggregate around fish farms (Sudirman et al., 2009; Dempster et al., 2010; Saksida et al., 2014). Infected escaped salmon are known to expose wild fish to SAV (Madhun et al., 2015). Disease outbreaks in wild fish species are usually due to emergencies that result from introduction of an exotic virus to a naïve host population (ie, anthropogenic drivers like aquaculture) (Hyatt et al., 1997; Gaughan et al., 2000; Skall et al., 2005; Moreno et al., 2014). Kurath and Winton (2011) list several examples of finfish virus transmission events between wild fish and farmed fish. In Australia, the initial outbreak of abalone viral ganglioneuritis caused by Abalone herpesvirus (AbHV) occurred on a farm during summer 2005/2006 and subsequently appeared to spread to wild populations, which experienced mortality throughout the following year during all seasons (Hooper et al., 2007; OIE, 2015b).

VHSV probably has the widest host range of all known fish viruses, having been isolated from at least 102 different freshwater and marine species in the Northern Hemisphere waters of Europe, North America and Asia (Hedrick et al., 2003; Skall et al., 2005; OIE, 2015a,b). Outbreaks in marine-farmed rainbow trout in France and Denmark (Castric and De Kinkelin, 1980; Hørlyck et al., 1984) and in farmed turbot *Scophthalmus maximus* in Germany (Schlotfeldt et al., 1991) and Scotland (Ross et al., 1994) are believed to have been due to virus from a local wild fish reservoir (Hill, 2005); for the outbreak in Germany, the virus was most likely introduced to the turbot production facility by contaminated seawater (Schlotfeldt et al., 1991). In Japan and Korea, VHSV originating from local wild Japanese flounder *Paralichthys olivaceus* (Watanabe et al., 2002) was shown to be responsible for the emergence of the disease in farmed Japanese flounder (Isshiki et al., 2001; Nishizawa et al., 2002) and olive flounder (Kim et al., 2009). In Japan, the disease was initially thought to be due to a new type of hirame rhabdovirus (HIRRV); HIRRV is widely distributed in fish in Japan (Nishizawa et al., 1991), and causes lesions very similar to those observed in VHSV infections. However, it was differentiated from HIRRV by lack of cross-neutralization with antisera against HIRRV and VHSV, and by western blot analyses and RT-PCR (Isshiki et al., 2001).

Aquaculture practices, such as the use of wild broodstock that may be healthy carriers of viral pathogens, provide opportunities for adapting new or endemic viruses to aquaculture; for example, the introduction of *Betanodavirus* to novel marine aquaculture species (Munday et al., 2002; Gomez et al., 2004, 2008). Aquaculture management procedures, including segregation of generations, "all in/all out," removal of infected populations, fallowing, disinfection of eggs, regulation of well boat traffic, surveillance of fish population, disposal of carcasses, by-products and waste, control of vectors and decontamination strategies, and the interventions used for the host, including vaccination, genetic selection or use of less susceptible species are all important for the outcome of an infection (Rimstad, 2011; Jones et al., 2015) and may lead to the evolution of increased virulence of viruses (Gandon et al., 2001; Kurath and Winton, 2011; Kennedy et al., 2016). The risk factors and causes of introduction of HSMI in the different areas of salmon aquaculture in Norway are largely undescribed (Rimstad, 2011), but it has been shown that transportation of fish during the seawater phase is a significant risk of spreading HSMI to new locations (Kongtorp and Taksdal, 2009; Garseth et al., 2013).

International trade in live aquatic animals and their products affect the geographical distribution of aquatic animal species and aquatic animal viruses and tend to promote viral emergence. Seafood (fish, crustaceans and mollusks) is the most widely traded animal protein globally, with a value of around US$140 billion in 2014 (IntraFish, 2015), and expected to reach US$202.96 billion by 2020 (Grand View Research Inc., 2014), which illustrates the large volume of international trade in fish and their products. The movement of live animals is the main route of disease spread. However, the introduction of nonnative species also drives the emergence of new diseases and known diseases in new hosts by bringing pathogens into contact with new potential hosts, thus facilitating host-switching (Peeler et al., 2011). Virus spread can occur through translocation of successfully farmed live aquatic animals to new destinations (eg, transfer of live nonindigenous fish for farming), particularly animals with low prevalence of clinical disease, subclinical infections or asymptomatic carrier hosts (Gaughan, 2002; Rodgers et al., 2011), including trade in ornamental aquatic animals (Whittington and Chong, 2007), or by the shipment of infected/contaminated fish eggs (McAllister and Reyes, 1984; Vike et al., 2009; Mutoloki and Evensen, 2011).

KHV *Cyprinid herpesvirus 3* (CyHV3) emerged in Israel in 1998 and spread globally via the ornamental fish trade (Haenen et al., 2004). For example, CyHV3 was introduced into Japan in 2004 and spread to wild common carp in Lake Biwa, where survivors have since become carriers and transmission to naïve common carp is still occurring (Uchii et al., 2009). Spring viremia of carp virus (SVCV) has also spread from Asia to the United States, United Kingdom and Canada

via the ornamental fish trade (koi carp imports without requirement for health certification of freedom from disease (Hill, 2005)) followed by fish escape or release or virus in effluent (Bernet and Wahli, 2002; Dikkeboom et al., 2004; Grimmett et al., 2006; Warg et al., 2007; Miller et al., 2007; Garver et al., 2007). Trade in ornamental fish has also been associated with the introduction of iridoviruses (Family *Iridoviridae*, Genus *Megalocytivirus*) into Australia (Whittington and Chong, 2007).

IHNV is considered to have spread from North America to Europe (initially to France and Italy and later to Germany) (Baudin-Laurencin, 1987; Bovo et al., 1987) and Japan (Yoshimizu, 1996; Nishizawa et al., 2006) via shipments of IHNV-contaminated rainbow trout eggs or fry (Bootland and Leong, 1999). The ISAV responsible for the Chilean outbreaks beginning in 2007 was shown to be genetically similar to Norwegian strains (Godoy et al., 2008; Kibenge et al., 2009), and was introduced from Europe to Chile likely through transport of fertilized salmon eggs (Vike et al., 2009) infected via vertical transmission (Marshall et al., 2014).

WSSV was not known prior to its emergence in China in 1992 (Zhan et al., 1998) and the original source of infection has not been determined (Walker and Mohan, 2009). The virus subsequently spread throughout Asia and to the Americas in 2001 (Walker and Mohan, 2009), and to Mozambique in 2011. This explosive spread is attributed to a prolific international trade in live shrimp and other crustacean seed and broodstock (Lightner et al., 1997). The rapid spread of TSV, another crustacean viral disease in the Americas and then to Asia is also attributed to the international trade in live shrimp (Walker and Winton, 2010). Similarly for IMNV, first detected in Brazil in 2002 and subsequently in Indonesia, Thailand and China (Senapin et al., 2007), its spread has almost certainly been due to the voluminous trade in *P. vannamei* broodstock (Walker and Winton, 2010), particularly since surviving shrimp have a persistent chronic infection. Moreover, although IMNV appears to cause disease only in white Pacific shrimp (Tang et al., 2005), the increasingly common practice in parts of Asia of cocultivation with black tiger shrimp is likely to present opportunities for adaptation and further spread of the disease (Walker and Winton, 2010). *Macrobrachium rosenbergii* nodavirus (MrNV) is considered endemic in south and southeast Asia, with geographic expansion likely due to the movement of giant freshwater shrimp *M. rosenbergii* stock for aquaculture purposes (Walker and Winton, 2010).

Examples of international trade in aquatic animal products leading to emergence of aquatic animal viruses have also been documented. VHSV infection in farmed rainbow trout *Oncorhynchus mykiss* (Smail, 1999) is considered to be of marine origin (Dixon, 1999; Skall et al., 2005), possibly introduced through use of raw (unpasteurized) marine fish as feed in the past 50 years (Einer-Jensen et al., 2004). An outbreak of VHSV in United Kingdom (Stone et al., 2008) was attributed to importation of rainbow trout carcasses. An outbreak of sleeping disease caused by SAV in the United Kingdom was due to introduction of the virus with rainbow trout carcasses from Europe (Branson, 2002; Graham et al., 2007). PHV was introduced into native populations of pilchard *S. sagax neopilchardus* in South Australia when pilchard imported as feed from America were fed to sea ranched tuna, resulting in massive die-offs in 1995 and again in 1998–99 (Whittington et al., 1997; Murray et al., 2003); PHV is now endemic in Australia (Whittington et al., 2008).

The anthropogenic factors unrelated to aquaculture that also have lead to emerging viral diseases in aquaculture include movement of pathogens or hosts via ballast water in ships, movement of bait by anglers, climate change, etc., (Daszak et al., 2001; Walker and Winton, 2010; Marcos-López et al., 2010). For example, it has been theorized that VHSV IVb was introduced into the Great Lakes basin by ballast water in ships (Elsayed et al., 2006; Bain et al., 2010); subclinical baitfish released by recreational anglers likely moved the virus from the Great Lakes to the inland lakes of Wisconsin and New York (Bowser, 2009). Artificial fish aggregating devices, such as wharfs and seawalls change the ecology of local areas by attracting wild fish similarly to aquaculture (Saksida et al., 2014).

Lastly, the improved diagnostic and surveillance efforts, which have accompanied the burgeoning aquaculture industry, have also been accompanied by the discovery of new and emerging viral diseases that are naturally enzootic in wild fish populations in a country (Anonymous 2, 2013; Batts et al., 2011, 2012; Haugland et al., 2011; Lorincz et al., 2011; Murray and Peeler, 2005; Palacios et al., 2010; Walker and Winton, 2010; Gjessing et al., 2015) (Table 6.1). Consequently, we are most likely to see more aquatic animal viral diseases in the future, and more knowledge of aquatic animal viruses is needed.

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Part II

DNA Viruses of Fish

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Chapter 7

Poxviruses of Fish

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7.1 INTRODUCTION

Poxviruses are among the largest viruses known with a wide host repertoire that comprises mammals, birds, reptiles, fish and insects. Unlike most other DNA viruses, poxviruses replicate entirely in the cytoplasm and have complex morphology (Moss, 2007). The viruses are ovoid or brick-shaped, depending on the genus, and have genomes that consist of double-stranded DNA (dsDNA). They belong to the family *Poxviridae*, which is divided into two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae* (Moss, 2007). There are 10 recognized genera in *Chordopoxvirinae* and 3 in *Entomopoxvirinae*. Both subfamilies also contain a number of unclassified virus species (King et al., 2012), for which new genera, including salmon gill poxvirus (SGPV), may be created in the future (Gjessing et al., 2015). Among the *Chordopoxvirinae*, the genus *Orthopoxvirus*, with the prototype vaccinia virus as the most studied, played a historical role in smallpox eradication. The vaccinia virus is also an effective tool for heterologous foreign protein expression.

Disease descriptions in fish are based on clinical manifestation, histopathology and transmission electron microscopy (TEM) of spontaneous cases and experimental studies and, more recently, with genome sequence analysis (Gjessing et al., 2015). Poxvirus-related diseases with high mortalities have been described in farmed Atlantic salmon (*Salmo salar* L.) (Gjessing et al., 2015; Nylund et al., 2008), koi and common carp (Lewisch et al., 2015; Ono et al., 1986) (*Cyprinus carpio* L.) and ayu (Wada et al., 2008, 2011) (*Plecoglossus altivelis* Temminck & Schlegel). In all cases, the poxviruses in fish have an affinity to gill and compromise gill function. The only published and characterized fish poxvirus genome is the SGPV (Gjessing et al., 2015). No fish poxvirus has been isolated in cell culture thus far. Hence, compared with poxviruses infecting warm-blooded vertebrates, the knowledge of poxviruses in fish is scarce and controlled experimental studies using cultivated virus are lacking.

7.2 SALMON GILL POXVIRUS DISEASE (SGPVD)

In 1995, a previously undescribed gill histopathology was discovered in Norwegian Atlantic salmon freshwater farms. Based on subsequent electron microscopy studies, a poxvirus was suspected to be involved (Dale and Kvellestad, unpublished results). Nylund et al. (2008) named the virus SGPV based on TEM studies. Finally, in 2015 the whole genome of the SGPV was sequenced and aspects of the disease it is associated with were characterized (Gjessing et al., 2015). The virus was proven to be the phylogenetically deepest representative of the *Chordopoxvirinae*. The most apparent disease manifestation is seen in the freshwater phase with acute, high mortality (up to 70%). The disease spreads from tank to tank and the fish suffer respiratory distress and crowd, unresponsive at the bottom of the tank. Other disease manifestations are still poorly characterized because specific diagnostic tools have just recently become available.

7.2.1 Structure and Composition of the Virus

SGPV share several morphological features with other poxviruses. The virus particle measure 360 × 270 × 250 nm and is brick to oval shaped depending of the plane of section (Gjessing et al., 2015; Nylund et al., 2008) (Fig. 7.1). It is composed of an electron-dense, centrally located core and a lateral body that is visible in TEM. Biochemical and physiological features of SGPV have not been analyzed.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00007-3
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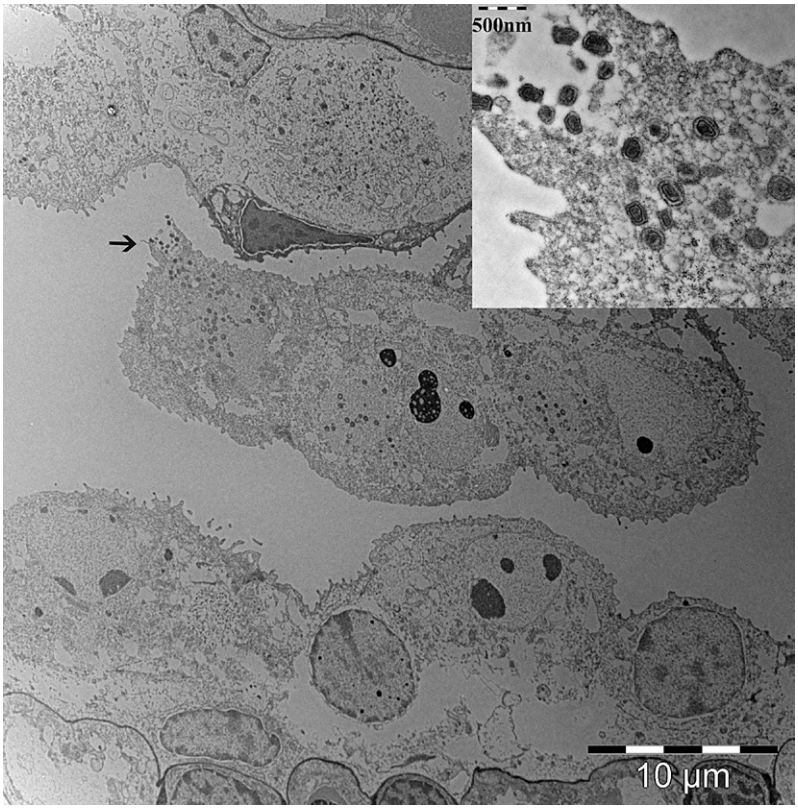

FIGURE 7.1 Transmission electron microscopy of salmon gill epithelial cell with poxvirus particles (*arrow*). Inset shows details of virus particles budding from an epithelial cell.

7.2.2 Classification and Virus Replication

Poxviruses are classified on the basis of original host, growth and morphological characteristics in the chorioallantoic membrane of embryonated eggs or cell cultures and on clinical manifestations. Based on morphology, size and molecular phylogenetic studies, SGPV was assigned to the base of the chordopoxvirus phylogenetic tree, indicating that the phylogeny of the chordopoxvirus follows the phylogeny of their hosts. Classification: Group 1 (dsDNA), Order *Megavirales* (proposed) (Koonin et al., 2015), Family *Poxviridae*, Subfamily *Chordopoxvirinae*, unclassified species SGPV (Gjessing et al., 2015).

SGPV have low G + C content and consist of single linear molecule of dsDNA of 241 kb that is predicted to encode for 206 unique proteins. SGPV contains most key elements needed for genome replication and expression, as shown in vaccinia virus. Like most poxviruses, its genome contains inverted terminal repeats (ITRs). However, gene arrangements of SGPV are different from other chordopoxviruses, and some highly conserved chordopoxvirus genes involved in viral membrane biogenesis and virus–host interaction are missing. Unique to the SGPV genome are a number of genes-encoding proteins with unknown function (Gjessing et al., 2015). The availability of SGPV genome has provided a unique resource to further our understanding of the general biology and more specifically, important genes of SGPV.

7.2.3 Epidemiology

Little is known of the epidemiology of SGPVD because specific diagnostic tools have been lacking until recently. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) (qRT-PCR) is now established and can be used to monitor the epidemiology of the virus in the farms (Gjessing et al., 2015). Archival samples and recent diagnostic cases show SGPVD to have a wide geographical distribution in Norwegian Atlantic salmon freshwater farms over a 20-year period. A recent study in the Faeroe Islands found SGPVD in some freshwater Atlantic salmon farms (Nolsøe et al., 2015) and it is also reported in farmed Atlantic salmon in Scotland (Rodger, H. personal communication). There are no available reports of the virus in wild Atlantic salmon and no available information on the origins and reservoirs of SGPV. The virus

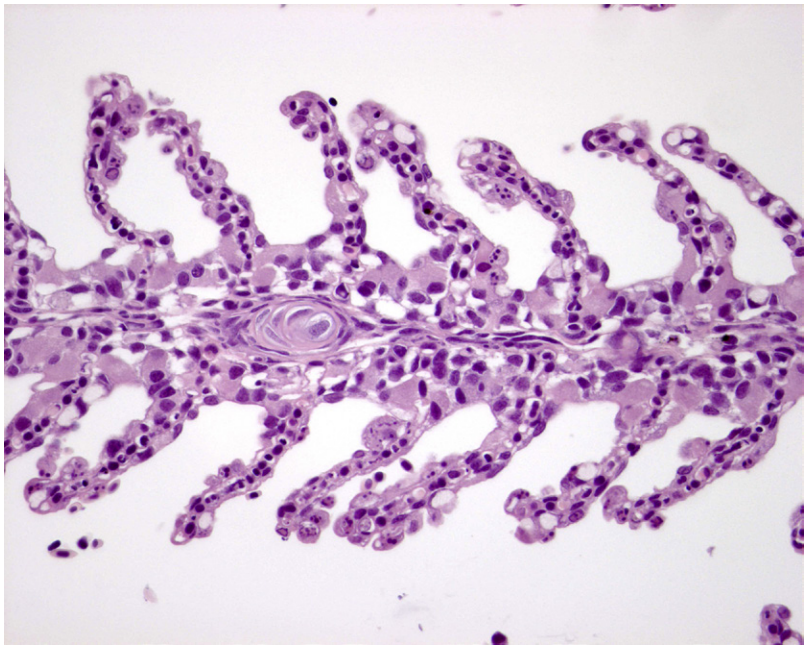

FIGURE 7.2 HE staining of Atlantic salmon gill infected with salmon gill poxvirus. The epithelial cells are detaching and have apoptotic morphology.

has mostly been found in connection with gill disease in freshwater, but has also been found concurrently with other gill pathogens in seawater-reared Atlantic salmon (Nylund et al., 2008).

In freshwater farms, the disease outbreaks are acute and spread rapidly within the farm. Morbidity seems high because most fish appear clinically affected, but mortalities can vary considerably. In large seawater Atlantic salmon farms, annual reports from a farm suggest losses from 2% to 70%.

The knowledge on SPGVD is based on spontaneous outbreaks because controlled studies on transmission mechanisms of SGPV is hampered by the lack of methods to isolate the virus in cell culture. In 2015, a transmission study was performed in a Faroese salmon farm (Nolsøe et al., 2015). In that study, naïve Atlantic salmon was infected after being transferred to a freshwater farm with an ongoing SGP outbreak.

7.2.4 Pathology and Immunity

Current understanding of pathology related to SGPV is based on assessment of fish obtained during outbreaks. In cases in which the disease is not complicated by other agents, only minor gross pathological changes are usually observed at necropsy. The gills and internal organs appear sparsely pale and the spleen is often moderately enlarged. The gut is usually empty, suggesting loss of appetite. In acute cases, histological examination of the affected fish show gill lesions with widespread adherences between lamella, limiting the respiratory surface significantly. This is accompanied by apoptosis of lamellar epithelial cells (Fig. 7.2). In more chronic cases, substantial proliferation of gill epithelial cells may occlude the otherwise thin water–blood interface. A typical feature of orthopoxvirus infections is inclusion bodies, but these are not reported for SGPVD. In hematopoietic organs like spleen and kidney, signs of significant blood cell breakdown are often present. In several cases of SGPVD, ensuing infections with fungi, bacteria and parasites may complicate the histopathological lesions. The knowledge of immunity against SGPVD is limited to genomic data, indicating that SGPV (like other viruses in this group) may also have immunomodulating properties. In farms with recurrent outbreaks in every year class, surviving fish appear not to experience further acute outbreaks of the same kind.

7.2.5 Diagnostic Methods

Infection with SGPV is detected primarily by a qPCR based on the SGPV genomic sequence and target a locus that is a homolog of the vaccinia virus D13L. Disease diagnosis relies on necropsy of fresh specimens, enabling histological demonstration of the characteristic gill pathology with apoptotic epithelial cells that if not pathognomonic, is still a

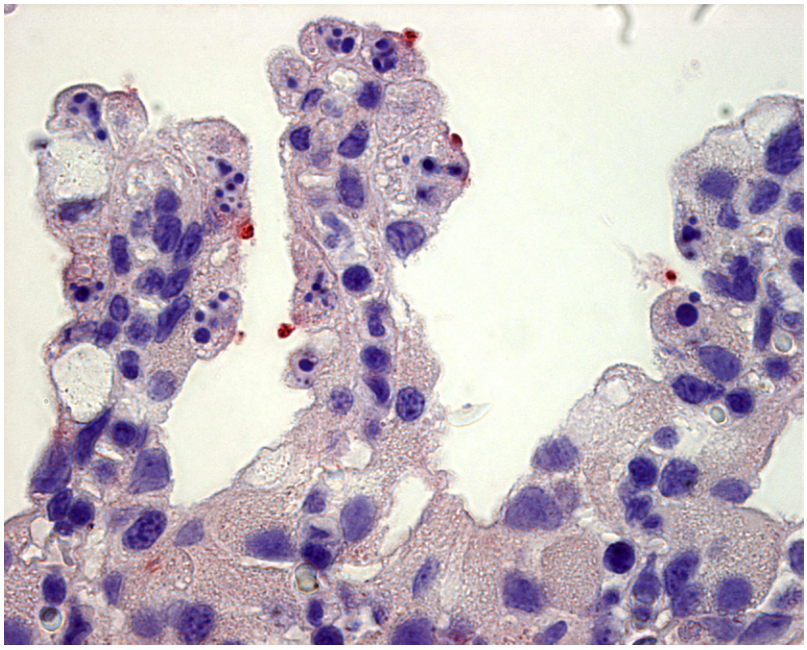

FIGURE 7.3 Immunohistochemical staining of Atlantic salmon gill infected with salmon gill poxvirus. Note red staining of budding virus particles from gill epithelial cells.

very characteristic trait (Fig. 7.2). Clinically diseased fish will have a severely reduced respiratory area. The presence of hemophagocytosis is not a consistent finding in every fish, but support the disease diagnosis on an outbreak level. An immunohistochemical method is established and stains budding or intracellular virus particles (Fig. 7.3).

7.2.6 Prevention and Control

Disease control is achieved efficiently when using best industry practices and basing regulatory rules on scientific knowledge and empirical experiences. Sufficient knowledge to implement epidemiological control measures is lacking because SPGVD is a new and emerging disease. The consequences of an SPGVD outbreak in a freshwater farm is minimized by immediately stopping feeding, raising oxygen levels and avoiding all stress on the first signs of clinical disease in a tank.

7.3 CARP EDEMA AND KOI SLEEPY DISEASE

Carp edema virus (CEV), affecting the treasured ornamental variety of *Cyprinus carpio* L. (known as Koi), was the first fish poxvirus when discovered in 1974 in Japan (Ono et al., 1986). Two distinct manifestations of disease caused by CEV have been described, carp edema (CE) and Koi sleepy disease (KSD). CE is seen in small (0.1–1.2 g) Koi carp in early summer. The fish seek close to the water surface or to the pond walls and in a few days mortality may reach 80% (Oyamatsu et al., 1997b; Seno et al., 2003). The small fish become generally edematous and have severely hyperplastic gill epithelial cells. KSD affects approximately 1-year-old fish (from 20 to 30 g) after their transfer from earthen ponds to pass the winter in cement-lined ponds. Severe outbreaks occur between 15°C and 25°C in the spring and autumn. High numbers of fish lie lethargic on the pond bottom, start dying after a few days and mass mortality ensues in the following 2 weeks (Miyazaki et al., 2005). For both disease manifestations a 0.5% salt treatment may alleviate mortality considerably (Miyazaki et al., 2005; Seno et al., 2003).

7.3.1 Structure and Composition of the Virus

The CEV has an oval to mulberry-like profile, one lateral body, one unilateral concave core and many membrane outward columnar units opposite the lateral body side. Presence of mature and immature virus particles is reported. The range of size estimates are: 245–400 × 300–400 and columnar units 33–50 nm (Miyazaki et al., 2005; Ono et al.,1986; Oyamatsu

et al., 1997b). While a 200-nm filter removes infectivity, a 400-nm filter has only slight effect on infectivity (Oyamatsu et al., 1997b).

7.3.2 Classification and Virus Replication

Based on morphology, size, DNA genome and the cytoplasmic replication, the CEV is classified as a poxvirus. Published genomic data are thus far limited to a cloned 1272-bp DNA fragment, which is used for diagnostic PCRs (Oyamatsu et al., 1997a).

7.3.3 Epidemiology

CEV has been widespread in Japanese Koi farms since the mid-1970s. An experiment indicates that CEV infection may become subclinical and that transport stress may elicit KSD in the already infected fish (Miyazaki et al., 2005). Thus, the worldwide trade with live Koi may spread the infection. In England in 2009, CEV was detected for the first time in imported Koi with KSD. Later, in 2012, CEV and a KSD-like disease was found for the first time in common carp from a cluster of fishery sites in southeast England (Way and Stone, 2013). In 2013–14, KSD was reported in Koi in Germany, France, the Czech Republic and Austria (Jung-Schroers et al., 2015; Lewisch et al., 2015). Cases of sleepy disease in common carp were also found (Lewisch et al., 2015; Way and Stone, 2013). In the United States, CEV has been found in diseased Koi at import sites and hobbyist ponds since 1996 (K. Way, pers. commun.). As several diseases in older Koi have signs that are quite similar to KSD, and opportunistic disease may be part of the KSD in older Koi (Lewisch et al., 2015), the PCR for CEV has been important to revealing the involvement of this virus agent in the reported cases.

In Japan, there is a seasonality of outbreaks because the edema form of the very young is seen in early summer (June to July), while the sleepy disease is seen in spring and autumn. The experimental edema challenges have been performed at 20–23°C (Oyamatsu et al., 1997b). KSD outbreaks are typically seen at water temperatures from 15°C to 25°C (Miyazaki et al., 2005). The KSD cases in Europe occurred over a temperature range of 7–22°C, while the KSD-like disease in common carp occurred at 6–9°C. Some outbreaks are complicated by mixed infections (Lewisch et al., 2015), while other cases are not (Jung-Schroers et al., 2015). It is presently unclear how outbreaks may be related to factors such as temperature, fish size and age, transportation stress, other management operations and concomitant other diseases.

Phylogenetic analysis of PCR products attained from different disease outbreaks showed the CEVs included were closely related. The sequences from common carp in the United Kingdom were most divergent, with approximately 93–95% similarity to the original Japanese CEV (Way and Stone, 2013). It is difficult to determine exactly when CEV have been introduced to new areas, but the spread is likely global, mirroring the trade with Koi.

7.3.4 Transmission

The disease has successfully been transmitted by immersing healthy Koi in filtered suspensions of gill homogenates obtained from sick fish. In these trials with small fish (0.10–0.26 g) developing CE, the fish started to die after 3–6 days. The mortality then lasted for 8–16 days, reaching 72–98% cumulative mortality, and this was repeated by serial passage 12 times (Oyamatsu et al., 1997b).

7.3.5 Pathology and Immunity

CE and KSD appear to be two different disease manifestations, depending on the age and size of the host. As the name implies, in CE the general edema is severe and results in a major swelling of the trunk of the small fry, and histologically disappearance of mucus cells, edema and atrophy of epidermal cells are reported (Oyamatsu et al., 1997b). In larger and older juveniles with KSD, there is less edema, but loss of skin mucus, epidermal erosions and ulceration, as well as sunken eyes occur (Miyazaki et al., 2005). Common to both manifestations are visibly swollen gills with histological changes comprising edema, hypertrophy and hyperplasia of lamellar epithelium, resulting in adhesion of lamellas to an extent that may severely hinder the osmoregulatory and respiratory function. Margination of nuclear chromatin in hypertrophic gill epithelial cells is described, a trait also seen with SGPV in Atlantic salmon where TUNEL staining indicates this to be an apoptotic change (Gjessing et al., 2015). Gill necrosis and detachment of gill epithelial cells are also seen. On histopathology, inclusion bodies are not reported, but TEM readily shows viral factories in many gill epithelial cells, and occasionally in a few skin epithelial cells. Viral particles have not been found in any other organs than skin and gill by TEM. Erythrophagocytosis in spleen and kidney appears to be common to both CE and KSD, as is also reported for SGPVD of Atlantic salmon (Gjessing et al.,

2015; Miyazaki et al., 2005; Oyamatsu et al., 1997b). Less clearly linked to the CEV infection are some other observed pathologies, such as degenerative changes in liver (Miyazaki et al., 2005) and vacuolated exocrine pancreas cells (Oyamatsu et al., 1997b). Little information is available on immunity, but a subclinical carrier state seems to occur in Koi surviving CE because the KSD manifestation can be elicited by stress in older fish (Miyazaki et al., 2005).

7.3.6 Diagnostic Methods

CE and KSD have some similarities to other diseases affecting carp in the spring, namely, disease associated with Koi herpesvirus (KHV) and spring viremia of carp (SVC) (Amita et al., 2002; Lewisch et al., 2015). Further, other diseases occurring at the same time may mask the presence of CEV-related pathology. At present, the best way to clarify involvement of CEV is by PCR on gill material, using a nested PCR developed in Japan or a further development of the assay at CEFAS (Way and Stone, 2013). Isolation in cell culture was not successful in any of the 31 different cell lines/cultures tested initially (Oyamatsu et al., 1997b) and still no cell culture has been reported to support the replication of CEV.

7.3.7 Prevention and Control

A transmission study in 0.14–0.74 g Koi showed that 0.5% salt water treatment could efficiently curb mortality of CE. The salt treatment is believed to counteract the pathophysiological consequences of the CE because infectivity of the tissues used were almost unaffected by salt treatment (Seno et al., 2003). In a study of 1-year-old Koi (20–30 g), the fish were transported to tanks with and without 0.5 % salt added to the fresh water. Only the fish in pure fresh water developed KSD and on day 4 most fish showed sleepy behavior. Mortality started day 6, peaked on day 8 and eventually reached 76% cumulative mortality on day 17. In the fish kept in water with 0.5% salt, no mortality was seen, but on histology at the end of the experiment, moderate hyperplastic gill epithelial cells were found. It appears that the fish population in this study was infected, and that a combination of transport stress and low water salinity was decisive for disease to develop. In keeping with this, it is recommended to treat Koi with 0.5% salt water before transportation and for at least 4 weeks at the new location to avoid KSD (Miyazaki et al., 2005). As the recent reports from other countries show, CEV may cause disease problems in Koi. However, the main concern now is what the spread of CEV may mean for the other variants of carp, including the common carp, which is the world leading aquaculture finfish species in terms of volume.

7.4 PROLIFERATIVE BRANCHITITS IN AYU (*P. ALTIVELIS* TEMMINCK & SCHLEGEL) "SWEETFISH"

Since 1998, severe mortalities have occurred in farmed Ayu in some prefectures in Japan. In two studies of spontaneous cases the fish, weighing from 9 to 136 g, congregated at water surface and displayed abnormal swimming behavior. The local farmers referred to the condition as "Boke" (Japanese expression used for disoriented, senile people). The mortality was acute and varied from 50% to 90% and tended to increase after feeding. Histology revealed that "Boke" comprised two seemingly different proliferative gill diseases, sometimes concurrently in a mixed infection. A Gram-negative rod seemed to induce proliferation of ordinary gill epithelium, while proliferation of enlarged, atypical gill epithelial cells could be related to Poxvirus-like particles found in large, basophilic inclusions in these cells. In both cases, clinical disease was probably similar due to a severe reduction of surface area of the gills, leading to osmoregulatory and respiratory problems (Wada et al., 2008, 2011).

7.4.1 Structure and Composition of Virus

The virus particles are reported to have a cocoon or smiling mouth shape, with structures resembling lateral bodies. Along the major axis, the virus particles ranged from 200 to 300 nm.

7.4.2 Classification and Virus Replication

Based on cytoplasmic assembly site, morphology and size, the virus is classified as a poxvirus.

7.4.3 Epidemiology

The disease is thus far only reported in farmed Ayu in Japan. The affected fish originate from prefectural fisheries stations, using brood fish captured in each prefecture.

7.4.4 Pathology and Immunity

At autopsy, the gills are reported to be pale and swollen with petechiae. Histology showed proliferation of hypertrophic gill epithelial cells that contained large vacuoles and basophilic inclusions in the cytoplasm as well as swollen nuclei. The proliferation appeared to start distally on the lamellas and progress to adhesion of the lamellas and even filaments. Moderate hemorrhaging and aneurysms, as well as a slight infiltration of lymphocytes, were present in the severely hyperplastic gills. No significant lesions were found in any other organs, including spleen, kidney, muscle, brain stomach, intestine and heart. TEM revealed that the basophilic inclusions found on histology in the enlarged gill epithelial cells contained abundant aggregations of Poxvirus-like particles (Wada et al., 2008, 2011).

7.4.5 Diagnostic Methods

In Ayu with "Boke" histology, demonstrating the proliferative branchitis with enlarged gill epithelial cells that contained basophilic inclusions appears pathognomonic for this disease, but TEM will be necessary to demonstrate the Poxvirus particles (Wada et al., 2011). The identity of the virus is unknown and no test for the virus infection has been reported.

7.4.6 Prevention and Control

No specific information is reported.

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Chapter 8

Iridoviruses of Fish

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8.1 INTRODUCTION

Viruses of the family *Iridoviridae* are large, double-stranded DNA viruses that have a host range restricted to insects and ectothermic vertebrates (Williams et al., 2005). Iridoviruses are emerging pathogens that are widespread in a diverse range of environments and hosts. The family includes numerous virus species that cause important diseases characterized by mortality with impacts on the production and welfare of fish in aquaculture. These pathogens are also a focus of biosecurity and regulatory controls because the different virus species present a threat to naïve fish populations with potential for an expanded geographic distribution. Three of the five genera of the family *Iridoviridae* are relevant to fish health: *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*; the other genera are restricted to invertebrate hosts. The nomenclature of this family is confusing and the taxonomy is under review with the benefit of expanded genetic sequence information. The names of viruses have conventionally been assigned by the host species or location in which they are discovered, with variable use of the family or genera designation (Fig. 8.1). Confusion in nomenclature and taxonomy of iridoviruses is a consequence of the broad host range and wide distribution. Several isolates that appear to be very similar using recently available sequencing techniques have been discovered and given different names. Classification at genus and species level is subject to review. The genus *Ranavirus* includes viruses that are pathogens of amphibians and reptiles and are emerging globally, but these virus species are rarely the cause of disease in fish. The ranaviruses of fish are pathogens of freshwater species; Epizootic hematopoietic necrosis virus (EHNV) and European catfish virus (ECV) are restricted to Australia and continental Europe, respectively. *Santee-Cooper ranavirus*, also referred to as Largemouth bass virus (LMBV), is the cause of disease in Largemouth bass (*Micropterus salmoides*) and subclinical infection of several species of freshwater fish in North America and Asia. Singapore grouper iridovirus (SGIV) is also currently classified as a ranavirus and causes disease in multiple species of marine fish in parts of Asia. Although currently classified within the genus *Ranavirus*, both *Santee-Cooper ranavirus* and SGIV differ significantly from the ranaviruses and are not correctly classified in this genus. Viruses of the genus *Megalocytivirus* are pathogens of marine and freshwater fish in Asia. Similar to the ranaviruses, they are the subject of biosecurity and regulation to reduce distribution to other parts of the world. The type species, *Infectious spleen and kidney necrosis virus*, is a pathogen mainly of freshwater fish and relevant in both ornamental and food fish. Red sea bream iridovirus causes disease with high mortality in marine fish. Viruses of the genus *Lymphocystivirus* cause the distinctive surface lesions of lymphocystis disease, a long recognized problem in many species of fish throughout the world. In addition, there are viruses within the family not yet assigned to a genus that are the cause of important diseases. Erythrocytic necrosis virus (ENV) is the cause of piscine erythrocytic necrosis (PEN) and White sturgeon iridovirus (WSIV) and related viruses are the cause of serious mortality events in aquaculture facilities rearing fish of conservation and economic significance.

8.2 STRUCTURE AND COMPOSITION OF VIRUS

Iridovirus virions are large, from 120 to greater than 300 nm in diameter, and structurally complex (Jancovich et al., 2012). The capsid constructed from the major capsid protein (MCP) has icosahedral symmetry with triangulation number, $T = 147$. The MCP comprises 40% of the structural proteins with approximately 36 additional polypeptides contributing to the virion structure. Fibrils of unknown function are present on the surface of the capsid and additional structural proteins are integrated with the inner lipid membrane that surrounds the inner surface of the capsid shell. An external membrane composed of lipid and glycoprotein is obtained by virions that are released by budding from the host cell membrane. This outer envelope is not formed in virions released during cell lysis and is not essential for infectivity.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00008-5
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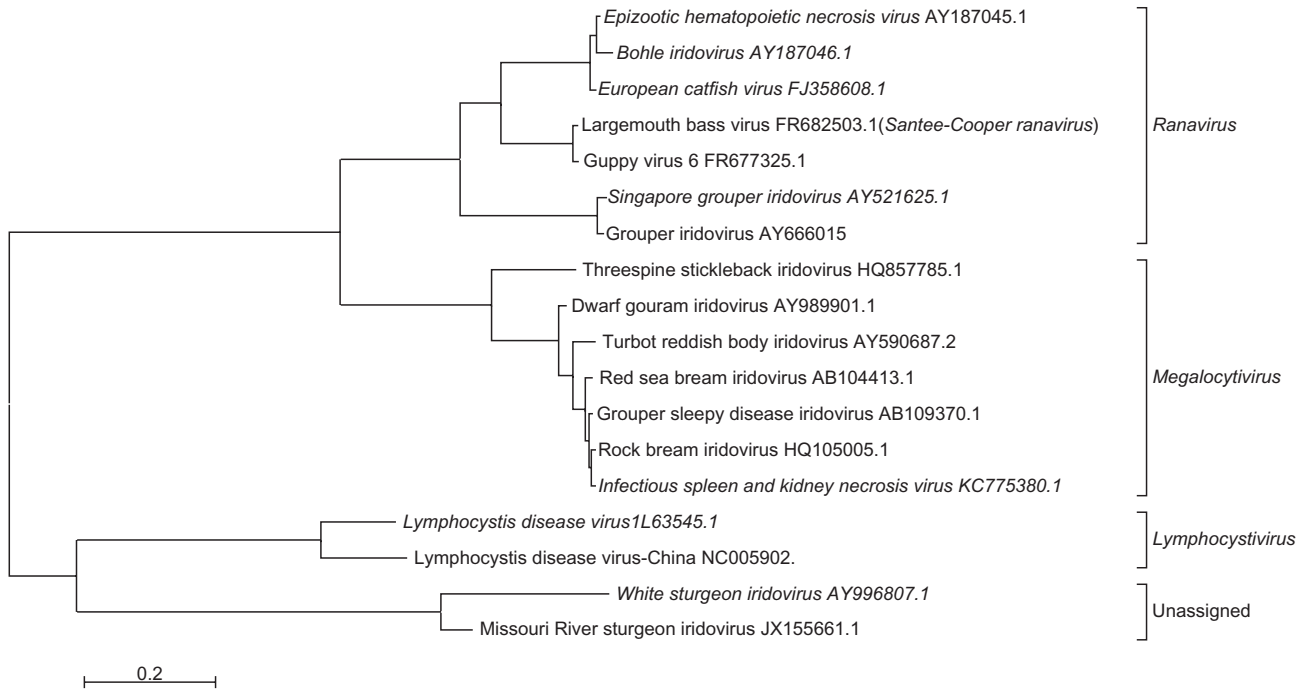

FIGURE 8.1 Molecular phylogenetic analysis by maximum likelihood method of the major capsid protein (MCP) gene of selected iridovirus sequences accessed from GenBank to represent the iridovirus genera relevant to fish health: *Ranavirus*, *Megalocytivirus* and *Lymphocystivirus*. Sequence was not available for the ENV MCP, which probably represents a separate genus within the family; the sturgeon iridoviruses are sufficiently divergent to be classified in a different genus. The tree was prepared according to the Tamura-Nei method (Tamura and Nei, 1993) using the program MEGA6 (Tamura et al., 2013). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 75% site coverage were eliminated; there were a total of 1334 positions in the final dataset.

Central to the inner core is the single, linear, double-stranded DNA genome that is packaged with associated proteins as a nucleoprotein filament. The genome is large, between 105 and 200 kilobase pairs (kbp), it is highly methylated, and has circular permutation and terminal redundancy. This genome encodes approximately 100 viral proteins, depending on the genus, the majority of which have unknown functions and are unique to the virus family (Grayfer et al., 2012). There are 26 core genes that are conserved across the family; the diversity in the remainder reflects a broad range of hosts and environments (Eaton et al., 2010).

The replication of iridoviruses is unique with a combination of nuclear and cytoplasmic nucleic acid synthesis that has been best characterized for Frog virus 3 (FV-3), the type species of the genus *Ranavirus* (Jancovich et al., 2012). Attachment of virus to a host cell receptor is followed by entry through receptor-mediated endocytosis. Fusion with the plasma membrane releases the DNA core into the host cell cytoplasm and transfers it into the nucleus, host macromolecular synthesis is shut down and viral polymerase enzymes synthesize viral DNA and transcripts. Replication is mediated by controlled expression of virus genes in three distinct phases: immediate-early, early and late (Chen et al., 2006; Zhang et al., 2013). Progeny DNA formed in the nucleus is transported to the cytoplasm where a second-stage DNA replication and methylation occurs. Virions are formed with packaging of DNA into virions in morphologically distinct cytoplasmic assembly sites that are formed under the influence of viral functions (Yan et al., 2014). Virions acquire an envelope when they exit the cell by budding from the cell membrane; alternatively if cell lysis occurs, infectious virions without an envelope will be released. SGIV is currently the subject of detailed studies of viral morphogenesis, protein function and molecular pathogenesis (Wang et al., 2014; Yan et al., 2014), including interactions with host cell immune mechanisms (Wei et al., 2012).

The nonstructural proteins with known functions include a large number involved in nucleic acid replication and modification (DNA and RNA polymerases, helicase and mRNA capping) and a complex array of additional enzymes, including those with kinase activities and ATPase (Jancovich et al., 2012). The range of proteins that interact with the host immune system is broad and incompletely characterized (Yan et al., 2014).

The family *Iridoviridae* is divided into genera based on morphological and biological distinctions. Each of the genera are serologically distinct and nucleotide sequence information indicates differences greater than 50% amino acid identity in the MCP gene (Williams et al., 2005). Species demarcation within genera is applied to isolates with less than 90% nucleotide sequence identity. The majority of phylogenetic analyses have used the MCP and ATPase gene sequences. The

increasing ease of determining complete genome sequence provides information about genomic structure and nonstructural elements, such as repeat elements, as well as much more genetic sequence information that sometimes reveals variable identity in different genes. The genetic sequence and genome structure are useful for improved taxonomy and elucidating evolutionary pathways, as well as being relevant to disease diagnosis and control because similar taxonomic units are involved in similar epidemiological situations (Jancovich et al., 2010).

8.3 SPECIFIC MAIN FISH DISEASES

8.3.1 Diseases of Fish Caused by Ranaviruses

8.3.1.1 Epizootic haematopoietic necrosis virus (EHNV) and European catfish virus (ECV)

Epizootic hematopoietic necrosis is a disease restricted to Australia caused by *Epizootic haematopoeitic necrosis virus* (EHNV). This is a systemic disease that causes high mortality in wild redfin perch (*Perca fluviatilis*), has impacted farmed rainbow trout (*Oncorhynchus mykiss*) and is a potential threat to native fish species (Becker et al., 2013; Langdon and Humphrey, 1987; Langdon, 1989). A related virus, *European catfish virus* (ECV) (then termed European sheatfish virus), was the first fish ranavirus isolated in Europe in 1988 from sheatfish (*Silurus glanis*) fry at a German recirculating aquaculture facility (Ahne et al., 1989).

8.3.1.2 Structure and Composition of Virus

Ranaviruses are approximately 150 nm in diameter and up to 200 nm when an envelope is present. The genome of EHNV is 127 kbp with 55% guanine-cytosine content (GC) content, which is larger than the amphibian ranavirus genomes that are typically 105 kbp (Jancovich et al., 2012).

8.3.1.3 Classification and Virus Replication

Complete genome sequence for EHNV and ECV identify them as distinct, closely related species within the genus *Ranavirus*, each with different piscine hosts.

8.3.1.4 Epidemiology

Of the two virus species within the genus *Ranavirus* that are pathogens of fish, EHNV is restricted to Australia and ECV to continental Europe (Whittington et al., 2010). Fish have been experimentally infected with amphibian ranaviruses, but reports of natural infection and disease are scarce (Moody and Owens, 1994; Waltzek et al., 2014).

The two natural hosts for ECV are considered to be *Ameiurus melas* and *Silurus glanis* because other fish species were not affected during outbreaks in the wild. ECV was reportedly the cause of epidemics in wild and farmed populations of bullhead catfish (*A. melas*) and farmed populations of brown bullhead catfish (*Ameiurus nebulosus*) (Whittington et al., 2010). After the initial isolation of ECV, few outbreaks of disease caused by ECV have been documented; most recently in wild Black bullhead catfish (*A. melas*) in France in 2007 (Bigarre et al., 2008) and *A. nebulosus* in Hungary in 2008 (Juhasz et al., 2013).

Experimental challenge trials with sheatfish (10 g) have shown high virulence with complete mortality observed by day 20, when fish were held at 15°C (Leimbach et al., 2014). Similar to other ranaviruses, water temperature can affect host susceptibility. Sheatfish of similar size exhibited 86% mortality when held in water at 25°C (Leimbach et al., 2014).

8.3.1.5 Pathology and Immunity

The ranaviruses cause diseases characterized by widespread systemic necrosis with effects usually most obvious in the hematopoietic tissues. These viruses cause similar lesions in all susceptible hosts due to a likely vascular endothelial tropism.

The primary site of EHNV infection is not known, but after a variable incubation period, which is negatively correlated with water temperature, viremia is likely to be responsible for dispersal of virus to many organ systems (Fig. 8.2). Grossly, there may be petechial hemorrhages at the base of fins, and excessive amounts of serosanguinous peritoneal fluid together with swelling of the kidney and spleen (Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). These changes are attributed to vascular damage and multifocal necrosis in the liver, spleen and hematopoietic tissue of the kidney (Langdon and Humphrey, 1987; Langdon et al., 1988; Reddacliff and Whittington, 1996; Whittington et al., 1994). In redfin perch, pale white to cream foci appear in the liver, which are found microscopically to be necrotic foci at the margins of which in some fish, basophilic intracytoplasmic inclusion bodies will be found within hepatocytes (Fig. 8.3).

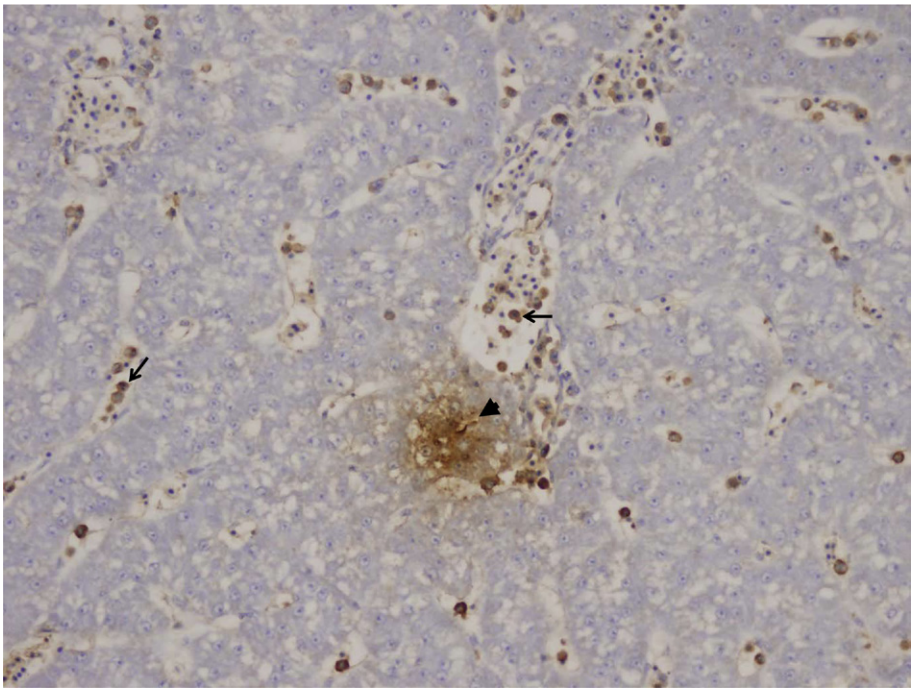

FIGURE 8.2 Epizootic hematopoietic necrosis virus. Liver, redfin perch (*Perca fluviatilis*). Early lesion with initial viral replication focus (*arrowhead*) and dissemination of virus in cells in liver sinusoids (*arrows*). EHNV antigen stained brown. Formalin-fixed, paraffin-embedded section, immunoperoxidase.

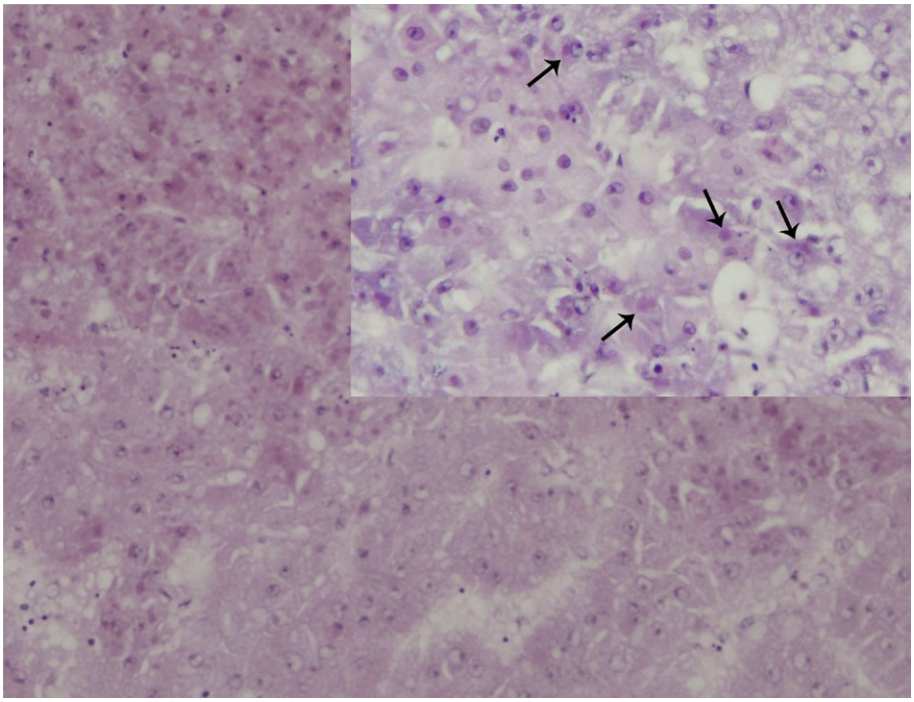

FIGURE 8.3 Epizootic hematopoietic necrosis virus. Liver, redfin perch (*Perca fluviatilis*). End-stage lesion, margin of large necrotic zone with amphophilic intracytoplasmic inclusion bodies in hepatocytes (higher power inset with *arrows*). Formalin-fixed, paraffin-embedded section, hematoxylin and eosin.

These inclusion bodies are difficult to observe in other tissues, but can sometimes be found within interstitial cells of the kidney or spleen. There is heavy staining of viral antigen within necrotic areas (Fig. 8.4). A feature of the histopathology in most affected fish is the presence of many scattered, individual necrotic cells within blood vessels (Fig. 8.5). A variety of other lesions have been reported in rainbow trout and redfin perch: diffuse necrosis of spleen, necrosis of gastrointestinal

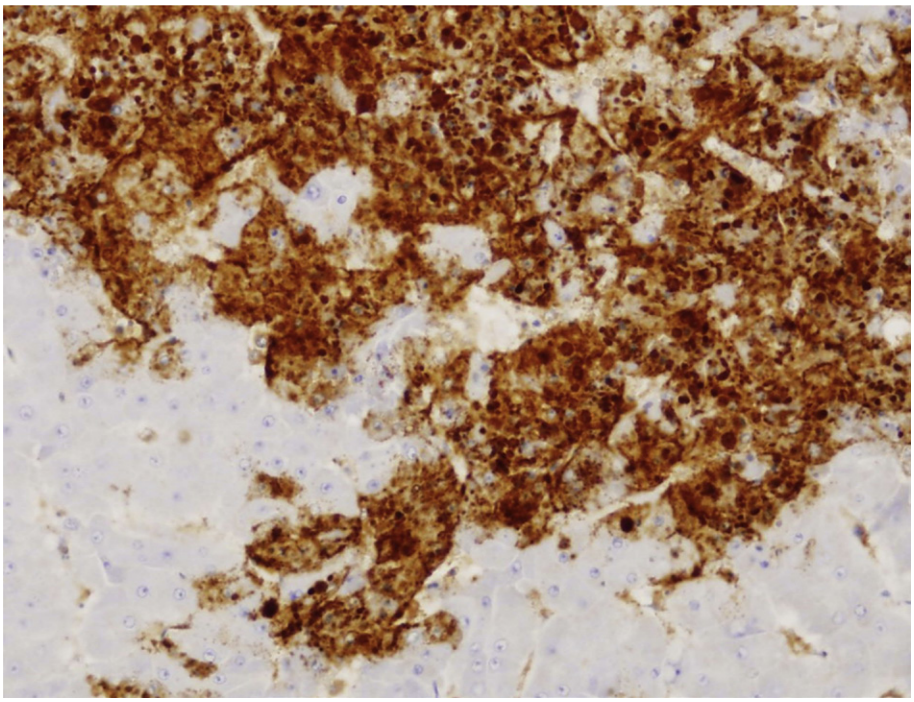

FIGURE 8.4 Epizootic hematopoietic necrosis virus. Liver, redfin perch (*Perca fluviatilis*). Same field as Fig. 8.2; EHNV antigen in necrotic area stained brown. Formalin-fixed, paraffin-embedded section, immunoperoxidase.

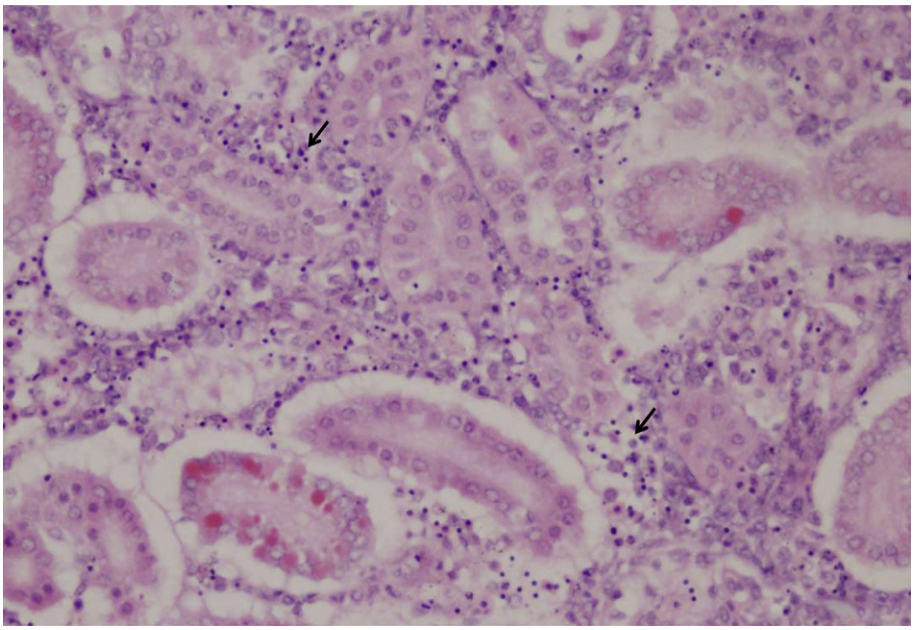

FIGURE 8.5 Epizootic hematopoietic necrosis virus. Kidney, redfin perch (*Perca fluviatilis*). There is interstitial (hematopoietic) necrosis characterized by karyorrhexis and karyolysis of individual cells throughout the section, creating a "starry sky" appearance (*arrows*). Formalin-fixed, paraffin-embedded section, hematoxylin and eosin.

epithelial cells, necrosis of atrial trabeculae, hyperplasia and multifocal necrosis of branchial epithelial cells, focal pancreatic necrosis, swim bladder edema and necrosis and ulcerative dermatitis (Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). Survivors of the infection can have perivascular mononuclear cell infiltrates (Langdon, 1989; Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). Experimental infections of Australian native finfish species, such as Macquarie perch and Murray Darling rainbow fish, with EHNV resulted in lesions similar to those described in redfin perch (Becker et al., 2013).

ECV infection in subadult to adult catfish leads to multifocal hemorrhage, renal hematopoietic and tubular epithelial cell necrosis, splenic necrosis, vascular necrosis, hepatic edema, and hyperplasia of branchial epithelial cells (Pozet et al., 1992). Infection of sheatfish fry with ECV similarly results in disseminated foci of hemorrhage and necrosis of the hematopoietic tissue of the kidney and spleen (Ogawa et al., 1990).

Although natural infections of fish with Bohle iridovirus (BIV), an amphibian ranavirus are not known, barramundi were experimentally infected and developed necrosis of hematopoietic tissue of the kidney and spleen (Moody and Owens, 1994).

8.3.1.6 Diagnostic Measures

Laboratory assays to identify the virus are required to confirm the diagnosis of EHN. The World Organization for Animal Health (OIE) recommends laboratory procedures for disease diagnosis and guidelines for certification of freedom from infection (OIE, 2012c). The most appropriate samples are visceral organs (kidney, liver and spleen) from moribund fish (Rimmer et al., 2012b). Numerous polymerase chain reaction (PCR) assays have been described. They most commonly target the MCP gene (Marsh et al., 2002; Pallister et al., 2007) or the polymerase gene (Holopainen et al., 2009). The real-time PCR assay which underwent validation by Jaramillo et al. (2012), was more sensitive than the methods recommended by the OIE. An antigen capture enzyme-linked immunosorbent assay (ELISA) was described for detection of EHNV (Whittington and Steiner, 1993). The same antibody reagents were suitable for use in immunohistochemical staining procedures to demonstrate EHNV in histopathology sections (Reddacliff and Whittington, 1996).

Virus isolation for EHNV can be performed in several fish cell lines, including fathead minnow (FHM), rainbow trout gonad (RTG), bluegill fry (BF-2) and Chinook salmon embryo (CHSE-214) at incubation temperatures between 15°C and 22°C (Crane et al., 2005; Langdon et al., 1986; OIE, 2012a). ECV can be isolated using Epithelioma papulosum cyprinid (EPC) cells, FHM, and channel catfish ovary (CCO) cells, with BF-2 cells providing the most sensitive means of isolation (Ahne et al., 1989; Pozet et al., 1992).

8.3.1.7 Prevention and Control

There is no practical cure or effective therapy for disease caused by EHNV or ECV and avoidance is the universal control measure. Certification of freedom from infection and movement restrictions based on zoning should be applied in areas where the viruses do not occur. Control measures including destocking, cleaning and disinfection using appropriate treatments will assist in eradication from an aquaculture facility; for example, sodium hypochlorite at 200 mg/L and other treatments, such as heating to 60°C (Bryan et al., 2009; Langdon, 1989). It is noted that EHNV is resistant to drying and can survive for months in water. Regions in which wild populations of fish have experienced an outbreak should be considered endemically infected. Protection of aquaculture facilities under these circumstances will require appropriate disinfection of equipment and intake water, for example, by exposure to UV irradiation together with control of vectors and fomites (Whittington et al., 2010).

8.3.2 Diseases of Fish Caused by Other Viruses That Are Currently Classified as Ranaviruses

8.3.2.1 Santee-Cooper Ranavirus (SCRV/LMBV)

Santee-Cooper ranavirus (SCRV) is a virus species currently classified within the genus *Ranavirus* that is also referred to as Largemouth bass virus (LMBV). In 1995, the initial fish kill attributed to LMBV disease occurred in wild adult largemouth bass in the Santee-Cooper reservoir, South Carolina (Plumb et al., 1996). Water temperatures were elevated at 30°C during the peak of mortality and moribund fish were observed floating at the surface, presumably due to an enlarged swim bladder. LMBV has since been identified as widespread in wild fish, but is rarely linked to serious disease (Grizzle and Brunner, 2003). The viruses referred to as Doctorfish virus (DFV) and Guppy virus-6 (GV6) were isolated from apparently healthy ornamental fish and belong to the Santee-Cooper ranavirus species (Ohlemeyer et al., 2011).

8.3.2.2 Structure and Composition of Virus

Virions are 145 nm in diameter with the typical appearance of an iridovirus (Deng et al., 2011).

8.3.2.3 Classification and Virus Replication

Phylogenetic analysis of the MCP gene suggests that a single virus species from multiple hosts and locations is referred to by multiple names (Ohlemeyer et al., 2011). The nucleotide sequence of the MCP gene of DFV and GV6 isolates shared 100% identity with each other and 99.21% identity with LMBV. An isolate from Largemouth bass in China was 100% identical to DFV and 98% similar to LMBV in partial MCP and ATPase genes (Deng et al., 2011). The identity of the partial MCP gene of all SCRV-like viruses with ranaviruses was 81%, suggesting they do not belong in this genus (Whittington et al., 2010).

8.3.2.4 Epidemiology

Infections with LMBV have been confirmed in major watersheds in 22 states across the eastern United States (Grizzle and Brunner, 2003). While LMBV more commonly infects largemouth bass (*M. salmoides*), it has been isolated from at least 16 teleosts, including nine species from the family Centrarchidae, three species from Moronidae and one each from Esocidae, Cyprinidae, Sciaenidae and Catostomidae (Iwanowicz et al., 2013). Investigations into periodic fish kills and declining numbers of Largemouth bass and Black crappie (*Pomoxis nigromaculatus*) in Lake Weir, Florida, resulted in the isolation of an iridovirus (Grizzle et al., 2002). A retrospective genetic study confirmed the Florida isolate as LMBV with identical DNA sequences for a 495-bp portion of the MCP gene and restriction fragment length polymorphisms compared with the Santee-Cooper ranavirus isolate (Grizzle et al., 2002).

Although historically associated with outbreaks in wild fish populations, LMBV has been associated with mortalities in farmed fish. Largemouth bass are routinely sourced from the wild to serve as broodstock in hatcheries for restocking local waterways and reservoirs (Woodland et al., 2002). In 2008, a ranavirus with high MCP gene sequence homology to LMBV was isolated from farmed Largemouth bass in China and associated with an outbreak of ulcerative syndrome with high mortality (Deng et al., 2011). The recent identification of LMBV infection in diseased Koi (*Cyprinus carpio*) in India was inconsistent with previous knowledge of the host range of this virus (George et al., 2015). There are previous reports of iridovirus detection in cyprinids, but these fish have not been susceptible to experimental challenge with ranaviruses (Berry et al., 1983; Shchelkunov and Shchelkunova, 1990).

8.3.2.5 Pathology and Immunity

Usually there is no histopathological evidence of infection in largemouth bass naturally infected with Santee-Cooper ranavirus, regardless of whether clinical signs of disease are present and even in animals that have died due to the infection. However, swim bladder lesions including over-inflation, hyperemia or yellow waxy exudate have sometimes been described (Hanson et al., 2001; Plumb et al., 1999). Attempts to reproduce the disease experimentally by intraperitoneal (IP) injection in largemouth bass have led to observations of pale liver, cutaneous hyperemia, inflammation at the injection site, peritonitis and focal necrosis of gastrointestinal mucosal epithelial cells (Grant et al., 2003; Zilberg et al., 2000). Disease in largemouth bass in China was associated with skin and muscle ulcers and splenomegaly (Deng et al., 2011). The pathogenesis of the disease in largemouth bass is unknown. No lesions have been described in the few ornamental fish in which DFV and GV6 infection was described (Hedrick and McDowell, 1995).

8.3.2.6 Diagnostic Methods

LMBV can be isolated using commonly available cell lines, including BF-2, FHM and EPC cells (McClenahan et al., 2005). Conventional and real-time PCR assays have been described for specific detection of LMBV (Getchell et al., 2007).

8.3.2.7 Prevention and Control

Application of biosecurity measures at the international as well as local level is the most important form of disease control. This was highlighted by the proposed pathway of introduction of LMBV to a naïve population of susceptible fish species in North America through trade in subclinically infected ornamental fish (Hedrick and McDowell, 1995). Biosecurity measures need to consider the potential for the virus to remain infective in water for up to 7 days.

8.3.3 Diseases of Fish Caused by Marine Ranaviruses

8.3.3.1 Singapore Grouper iridovirus (SGIV) and Grouper Iridovirus (GIV)

Sleepy grouper disease is a syndrome with high mortality in net-pen culture of grouper. Initial reports of sleepy grouper disease were attributed to iridovirus infection without detailed classification. In Singapore, recurrent disease outbreaks with high cumulative mortality and no antecedent clinical signs other than lethargy and anorexia were reported in brown-spotted grouper (*Epinephelus tauvina*) in 1992 (Chua et al., 1994) and Malabar grouper (*Epinephelus malabaricus*) in 1998 (Chang et al., 2002). SGIV was initially characterized as a ranavirus by sequence of the MCP gene and later the complete genome from further outbreaks of grouper sleepy disease in these species (Qin et al., 2002; Song et al., 2004). A similar disease with clinical signs, including circling and anemia and 60% mortality of yellow grouper (*Epinephelus awoara*) occurred in Taiwan (Chou et al., 1998). Grouper iridovirus (GIV) was characterized from further outbreaks as a ranalike virus based on complete genome sequence (Tsai et al., 2005).

Both SGIV and GIV are very closely related, and although currently classified in the genus *Ranavirus*, the distinction of causing disease in marine fish and the phylogenetic differences from other ranaviruses are sufficient to warrant classification in a separate genus (Whittington et al., 2010). There is confusion in the literature because of a disease of grouper with a similar nonspecific clinical presentation first described in brown-spotted grouper in Thailand in 1993. The causative virus was designated Grouper sleepy disease iridovirus (GSDIV). This virus was different to SGIV and GIV, and later determined to be very closely related to the Red sea bream iridovirus (RSIV) group within the genus *Megalocytivirus*, discussed next (Sudthongkong et al., 2002b).

8.3.3.2 Structure and Composition of Virus

The genome of SGIV and GIV is approximately 140 kb, much larger than megalocytiviruses (~110 kb) and amphibian ranaviruses (~105 kb), but closer to the fish ranavirus EHNV (~130 kb). The similar genomic structure of SGIV and GIV produces complete colinearity. Dot-plot analysis indicates that these viruses were more divergent from EHNV than EHNV is from the amphibian ranaviruses (Jancovich et al., 2010).

8.3.3.3 Classification and Virus Replication

SGIV and GIV share 99% nucleotide similarity, but the available complete genome sequences distinguish the isolates as sharing only 50% of nucleotide repeat elements (Eaton et al., 2010). The partial MCP sequences of SGIV and GIV share 70% amino acid similarity with FV-3 (the *Ranavirus*) and 36% with *Infectious spleen and kidney necrosis virus* (ISKNV) (the *Megalocytivirus* type species) (Qin et al., 2003). There are antigenic similarities with ranaviruses that were indicated by cross-reactivity of polyclonal rabbit antisera to SGIV with FV-3 (Qin et al., 2002).

8.3.3.4 Epidemiology

Grouper sleepy disease in Malabar grouper and brown-spotted grouper resulted in total cumulative mortality of up to 90% over approximately 4 weeks, or as a mass event that was exacerbated by handling, transport or overfeeding (Qin et al., 2003). Typically the mortality associated with diseases caused by SGIV or GIV is between 30% and 100%, influenced by multiple factors, including the age of the fish. Disease has been recorded in 100–200 g fish and with 50% mortality of 2–4 kg adults and broodstock. Outbreaks have lasted up to 12 weeks in the absence of emergency harvest or culling (Tsai et al., 2005).

A standard dose of GIV resulted in higher mortality of orange-spotted grouper (*Epinephelus coioides*) compared with Taiwan grouper iridovirus (TGIV) (a megalocytivirus) in an experimental setting (Leu et al., 2013). Longitudinal analysis of isolates obtained from grouper diseases in Taiwan indicated that GIV was predominant between 2001 and 2005, with megalocytiviruses more common until 2009 (Huang et al., 2011).

8.3.3.5 Pathology and Immunity

Many of the features of grouper sleepy disease caused by SGIV are similar to the disease caused by megalocytiviruses. Clinical signs include lethargy, absence of response to stimulation, inappetence and hanging at the surface or bottom of the net.

The histopathological lesions are more similar to those caused by ranaviruses, which is systemic necrosis in multiple organs, particularly the spleen, instead of hypertrophied inclusion-bearing cells (IBC) (Whittington et al., 2010). Disruption of ellipsoid sheaths with degeneration of cells, pyknosis, karyorrhexis and reduction of hemopoietic tissue with basophilic intranuclear inclusion bodies in some cells were consistent findings (Chua et al., 1994). Endocardiosis and necrosis of reticuloendothelial elements of the kidneys and macrophage infiltration were also observed.

8.3.3.6 Diagnostic Methods

SGIV causes splenomegaly to two to three times the organ's normal size in fish that are in good body condition with occasional pale gills and enlarged kidneys (Qin et al., 2002). The presence and location of the virus concurrent with histopathological lesions can be visualized using in situ hybridization staining of nucleic acids (Huang et al., 2004) or by immunohistochemistry using monoclonal antibodies to visualize viral antigens (Lin et al., 2014).

The virus can be isolated using newly described cell lines including one derived from grouper spleen, *Epinephelus akaara grouper kidney* (EAGK) (Gong et al., 2011). Techniques to confirm the identity of the cultured virus include staining with virus-specific monoclonal antibodies and molecular methods. Loop-mediated isothermal amplification (LAMP) assays are the most commonly described molecular tests specific for these viruses. Sensitive and specific detection of SGIV (Mao et al., 2008) and GIV (Sung et al., 2010) have been claimed based on evaluation of the analytical characteristics of these assays. Commercial diagnostic molecular assays are also available. Antibody ELISA and serum neutralization tests for SGIV can demonstrate a serological response that indicates prior exposure to the virus (Ou-yang et al., 2012a).

8.3.3.7 Prevention and Control

Commercial vaccines are not available, but are a focus of research. Vaccination for SGIV using cultured virus inactivated with formalin or beta-propiolactone provided protection from challenge in orange-spotted grouper (*E. coioides*) in a laboratory experiment (Ou-yang et al., 2012a). Recombinant proteins identified using a bioinformatic approach provided approximately 60% relative survival compared with control fish in a laboratory challenge study of *E. coioides* (Ou-Yang et al., 2012b). Mortality due to SGIV in orange-spotted grouper (*E. coioides*) in a laboratory disease model was reduced by 60% in a preliminary therapeutic treatment evaluation of antiviral ssDNA aptamers (Li et al., 2014). In the absence of these treatment and prevention strategies, more generic disease control measures using biosecurity and husbandry modifications specific to the context are suitable to reduce the impact of these important pathogens (Chew Lim et al., 1996).

8.3.4 Diseases of Fish Caused by Megalocytivirus

A fifth genus within the family *Iridoviridae* called *Megalocytivirus* was accepted by the International Committee on Taxonomy of Viruses (ICTV) in 2005 based on the appearance of enlarged cells observed on histological examination of infected fish (Chinchar et al., 2005). Megalocytiviruses have been recognized as the cause of high mortality diseases that have seriously impacted aquaculture since they were first recognized in 1990 (Inouye et al., 1992). Disease has occurred most frequently in east and southeast Asia (Subramaniam et al., 2012). Three groups of viruses within the genus each have a broad host range, including fish from 50 species, both freshwater and marine. Disease has been seen in a range of tropical and temperate regions (reviewed by Kurita and Nakajima, 2012). Trade in juvenile fish for aquaculture and international trade in ornamental fish threatens to increase the distribution of megalocytiviruses, as evidenced by disease in fish exported to Europe, Japan and Australia (Go et al., 2006; Sriwanayos et al., 2013; Tanaka et al., 2014). Megalocytiviruses are subject to regulation and notification requirements in many countries to protect regions that remain free of disease. Red sea bream iridoviral disease is listed by the World Organization for Animal Health; it is defined by infection with either RSIV or ISKNV as causative agents (OIE, 2012b).

All disease syndromes caused by the various megalocytiviruses present with nonspecific signs of systemic disease, preceding death. Mortality is typically between 20% and 60% and sometimes reaches 100% of fish in intensive aquaculture (Kurita and Nakajima, 2012). Disease is more severe in juvenile fish, but mortality also occurs in market-size fish. Several disease names reflect the different virus species and host fish and location of first description.

Red sea bream iridoviral disease was first described in Japan in 1990 in red sea bream (*Pagrus major*) (Inouye et al., 1992). Forty species are listed by the OIE as being susceptible to disease, including groupers (*Epinephelus* spp.), sea bass (*Lates calcarifer*) and families Seriola, Oplegnathus and Acanthopagrus (OIE, 2012b).

Grouper sleepy disease was used to describe an outbreak in brown-spotted grouper (*E. tauvina*) in Thailand in 1993 by a virus designated GSDIV. Although this disease syndrome is very similar to that caused by SGIV (ranalike iridoviruses,

see preceding section) this disease was caused by a megalocytivirus that was very similar to RSIV (Sudthongkong et al., 2002b). A disease characterized by inactivity, loss of appetite and mortality caused by GSDIV (megalocytivirus) is reported to affect several grouper species cultured in Indonesia, Taiwan, Malaysia and neighboring countries (Mahardika et al., 2008).

Disease caused by ISKNV has been recognized in mandarin fish (*Siniperca chuatsi*), an economically important freshwater species in China since 1994 (He et al., 2001). High mortality is associated with clinical signs of reduced activity and feeding, pale body coloration and pale gills (He et al., 2002). Similar signs were reported in freshwater ornamental fish, including dwarf gourami (*Colisa lalia*) and Murray cod (*Maccullochella peelii peelii*) (Go and Whittington, 2006; Lancaster et al., 2003). Affected marine ornamental fish have lethargy, darkened body and increased respiration rate preceding death (Sriwanayos et al., 2013; Weber et al., 2009).

Turbot reddish body iridovirus (TRBIV) disease is caused by megalocytiviruses belonging to genotype 3 and affects marine flat fish, including turbot (*Scophthalmus maximus*) and flounder (*Paralichthys olivaceus*) in Korea and China (Do et al., 2005; Shi et al., 2004). Mortality is accompanied by clinical signs of reduced feeding activity, pale coloration and increased respiratory effort.

8.3.4.1 Structure and Composition of Virus

Megalocytivirus virions have the typical morphological structure of iridoviruses with a diameter of 160–200 nm. The genome is short relative to the other fish iridoviruses, approximately 110 kb with a GC content of 53–55%. There are approximately 120 open reading frames of which approximately 70% are unique to the genus.

8.3.4.2 Classification and Virus Replication

The large number of megalocytivirus isolates named for the host species from which they were obtained are closely related; there is greater than 93% nucleotide sequence identity (Jancovich et al., 2012). Phylogenetic analysis of the MCP and ATPase genes of approximately 100 isolates resolved three genotypes or tentative virus species (Kurita and Nakajima, 2012; Song et al., 2008). These three groups were supported by the complete genome sequence and structure that is available for fewer isolates (Shi et al., 2010; Zhang et al., 2013). The subclassification of the genus into three virus species is consistent with differences in host range and geographic distribution of each group (Song et al., 2008).

The type species, ISKNV and related viruses are predominately detected in freshwater species, including isolates from ornamental fish such as Dwarf gourami iridovirus (DGIV) and African lampeye iridovirus (ALIV) (Subramaniam et al., 2014a). The group has also been detected in marine fish, including sea bass (*L. calcarifer*) and grouper (*Epinephelus* spp.) and includes isolates referred to as TGIV (Huang et al., 2011; Kurita and Nakajima, 2012). RSIV likely represents a second species in the genus including phylogenetically similar viruses from marine species including rock bream iridovirus (RBIV), Orange-spotted grouper iridovirus (OSGIV) and viruses associated with grouper sleepy disease, such as GSDIV. TRBIV was isolated from turbot (*S. maximus*) in Korea in 2003 and was the cause of disease primarily in fish of the order *Pleuronectiformes*. Isolates related to TRBIV have been a cause of disease in knifejaw (*Oplegnathus* spp.) and sea perch (*Lateolabrax* spp.). A fourth tentative species within the megalocytivirus group was proposed for Threespine stickleback iridovirus (TSIV) detected in Canada from 2008 (Waltzek et al., 2012).

Megalocytiviruses cause systemic disease due to tropism for cells adjacent to blood vessels in visceral organs and possibly fibroblastic cells of the connective tissue (Tanaka et al., 2014).

8.3.4.3 Epidemiology

The host range for megalocytiviruses is broad. More than 50 species, primarily marine, have been identified as being susceptible to disease caused by RSIV; five marine species were susceptible to TRBIV and 35 primarily freshwater species were susceptible to ISKNV (Kurita and Nakajima, 2012). Many species important to aquaculture are susceptible to infection and in surveys, PCR test results have revealed an average prevalence of 15% across 100 species of apparently healthy wild caught marine fish in China (Wang et al., 2007). These viruses have also been detected in apparently healthy freshwater fish in Australia, Malaysia and Korea, including important ornamental species from families: Cichlidae and Poeciliidaes (Jeong et al., 2008; Rimmer et al., 2015; Subramaniam et al., 2014a). Although extensive surveys have not been reported for TRBIV, the potential for persistent subclinical infection was indicated by a transmission study with several susceptible species of fish (Oh et al., 2006).

Movement of subclinically infected fish is a pathway for introduction of disease to new regions, and regulation of trade requires strategies to detect this state of infection. For example, DGIV from subclinically infected gourmai was transmissible to Murray cod; these ornamental fish had passed quarantine inspection prior to importation into Australia, and the same virus caused a disease outbreak in farmed Murray cod (Go and Whittington, 2006; Lancaster et al., 2003; Rimmer et al., 2015). Subclinical infection of marine ornamental fish originating from Indonesia, Banggai cardinalfish (*Pterapogon kauderni*) and orbiculate batfish (*Platax orbicularis*) also succumbed to disease shortly after international transport (Sriwanayos et al., 2013; Weber et al., 2009).

The subdivision of *Megalocytivirus* into three phylogenetic genotypes based on the sequence of the MCP gene provides useful correlations with different disease situations, although genotype designations have not been consistent (Song et al., 2008; Kurita and Nakajima, 2012). Fine-level phylogenetic analysis of the MCP gene distinguished clades within the genotypes that can be useful for molecular epidemiology (Sriwanayos et al., 2013). Viruses phylogenetically related with ISKNV were widely distributed in Asia, whereas the range of RSIV and TRBIV had a more limited geographic range (Song et al., 2008, Subramaniam et al., 2014a). Isolates characterized from Thailand (GSDIV), South Korea (RBIV), and Japan (RSIV) were given different names, but they are the same virus species as RSIV, indicating a wider geographic and host range for this genotype (Kurita and Nakajima, 2012).

Megalocytiviruses are widely distributed in Asia, with only a few apparently contained incursions into other regions. An exception was a new species of megalocytivirus, which was identified as the cause of disease in wild caught three spine stickleback (*Gasterosteus aculeatus*) in Canada in 2008 (Waltzek et al., 2012).

Disease occurs more commonly in summer when the water temperature is higher; market-sized fish are susceptible, but disease is more severe in juveniles where mortality reaches 100% in some intensive aquaculture settings (Kurita and Nakajima, 2012). Horizontal transmission is evident, and may be directly by water, based on susceptibility of some fish to immersion challenge in laboratory experiments (eg, Go and Whittington, 2006). There have been no reports of vertical transmission.

8.3.4.4 Pathology and Immunity

The unifying pathology of megalocytivirus infections is the presence of megalocytes in a variety of tissues, with or without degeneration and necrosis. Megalocytes are distinctive hypertrophied cells that contain large foamy to granular basophilic inclusion bodies that grossly distend the cytoplasm and displace the nucleus to the periphery of the cell (Figs. 8.6 and 8.7).

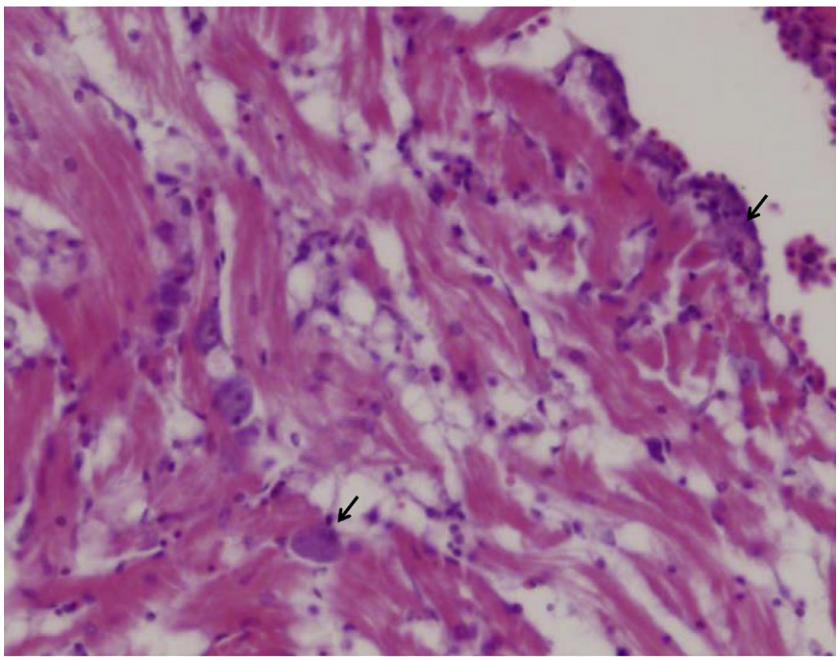

FIGURE 8.6 Red sea bream iridovirus. Myocardium, red sea bream (*Pagrus major*). Inclusion-bearing cells are located on the lining of vessels and myofibers (*arrows*). The nucleus is displaced to the edge of the cytoplasm. Formalin-fixed, paraffin-embedded section, hematoxylin and eosin. (Original glass slide kindly provided by Dr. K. Nakajima, Japan.)

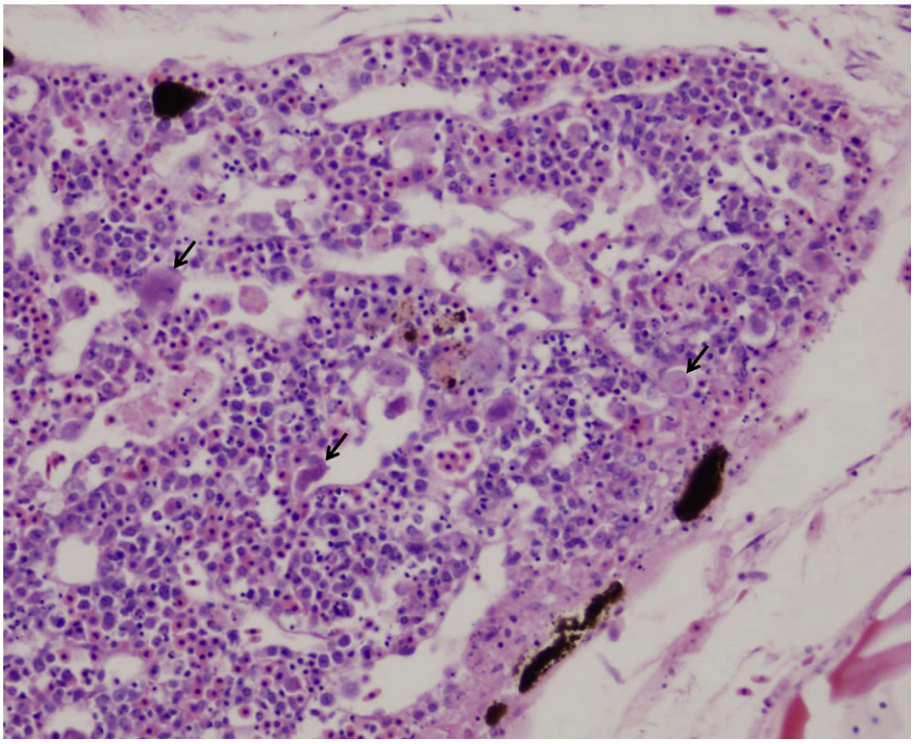

FIGURE 8.7 Dwarf gourami iridovirus. Anterior kidney, Murray cod (*Maccullochella peelii peelii*). Eosinophilic to basophilic inclusion-bearing cells (*arrow*) are located throughout the section. Formalin-fixed, paraffin-embedded section, hematoxylin and eosin.

Megalocytes are sometimes present in large numbers in clinically affected fish, particularly those infected with RSIV, but often they are not numerous and may be absent in some cases (Go and Whittington, 2006). The large size, granular foamy appearance of the inclusion body and distorted cell outline has led to the description of these megalocytes as amoeba-like organisms (Anderson et al., 1993) and their designation as IBC (Miyazaki, 2007). They can be found in many tissues such as spleen, kidney and gastrointestinal tract, and to a lesser extent, liver, heart and gills, but can likely be found in all connective tissues (Chao et al., 2004; Gibson-Kueh et al., 2003; Go and Whittington, 2006; Lancaster et al., 2003; Paperna et al., 2001; Sudthongkong et al., 2002a). These cells may localize to perivascular areas, may block blood flow, and thus, may lead to foci of ischemic necrosis within affected organs. The distorted morphology of infected cells has confounded identification of the target cell type, but recent evidence suggests that they are leukocytes (Lee et al., 2009). The appearance of acutely infected fish, which characteristically have splenomegaly and splenic necrosis (Miyazaki, 2007), suggests other pathogenic mechanisms.

8.3.4.5 Diagnostic Methods

Enlargement of the spleen is a key finding for disease caused by megalocytivirus. The anterior kidney may also be involved and the gills can be pale (Tanaka et al., 2014). Giemsa staining of impression smears of the spleen and heart can demonstrate enlarged cells without requiring histopathology. There are numerous techniques available to confirm the presence of viruses, and different diagnostic guidelines are provided in the Manual of Diagnostic Tests for Aquatic Animals for the purposes of confirming a diagnosis or certification of freedom from infection for disease for control purposes (OIE, 2012b). For diagnosis, samples are best taken from moribund fish with the spleen being the most important target organ, but other relevant tissues include the kidney, gill, heart, liver and intestine. In the case of detecting subclinical fish a broader range of samples should be considered for testing. Monoclonal antibodies are available for fluorescent antibody tests to demonstrate the presence of the virus in histopathology slides or cell cultures.

RSIV can be cultured in several cell lines including Bluegill fry (BF-2), Kelp and Red spotted grouper embryo (KRE-3), and grunt fin (GF). The GF cell line is recommended by OIE for RSIV and ISKNV, but isolation of the virus from freshwater fish is difficult. The ISKNV-like virus, DGIV, has not yet been cultured (OIE, 2012b).

The current OIE recommendation for molecular detection of RSIV and ISKNV is a conventional PCR assay targeting the *Pst* 1 restriction fragment of the viral genome, described by Kurita et al. (1998). False negative tests due to sequence mismatch can occur across the range of viruses within the genus, for example, an isolate of RSIV from sea perch in China failed to amplify due to sequence mismatch (Jeong et al., 2004). Real-time PCR assays are desirable for increased sensitivity and to accommodate high throughput testing for certification testing. The assay validated by Rimmer et al. (2012a) detected DGIV (ISKNV virus species) with much greater sensitivity compared with the conventional PCR assay. Other assays have been developed that are capable of detecting both RSIV and DGIV using conserved amplification targets in the MCP gene (Gias et al., 2011). LAMP assays have been described for ISKNV (Subramaniam et al., 2014b; Sung et al., 2010) and TRBIV (Zhang et al., 2009). The analytical characteristics of these assays are described, but there was limited information for diagnostic validation.

8.3.4.6 Prevention and Control

Vaccination using formalin-inactivated RSIV was demonstrated to provide increased survival of red sea bream after field challenge (Nakajima et al., 1999). Efficacy of the vaccine was demonstrated using RSIV in a laboratory trial in additional species including yellow tail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), kelp grouper (*Epinephelus moara*) and striped jack (*Pseudocaranx dentex*), and GSDIV in humpback grouper (Mahardika et al., 2008; Nakajima et al., 2002). This was one of the first vaccines available commercially. Newer vaccine technologies under evaluation include a DNA vaccine for ISKNV in Chinese perch (Fu et al., 2014) and a recombinant RBIV MCP protein expressed in genetically modified rice plant for oral administration (Shin et al., 2013). A novel approach of exploiting the pathogenesis of the disease involves vaccination of grouper by exposure to RSIV at decreased rearing temperature where infection is not expressed as disease (Oh et al., 2014).

Nonspecific measures to reduce the expression of disease exploit the impact of stress factors on disease expression; improved water quality, reduced stocking density and appropriate nutrition can be beneficial (OIE, 2012b). Biosecurity measures require pathogen-free stock and food fish. Aquaculture hygiene is applied based on predicted disinfection protocols for megalocytiviruses, for example, susceptibility to acid (pH 3.0) and heat (56°C 30 min) (He et al., 2002; Kasai et al., 2002).

8.3.5 Diseases of Fish Caused by Lymphocystivirus

8.3.5.1 Lymphocystis Disease Virus

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease (LCD), affecting more than 140 species of marine and freshwater fish worldwide (Anders, 1989). Currently, the ICTV recognizes *Lymphocystis disease virus 1* (LCDV-1) and tentative species include Lymphocystis disease virus 2 (LCDV-2) and Lymphocystis disease virus China (LCDV-C) (Williams et al., 2005). This cosmopolitan virus has a global distribution and the benign wartlike infection has been well described over many decades (Anders, 1989; Roberts, 2012; Wolf, 1988). Farmed fish affected by LCD are considered susceptible to secondary infections, exhibit reduced growth rates and have an unsightly appearance, leaving the fish unmarketable (Cano et al., 2013; Kitamura et al., 2006a).

8.3.5.2 Structure and Composition of Virus

LCDV is a large iridovirus with virions of heterologous size; the diameters range from 200 up to 230 nm, depending on fish host (Jancovich et al., 2012). LCDV is distinguished from other iridoviruses of fish by a genomic GC content of 29% compared with 48–55%. The genome of LCDV-1 is 102 kbp. LCDV-C is the largest vertebrate iridovirus with a 186 kpb genome and 178 potential genes compared with 108 for LCDV-1.

8.3.5.3 Classification and Virus Replication

The tentative species LCDV-C shares 78% nucleotide sequence identity with LCDV-1 in the MCP gene. Finer level phylogenetic differentiation based on the MCP gene sequence has identified genotypic variants with sequence identity less than 85% that correspond with the host fish species (Hossain et al., 2008). LCDV has a tropism for fibroblasts and is limited to the dermis, rather than causing a systemic infection. The viruses cannot be readily cultivated in cell cultures (Essbauer and Ahne, 2001).

8.3.5.4 Epidemiology

LCD has been described since the 19th century, with a viral etiology first suggested in 1914 (Wolf, 1988). Although highly contagious, LCDV is often considered a benign infection usually affecting wild caught fish living in both freshwater and marine environments. More than 140 fish species are known to be infected and comprehensive lists of known fish hosts were presented in Wolf (1988) and Anders (1989). Ornamental fish in culture are also affected by LCD (Hossain et al., 2008; Weber, 2013; Xu et al., 2014); in this industry, any external blemish can render the fish a complete economic loss. Significant ornamental fish species affected by LCD include *Chanda baculis, Trichopodus trichopterus, Trichopodus leerii* and *Macropodus opercularis*.

Mortality associated with LCD was once considered to be rare (Essbauer and Ahne, 2001) because the lymphocysts typically resolve within a few weeks of onset, often healing with little or no scar tissue (Paperna et al., 1982). However, more recently LCDV has been associated with significant mortality events in farmed fish (Dezfuli et al., 2012; Haddad-Boubaker et al., 2013). Evidence from outbreaks at farms suggests that juveniles are considerably more susceptible than larger, market-sized fish (Tak Seng and Colorni, 2002). Currently, LCDV-1 is the main viral pathogen in cultured gilthead sea bream (*Sparus aurata*), which is one of the most important fish species in the Mediterranean (Cano et al., 2006, 2013). It is also considered a major disease in both Korea and Japan for culture of warmwater marine fish including Japanese flounder (*P. olivaceus*) and Japanese seabass (*Lateolabrax japonicus*) (Tak Seng and Colorni, 2002).

The disease process is relatively slow and high prevalences of subclinically infected carriers are common in natural infections (Cano et al., 2006; Palmer et al., 2012). Although LCDV-1 is known to infect 30 families of marine fish, natural infections with LCDV-1 are associated with high host specificity even though experimental infections have shown a wider host range (Wolf, 1988). Natural infections tend to occur in one or two fish species from a family, even though other members are sharing the environment and have been shown to be susceptible under laboratory conditions (Anders, 1989). This has been demonstrated in centrarchids, in particular in *Micropterus* and *Lepomis*. Kitamura et al. (2006a) demonstrated that strains isolated from rockfish (*Sebastes schlegeli*) and Japanese flounder (*P. olivaceus*) were only able to infect the host species from which they were isolated. In addition, a homologous strain of LCDV-1 required 40 days to produce visible lesions in a percid compared with 10 days in a centrarchid (Wolf, 1988).

The mechanisms of pathogenesis of this virus remain largely unknown (Palmer et al., 2012). The virus can be transmitted horizontally through the water primarily from external surfaces and is presumed to enter the host through gills and breaks in the skin (Essbauer and Ahne, 2001; Wolf, 1988). The incubation time and lesion duration are dependent on host species, virus and the appearance and resolution of visible lymphocysts is water temperature dependent. For example, in centrarchids the disease appears and resolves after 10 days when held at 25°C compared with up to 6 weeks at 12°C (Wolf, 1988). Factors associated with the appearance and recrudescence of disease include water contamination, oxygen depletion, overcrowding and nutritional stress (Cano et al., 2006, 2007). The virus can be detected in fish skin, caudal fin and eyeballs up to 4 weeks after all clinical signs of LCD have resolved (Cano et al., 2006).

8.3.5.5 Pathology and Immunity

The tropism of LCDV-1 for fibroblasts, combined with characteristic cytopathology of cell enlargement, results in classical lesions of the skin in wide range of marine finfish species. Clinically, there are multinodular tumorlike masses in the skin (Figs. 8.8 and 8.9). Nodular lesions may also be observed on the surfaces of viscera (Roberts, 2012). Each nodule, up to 1 mm in diameter, consists of a single enlarged dermal fibroblast, which is macroscopically visible. This incredible degree of cell hypertrophy is pathognomonic and can be confirmed in tissue biopsies, by direct microscopy using a wet mount (Fig. 8.10), or by histopathology (Fig. 8.11). The cytoplasm of lymphocystis-affected cells contains multiple, highly reticulate, basophilic, inclusion bodies (Miyazaki, 2007) and the nucleus and nucleolus are also hypertrophic (Reddacliff and Quartararo, 1992). In some species, such as yellowtail, nodules do not form and the affected fibroblasts are disguised by overlying melanocytes, resulting in pigmented lesions (Miyazaki, 2007).

8.3.5.6 Diagnostic Methods

The characteristic gross lesions and histopathology associated with LCD are pathognomonic for the LCD. Although the agent is not readily cultured, it can be detected using rapid and sensitive molecular assays. A conventional PCR assay for LCDV-1 was sufficiently sensitive to detect subclinical infection using a sample of fin tissue (Cano et al., 2007); a multiplex PCR was suitable to detect multiple genotypes (Kitamura et al., 2006b). Assays using real-time PCR or LAMP technology to amplify a conserved region of the MCP gene were suitable for detection of the MCP region of the three tentative species (Ciulli et al., 2015; Li et al., 2010).

FIGURE 8.8 Lymphocystis nodules (*arrows*) on skin, clown fish, Australia. *Image courtesy LA Reddacliff.*

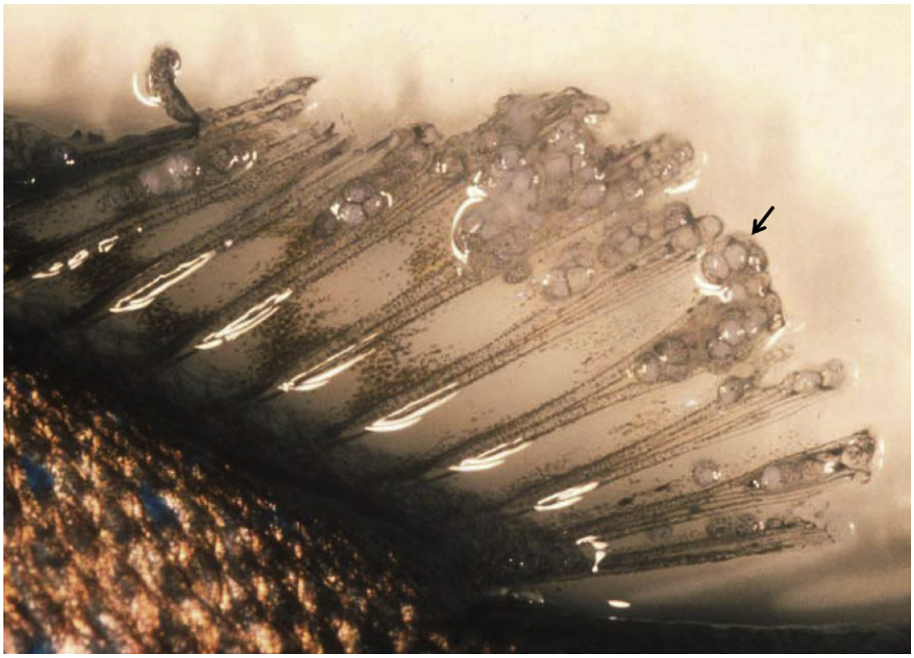

FIGURE 8.9 Lymphocystis nodule (*arrow*), dorsal fin, snapper (*Pagrus auratus*) Australia. *Image courtesy LA Reddacliff.*

8.3.5.7 Prevention and Control

There is no practical cure or effective therapy for LCD and avoidance is the universal control measure. Even though the lymphocysts will eventually slough off and epidemics causing mortality are unusual (Tak Seng and Colorni, 2002; Roberts, 2012), maintaining LCDV-free populations should be prioritized for sustainable aquaculture in endemic areas. In populations of farmed fish, it is paramount that affected individuals are isolated or preferably culled to prevent spread of the virus. Reducing stocking densities can also reduce the chronic production loss associated with the disease. Subclinically infected broodstock can vertically transmit the virus on the surface of eggs (Cano et al., 2013). As such, egg disinfection with iodine (50 mg/L active iodine solution for 10 min) to prevent transmission of LCDV is recommended. Vaccine research has focused on the development of a recombinant plasmid DNA vaccine with a fragment of the MCP cloned into an expression vector (Zheng et al., 2006). Although not tested in clinical trials, the plasmid induced a specific immune response for at least 90 days following intramuscular injection. Another experimental vaccine using encapsulated microspheres loaded with pDNA coded for LCDV-1 showed efficacy after oral administration (Tian et al., 2008).

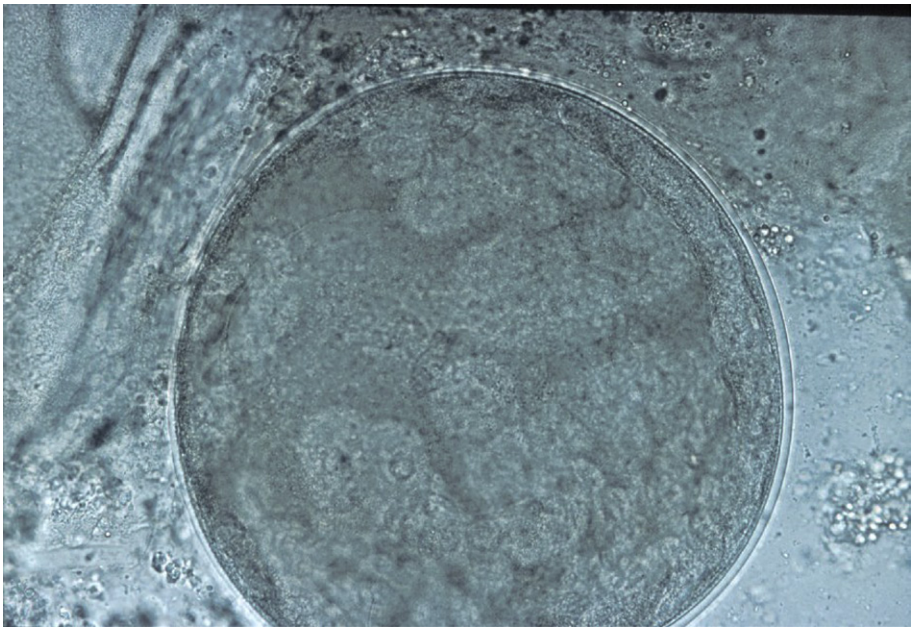

FIGURE 8.10 Lymphocystis nodule, dorsal fin, snapper (*Pagrus auratus*) Australia. Wet-mount preparation of a single nodule, which is a single enlarged cell. *Image courtesy LA Reddacliff.*

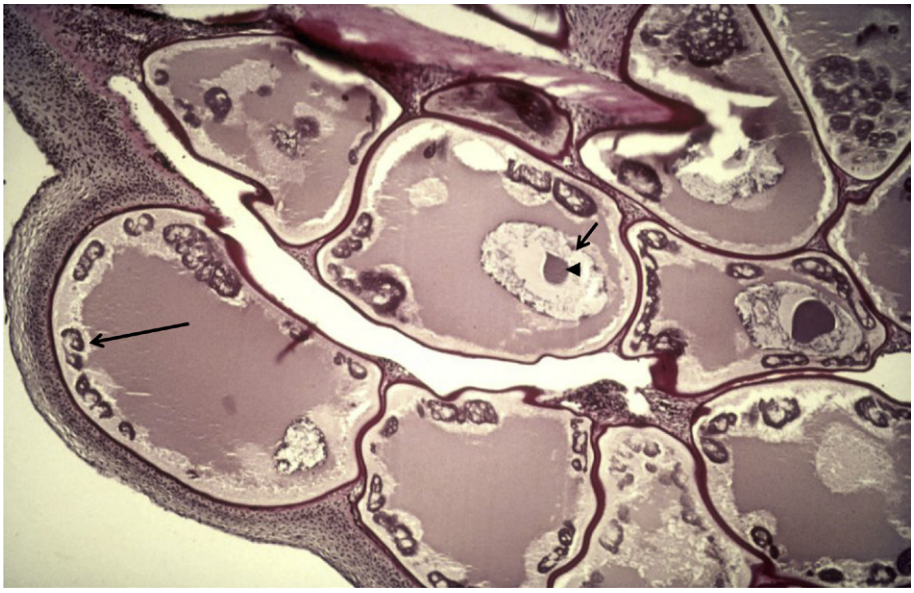

FIGURE 8.11 Lymphocystis, fin ray of snapper (*Pagrus auratus*). Formalin-fixed, paraffin-embedded section, periodic acid Schiff. Hypertrophied nucleus (*short arrow*) and nucleolus (*arrow head*), peripheral intracytoplasmic inclusion bodies (*long arrow*). *Image courtesy LA Reddacliff.*

8.3.6 Diseases of Fish Caused by Erythrocytic Necrosis Virus (ENV)

Viral erythrocytic necrosis (VEN) or piscine erythrocytic necrosis (PEN) is a disease of fish caused by infection with ENV, a currently unassigned iridovirus (Emmenegger et al., 2014). The disease is characterized by destruction of red blood cells, leading to anemia. It has been recognized since the 1960s (Laird and Bullock, 1969) and is most commonly reported from cool water species, including herrings and salmonids in the Atlantic and Pacific oceans (Wolf, 1988). The host range and geographic distribution is diverse, with more than 20 species of marine and anadromous fish species known to be

susceptible to VEN (Dannevig and Thorud, 1999). Infection with ENV is a cause of disease and mortality in aquaculture and in wild fish populations (Hershberger et al., 2009).

8.3.6.1 Structure and Composition of Virus

ENV was identified as an iridovirus based on the ultrastructural appearance of virions in infected host cells (Walker and Sherburne, 1977). Virions are commonly reported to be large, with a diameter greater than 300 nm. Samples of diseased fish of different species have been examined by electron microscopy (EM) and there has been considerable variation between the reports of the morphology of the ENV virions. Sizes ranged from 145 to 330 nm in diameter, with variation in the electron density of the core and the appearance of envelope structures (Nicholson and Reno, 1981).

8.3.6.2 Classification and Virus Replication

ENV has not been isolated in cell culture and very little nucleotide sequence information is available. However, recent molecular characterization of an isolate of ENV from Pacific herring from the west coast of North America confirmed that this virus is an iridovirus (Emmenegger et al., 2014). A portion of the DNA polymerase gene was most closely aligned to erythrocytic iridoviruses that infect terrestrial reptiles, a group of viruses proposed to represent a new genus within the family *Iridoviridae*. The diversity of ENV from the range of hosts, disease conditions and geographic locations has not been determined.

ENV replicates in erythrocytes. In-vivo studies demonstrated that viral load peaked 7 days after experimental infection of Pacific herring (*Clupea pallasii*) and the virus persisted in surviving fish for 28 days (Glenn et al., 2012). Persistent infection of an individual host was proposed to be maintained though erythropoiesis because virions released through destruction of senescent cells infected the new erythrocytes.

8.3.6.3 Epidemiology

Disease caused by ENV has been reported most commonly from the northern Atlantic and Pacific oceans, but the geographic range includes Spain, Japan and Australia (Davies et al., 2009; Lamas et al., 1996; Yoshimizu et al., 1988). Although primarily reported from salmonoids and herring species, the known host range extends to more than 20 species from multiple families including an elasmobranch (Dannevig and Thorud, 1999).

Red blood cell lesions consistent with VEN have been detected at greater than 10% prevalence amongst apparently healthy wild fish (Eiras, 1984; Reno et al., 1978; Smail, 1982). Endemic ENV caused recurrent outbreaks of VEN in wild stocks of young Pacific Herring that resulted in population level effects (Hershberger et al., 2009). Adverse conditions associated with holding wild-caught Pacific herring in tanks resulted in precipitation of VEN at 100% prevalence in 12 days (Hershberger et al., 2006). Similarly, fish affected by VEN responded unfavorably to stress tests under experimental conditions (Haney et al., 1992). This is consistent with field observation of VEN in association with stressors such as secondary disease, low dissolved oxygen and low salinity (Meyers et al., 1986). Mortality from VEN is most common in immature fish, but lesions can be present at low intensity in older fish (Hershberger et al., 2013).

Horizontal transmission is presumed to be the most important method of spread of ENV, even though most reported experimental trials have used injection of material from infected fish and not cohabitation (Glenn et al., 2012; MacMillan et al., 1989; Smail, 1982). The observation of VEN-affected fish erythrocytes in an isopod suggests that vector-borne transmission should be considered (Davies et al., 2009). Vertical transmission of ENV has not been demonstrated, but a high incidence of disease was found in the fry of infected broodstock irrespective of the mechanism of transmission (Rohovec and Amandi, 1981).

8.3.6.4 Pathology and Immunity

Replication of ENV in erythrocytes results in destruction of these cells. The clinical signs in affected fish reflect anemia and subsequent disruption of physiology and hemodynamic homeostasis (Smail, 1982). A disruption of normal hemostasis can occur (MacMillan et al., 1989). Pale gills are evident on examination and the packed cell volume can be reduced to below 10% over 2–4 weeks.

Blood smears stained with conventional stains reveal characteristic darkly stained intracytoplasmic inclusion bodies in erythrocytes and erythroblasts (Smail, 1982). The inclusion bodies are round, with variable size approximately 0.8–4 μm in diameter, and they are singular with a paranuclear location. Up to 90% of red blood cells are affected at the peak of clinical

disease, but intensity can be less than 1% in persistent infections (Glenn et al., 2012). Affected erythrocytes sometimes have degeneration of the cell membrane and the nucleus with pyknosis and karyorrhexis.

8.3.6.5 Diagnostic Methods

A presumptive diagnosis of VEN is obtained using light microscopy to examine a stained blood smear. Conventional stains, such as Geimsa, are appropriate for identification of the characteristic eosinophilic inclusion bodies and accompanying pathology lesions. Ghost cells may be observed and there is responsive erythroblastosis (Hershberger et al., 2006). The red blood cell lesions can also be observed in fixed tissues examined by histology and in blood-rich organs such as the spleen if a blood sample is not available.

Transmission EM is used to confirm the presence of an iridovirus in the cytoplasm of affected erythrocytes (Glenn et al., 2012). This differentiates the inclusion bodies from protozoan parasites of blood cells or staining artifacts in the cells. A differential diagnosis is erythrocytic inclusion body syndrome (EIBS), an anemia-inducing disease of salmonoids caused by an unclassified RNA virus with icosahedral virions that are 80 nm in diameter (Graham et al., 2002). Piscine orthoreovirus causes these lesions in experimental infections and may be the cause of at least some cases of EIBS (Finstad et al., 2014). Acridine orange staining provides an indirect differential test. It emits a green fluorescence when bound to the double-stranded DNA of ENV in the inclusion bodies, rather than a red fluorescence if bound to RNA (Davies et al., 2009). Piscine orthoreovirus can also be detected using a specific PCR assay (Finstad et al., 2014).

Sequence information for ENV enabled the development of PCR tests for rapid, sensitive and specific identification ENV (Emmenegger et al., 2014). These tests must be interpreted with caution until they are further validated, particularly as they were designed with a paucity of ENV sequence information. Determination of the nucleotide sequence of many isolates of ENV will enhance the diagnostic repertoire in addition to providing greater understanding of VEN.

8.3.6.6 Prevention and Control

Current understanding of the epidemiology supports changes to general fish husbandry as a method of reducing the impact of VEN. This includes minimizing handling or transportation, adverse environmental conditions, particularly low dissolved oxygen, low salinity and other stressors. The impact of VEN may be reduced using appropriate diagnosis, treatment and control of secondary infections.

Nonspecific biosecurity measures are applicable based on the presence of ENV in wild fish and the potential for horizontal transmission by currently poorly understood mechanisms. International trade in salmonoids is regulated in part by the risk of transferring ENV to countries in the southern hemisphere that are free of VEN. Possible detection of subclinical infections using survival samples permits screening and exclusion of fish, including broodstock with VEN lesions (Hershberger et al., 2006). However, an infected population will not be detected without an adequate sample size.

8.3.7 Diseases of Fish Caused by White Sturgeon Iridovirus (WSIV)

White sturgeon iridoviral disease is an impediment to the culture of several species of sturgeon that are valuable for conservation and aquaculture. The disease has a prolonged course with chronic lethargy and cessation of feeding, leading to emaciation and high mortality in young sturgeon (Adkison et al., 1998; Hedrick et al., 1990; LaPatra et al., 1994). Clinical signs result from epithelial lesions affecting the skin, oral mucosa and gills. Total cumulative mortality varies between 50% and 100% over a period of 2–4 weeks. The causative agent, WSIV was first isolated from white sturgeon (*Acipenser transmontanus*) affected by disease events in California in the late 1980s (Hedrick et al., 1992). A similar disease associated with an iridovirus infection caused recurrent annual mortality of Russian sturgeon (*Acipenser gueldenstaedtii*) in northern Europe (Adkison et al., 1998). More recently, Missouri River sturgeon iridovirus (MRSIV) was identified as the cause of a disease with similar clinical appearance in juvenile hatchery-reared pallid sturgeon (*Scaphirhynchus albus*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) (Kurobe et al., 2011). A related virus that caused a similar high mortality disease in Manitoba lake sturgeon (*Acipenser fulvescens*) was designated Namao virus.

8.3.7.1 Structure and Composition of Virus

WSIV is currently unassigned within the family *Iridoviridae* (Jancovich et al., 2012). The large size and larger, unique inner membrane of virions more closely resemble LCDV than other iridoviruses. The diameter of the outer capsid is approximately 300 nm between vertices when observed by EM (Raverty et al., 2003; Watson et al., 1998a). The outer capsid of MRSIV was smaller 250–260 nm (Kurobe et al., 2011), with Namao virus intermediate at 280 nm (Clouthier et al., 2013).

8.3.7.2 Classification and Virus Replication

Phylogenetic relationships have been determined using limited regions of viral sequence. The amino acid identity in the MCP gene of Namao virus and WSIV was 83.5% and these aligned more closely with viruses of the family *Mimiviridae* compared with *Iridoviridae* (Clouthier et al., 2013). Inability to culture MRSIV *in vivo* and sequence determined only for the serpin gene has limited the characterization of this virus (Kurobe et al., 2010).

8.3.7.3 Epidemiology

Disease caused by WSIV has occurred recurrently in North America and Europe since the late 1980s (OIE, 2003). Reports of disease in sturgeon reared on farms in China are suggestive of a broader geographical range (Yu-ping and Di, 2005). The host range for natural disease caused by WSIV-like viruses is limited to sturgeon species, and neither striped bass (*Morone saxatilis*) or channel catfish (*Ictalurus punctatus*) were susceptible to experimental transmission (Hedrick et al., 1992).

Disease caused by WSIV occurs in fish less than 1 year of age and mortality is lower in older fish (Adkison et al., 1998; Georgiadis et al., 2001; LaPatra et al., 1994). Survivors of outbreaks were reported to be resistant to disease during subsequent outbreaks. There is considerable evidence for a WSIV carrier state in apparently healthy wild fish (LaPatra et al., 1994). A carrier state was demonstrated for MRSIV in experimentally infected fish (Kurobe et al., 2011) and a high prevalence of positive PCR tests were obtained from wild adult specimens (Kurobe et al., 2010).

Experimental transmission of WSIV and MRSIV by cohabitation indicates the importance of water-borne horizontal transmission, including from fish with subclinical infection (Drennan et al., 2007a; Kurobe et al., 2011). Epidemiological studies of disease in hatcheries indicate that vertical transmission might also occur from wild-caught broodstock (Georgiadis et al., 2001) and the source of eggs was the most important risk factor for disease (Georgiadis et al., 2000).

Water temperature strongly influenced experimental infection of white sturgeon. The course of disease was slower, but greater cumulative mortality occurred at 10°C compared with higher temperatures (Watson et al., 1998b). Stressful events can influence the timing and severity of outbreaks (Georgiadis et al., 2001). Mortality was higher at increased stocking density and fluctuating water temperature, low water flow, chlorine insult and the handling of fish might influence expression of disease (Georgiadis et al., 2000; LaPatra et al., 1994, 2014).

8.3.7.4 Pathology and Immunity

Infection with WSIV differs from many other iridoviruses because it does not cause a systemic infection. The virus has tropism for the superficial epithelium, causing hemorrhagic and ulcerative lesions in the oral mucosa and olfactory organs as well as the skin, gills and alimentary tract. The lesions are often multifocal and severe, resulting in cessation of feeding and loss of body condition (Adkison et al., 1998; Hedrick et al., 1990; Kurobe et al., 2011). Disruption of sensory epithelium, in particular, might be the cause of cessation of feeding that contributes to morbidity and mortality (Watson et al., 1998a). Mortality and clinical signs can be exacerbated by secondary infections.

There is variable hypertrophy, degeneration and necrosis of the integument and mucosa. Hyperplastic epithelial cells have evidence of dysplasia and karyorrhexis (Hedrick et al., 1990; LaPatra et al., 1994; Raverty et al., 2003). There are associated clusters of enlarged Malpighian and mucosal cells, although cell enlargement is less marked compared with lymphocystis. Affected cells have irregular or eccentric nuclei and a finely granular amphophilic to basophilic-staining cytoplasm that might include intracytoplasmic "crystalline rod-like" inclusion bodies (Raverty et al., 2003).

Survivors of a disease outbreak were refractory to further disease events, although it was not clear if this was due to being larger and older or the presence of specific immunity (Adkison et al., 1998). Antibodies specific for WSIV were detected in the serum and mucosa of white sturgeon after immunization (Drennan et al., 2007b).

8.3.7.5 Diagnostic Methods

The target tissues for sampling to detect lesions or the virus are the gill, the fleshy part of the mouth and the fleshy portion of the fins. These tissues are fixed in formalin and prepared for histologic examination and portions are used fresh for virology. Nonlethal samples to test for subclinical infection can be obtained by punch biopsy of pectoral fin, and these compared favorably to mucosal tissues from the head collected from experimentally infected fish (Drennan et al., 2007a).

The histopathological appearance of lesions in epithelial cells in hematoxylin and eosin (H&E)-stained tissue sections is characteristic of the disease. Confirmation of the presence of WSIV in association with the lesions requires EM (Raverty et al., 2003) or an immunohistochemical test, using polyclonal rabbit antisera or monoclonal antibodies (OIE, 2003). Histopathology does not identify the subclinical state indicated by positive results in a PCR assay (Kurobe et al., 2010).

Culture of WSIV using numerous primary and established sturgeon cell lines including WSS-2 (white sturgeon spleen) or WSSK-1 (white sturgeon skin) enables this virus to be isolated with cytopathic features, including pseudosyncytia (Watson et al., 1998a). The cause of cytopathic effect is confirmed using neutralization or fluorescence-staining tests with WSIV-specific antibodies (OIE, 2003). MRSIV is refractory to culture.

A conventional PCR assay specific for WSIV was described by Kwak et al. (2006) and different conventional and real-time PCR assays were required for MRSIV (Kurobe et al., 2010). A conventional PCR targeting the MCP gene of Namao virus also detected WSIV, but the qPCR assays for each virus were not cross-reactive (Clouthier et al., 2013). Strain differences causing false negative PCR results are reported by Drennan et al. (2007a), indicating the need for the EM in diagnostic cases where characteristic histopathology is observed. Other viral infections are important in the differential diagnosis of disease in sturgeon, including another iridovirus: the amphibian ranavirus FV-3, which has caused disease with lesions in the hematopoietic system and mortality up to 95% in pallid sturgeon (Waltzek et al., 2014).

8.3.7.6 Prevention and Control

Nonspecific methods of reducing the impact of disease through improved culture conditions and general husbandry, including reduced handling and stocking density, are applicable (LaPatra et al., 1994). As noted, the impact of the disease may be reduced by increasing the water temperature above 10°C.

The disease was previously listed by the World Organization for Animal Health (OIE, 2003) and local regulations will protect against spread of the viruses. In areas where the disease is endemic, control of transmission requires identification of broodstock that are free from infection and water that is adequately disinfected (LaPatra et al., 1999). Disease-free broodstock cannot be guaranteed, but fish should be excluded if they test positive using sensitive molecular tests on biopsies of epithelial tissue.

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Chapter 9

Alloherpesviruses of Fish

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9.1 INTRODUCTION

Herpesviruses are a diverse group of viruses with large DNA genomes. Genetic analysis has shown that there are three distinct groups (families) of herpesviruses: *Herpesviridae* infects the reptiles, birds and mammals; *Malacoherpesviridae* infects mollusks; and *Alloherpesviridae* infects fish and amphibians (Davison et al., 2009; McGeoch et al., 2006; Waltzek et al., 2009a,b). The possible exception to these general trends is tilapia larvae encephalitis virus, which showed more sequence similarity in a small fragment of the polymerase gene to *Herpesviridae* than *Alloherpesviridae* (Shlapobersky et al., 2010). Sequence similarity between herpesvirus families is almost nonexistent. In fact, in large-scale comparisons between the deduced amino acid sequences of the genes of the DNA viruses, the *Herpesvirales* did not form a monophyletic group (Wu et al., 2009). The only gene with clear homology is the ATPase subunit of the terminase, a protein involved in packaging genome into the capsid during virion assembly (Davison, 2002; Davison et al., 2005, 2009). The encoding gene also appears to be related to a similar gene in T4-like bacteriophages. Within *Alloherpesviridae* there are 12 genes that are consistently conserved (van Beurden et al., 2010). The more highly conserved regions with these genes can be used to design degenerate primers for polymerase chain reaction (PCR)-based targeted gene sequence amplification and for sequence comparisons (Hanson et al., 2006; Kelley et al., 2005; Waltzek et al., 2009a). This method was used by Waltzek et al. (2009a) to amplify and sequence a portion of the DNA polymerase gene, and ATPase subunit of the terminase gene and allow phylogenic assessment of 13 fish and amphibian herpesviruses. This study showed that there were two monophyletic clades within *Alloherpesviridae* with Anguillid herpesvirus 1 (AngHV1), Cyprinid herpesvirus 1 (CyHV1), Cyprinid herpesvirus 2 (CyHV2), Cyprinid herpesvirus 3 (CyHV3) in Clade 1, and Ictalurid herpesvirus 1 (IcHV1), Ictalurid herpesvirus 2 (IcHV2), Acipenserid herpesvirus 1 (AciHV1), Acipenserid herpesvirus 2 (AciHV2), Salmonid herpesvirus 1 (SalHV1), Salmonid herpesvirus 2 (SalHV2), Salmonid herpesvirus 3 (SalHV3), Ranid herpesvirus 1 (RaHV1), and Ranid herpesvirus 2 (RaHV2) in Clade 2 (Table 9.1) (Waltzek et al., 2009a). A later study highlighted the uniqueness of the ranid herpesviruses from clade 2 and suggested three subfamilies should be considered within the *Alloherpesviridae* (Doszpoly et al., 2011a). Currently, the International Committee on Taxonomy of Viruses (ICTV) recognizes the following genera and associated species: *Batrachovirus* containing *RaHV1* and *RaHV2*; *Cyprinivirus* containing *CyHV1, CyHV2, CyHV3*, and *AngHV1*; *Ictalurivirus* containing *IcHV1, IcHV2* and *AciHV2* and *Acipenserid herpesvirus 2*; and *Salmonivirus* containing *SalHV1, SalHV2*, and *SalHV3* (ICTV, 2014).

Although there is almost no sequence conservation between the families, there is structural and biological conservation. Structurally, a herpesvirus virion contains a linear, double-stranded DNA genome packaged within an icosahedral nucleocapsid (capsid diameter ~100 nm) that is surrounded by a proteinaceous tegument layer and finally a host-derived envelope (Minson et al., 2000). The linear genome is packed in an electron-dense staining core in transmission electron microscopy (TEM). Cryoelectron microscopy and computer-based image reconstruction demonstrates that the physical structure of the capsids of *Human herpesvirus 1* (*Herpesviridae*), *Ictalurid herpesvirus 1* (*Alloherpesviridae*) and *Ostreid herpesvirus 1* (*Malacoherpesviridae*) are remarkably conserved. All three are composed of 162 capsomers (150 hexons, 12 pentons) and each capsomer has a chimney-like protrusion with an axial channel in the middle (Booy et al., 1996; Davison et al., 2005).

The replication scheme of these viruses also appear to be conserved and have been best characterized in the *Herpesviridae* (Mettenleiter et al., 2009). The glycoproteins on the envelope of virions attach to the cellular receptors, and envelope-membrane fusion releases the nucleocapsid and tegument into the cytoplasm. The nucleocapsid is transported to a nuclear pore and the linear genome is released into the nucleus. The genome is then circularized in the nucleus and genes are expressed using the cell's transcription and translation machinery. Replication using the viral DNA polymerase generates branching concatamers. Empty capsids assemble in the nucleus, then DNA is loaded into them and cleaved at specific sites

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00009-7
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TABLE 9.1 Alloherpesviruses and Associated Diseases That Impact Aquaculture and Wild Fisheries

Aquaculture	Virus Name (Abbreviation)	Common Name (Abbreviation)	Host(s)	Clinical Presentation	References
Eels	Anguillid HV 1 (AngHV1)	HV anguillae (HVA)	Japanese eel *Anguilla japonica*, European eel *Anguilla anguilla*	Hemorrhages of skin, fins, gills, liver	Sano et al. (1990), van Beurden et al. (2010)
Cyprinids	Cyprinid HV 1 (CyHV1)	HV cyprini, carp pox HV, carp HV(CHV)	Common carp *Cyprinus carpio*	High losses in fry-exophthalmia hemorrhages, survivors develop papillomas below 20°C	Sano et al. (1985a,b), Sano et al. (1991)
	Cyprinid HV 2 (CyHV2)	Goldfish hematopoietic necrosis virus (GFHNV)	Goldfish *Carassius auratus*, Prussian carp *Carassius gibelio*	High mortality all ages classes, necrosis of hematopoietic tissue, spleen, pancreas, intestine	Goodwin et al. (2006a,b), Jung and Miyazaki (1995)
	Cyprinid HV 3 (CyHV3)	Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)	Common carp	Gill inflammation, hyperplasia, and necrosis, hematopoietic tissue necrosis, high mortality, 18–26°C, all ages	Hedrick et al. (2000)
Catfish	Ictalurid HV 1 (IcHV1)	Channel catfish virus (CCV), Channel catfish herpesvirus	Channel catfish *Ictalurus punctatus*	Kidney, liver and intestinal necrosis, hemorrhages, high mortality in young fish at above 27°C	Fijan et al. (1970), Wolf and Darlington (1971)
	Ictalurid HV 2 (IcHV2)	Black Bullhead HV Ictalurus melas HV (IcmHV)	Black bullhead *Ameiurus melas*	Kidney necrosis, hemorrhages, high mortality all ages	Alborali et al. (1996), Hedrick et al. (2003)
Sturgeon	Acipenserid HV 1 (AciHV1)	White sturgeon HV 1	White sturgeon *Acipenser transmontanus*	Diffuse hyperplastic dermatitis, moderate losses in juveniles	Hedrick et al. (1991)
	Acipenserid HV 2 (AciHV2)	White sturgeon HV 2	White sturgeon Siberian sturgeon *Acipenser baeri*, Shortnose sturgeon, *Acipenser brevirostrum*, Lake sturgeon *Acipenser fulvescens*	Diffuse hyperplastic dermatitis, high losses in juveniles	Watson et al. (1995), Shchelkunov et al. (2009)
Salmonids	Salmonid HV 1 (SalHV1)	HV salmonis (HPV) Steelhead herpesvirus (SHV)	Rainbow trout *Oncorhynchus mykiss*	Mild disease low losses at 10°C, asymptomatic brood stock carry the virus and shed it in ovarian fluid	Wolf et al. (1978)

	Salmonid HV 2 (SalHV2)	Oncorhynchus masou virus (OMV), Nerka virus Towada Lake Akita and Amori Prefecture (NeVTA), yamame tumor virus (YTV)	Cherry salmon *Oncorhynchus masou*, coho salmon *Oncorhynchus kisutch*, sockeye salmon *Oncorhynchus nerka*, coho salmon *Oncorhynchus keta*, rainbow trout	Viremia, external hemorrhages exophthalmia, hepatic necrosis with high losses in young. Survivors develop oral papillomas, and shed virus in ovarian fluid	Kimura et al. (1981), Sano (1976)
	Salmonid HV 3 (SalHV3)	Epizootic epitheliotropic disease virus (EEDV)	Lake trout *Salvelinus namaycush*, lake trout X brook trout *Salvelinus fontinalis* hybrids	Epithelial hyperplasia, hypertrophy, hemorrhages on eye and jaw. High losses in juveniles at 6–15 °C	Bradley et al. (1989), McAllister and Herman (1989)
	Salmonid HV 4 (SalHV4)	Atlantic salmon papillomatosis virus (ASPV), Atlantic salmon herpesvirus	Atlantic salmon *Salmo salar*	Papillomas especially on smolts	Doszpoly et al. (2013), Shchelkunov et al. (1992)
Cod	*Gadid herpesvirus* 1 (GaHV1)	Atlantic cod herpesvirus (ACHV)	Atlantic cod *Gadus morhua*	Hypertrophy of cells in gills.	Marcos-Lopez et al. (2012)[a]
Pilchards (wild)	Pilchard HV		Australian pilchard *Sardinops sagax*	Acute losses with gill inflammation, epithelial hyperplasia and hypertrophy	Crockford et al. (2005), Doszpoly et al. (2011a), Hyatt et al. (1997), Whittington et al. (1997)[a]
Tilapia	tilapia HV	Tilapia larvae encephalitis virus (TLEV)	Blue tilapia (*Oreochromis aureus*)	Encephalitis and high loses in larvae	Shlapobersky et al. (2010)

[a]*Crockford, M., Jones, J.B., Crane, M.S.J., Wilcox, G.E., 2005. Molecular detection of a virus, Pilchard herpesvirus, associated with epizootics in Australasian pilchards Sardinops sagax neopilchardus. Dis. Aquat. Organ. 68, 1–5.*
Hyatt, A.D., Hine, P.M., Jones, J.B., Whittington, R.J., Kearns, C., Wise, T.G., et al., 1997. Epizootic mortality in the pilchard Sardinops sagax neopilchardus in Australia and New Zealand in 1995. II. Identification of a herpesvirus within the gill epithelium. Dis. Aquat. Organ. 28, 17–29.
Marcos-Lopez, M., Waltzek, T.B., Hedrick, R.P., Baxa, D.V., Garber, A.F., Liston, R., et al., 2012. Characterization of a novel alloherpesvirus from Atlantic cod (Gadus morhua). J. Vet. Diagn. Invest. 24, 65–73.
Whittington, R.J., Jones, J.B., Hine, P.M., Hyatt, A.D., 1997. Epizootic mortality in the pilchard Sardinops sagax neopilchardus in Australia and New Zealand in 1995.1. Pathology and epizootiology. Dis. Aquat. Organ. 28, 1–16.

using a portal complex. These newly formed nucleocapsids then bud out of the nucleus and are released into the cytoplasm where they acquire the tegument and then bud into the vesicles of the trans-Golgi network, acquiring the glycoprotein-studded envelope, after which they are exocytosed as infectious virions.

The genomes contain more than 70 genes. The smallest characterized alloherpesvirus, *Ictalurid herpesvirus 1*, is 134 kb and contains 76 deduced genes (Davison, 1992). The largest, *Cyprinid herpesvirus 3*, is 295 kb and contains 155 genes (Aoki et al., 2007; Davison et al., 2013). The gene structures are generally simple with only 1 out of 10 genes in the genome containing introns. However, transcriptome analysis demonstrates more frequent splicing and alternate splicing than was predicted from genomic data (van Beurden et al., 2012a). In addition, overlapping transcription is relatively rare and transcription occurs from both strands. These include genes that encode proteins involved in basic structural components of the virion, virus replication and a variety of genes that encode proteins that intricately interact with the host's physiology. A common characteristic of herpesviruses is that they establish a latent state in the host after an initial replicative infection. During this latent state, no infectious virus is produced and very little viral protein is expressed. The triggers that regulate latency and productive replication are intricately tied to the physiology of the host. Most often these viruses will not infect or will establish a limited infection in the nonnatural host, but occasionally herpesviruses can cause lethal disease in non-native hosts. For example, herpes B virus, *Cercopithecine herpesvirus 1*, is a common pathogen causing mild disease in macaque monkeys but is highly lethal to humans, causing more than 70% mortality if untreated.

Although herpesviruses have large complex genomes, the viral DNA is infectious and can be manipulated, allowing for intricate reverse genetics experiments and recombinant vaccine development. One of the most promising developments is the establishment of infectious clones in bacterial artificial chromosomes (BAC) because genetic manipulation can be done using highly efficient bacterial systems. Recombinant BAC technology has been applied to IcHV1 and CyHV3 (Costes et al., 2008; Kunec et al., 2008, 2009).

The diseases produced by herpesviruses vary substantially and a latent or recrudescent virus can cause different disease signs and outcomes than the primary infection. They undergo productive replication during primary infection. This often involves replication in a variety of cell types and may result in viremia and acute disease. Disease outcome from a primary infection is often controlled by the dose of the virus and the immune status of the fish. In the natural host, primary infection often results in mild disease and dissemination within a population, but under optimal conditions that often occur in high-density aquaculture, several alloherpesviruses can cause severe epizootics with high morbidity and mortality. Typical lesions seen in primary herpesvirus infections include epithelial proliferation of the skin and gills (Fig. 9.1C,D), hemorrhages (Fig. 9.1E), ascites (Fig. 9.1F), and kidney and liver necrosis. The most common manifestation of disease associated with recrudescent virus expression is dermal or epidermal cell proliferation (Fig. 9.1A). These proliferative lesions are often unsightly, but are rarely fatal. Cellular changes that are often seen with alloherpesviruses include hypertrophy and karyomegaly, margination of the chromatin with eosinophilic or light-staining intranuclear inclusions (Fig. 9.2A,C,D).

Alloherpesviruses cause economically important diseases in catfish, carps, sturgeon, eel and salmonid aquaculture industries (Table 9.1). In addition, alloherpesviruses cause disease in amphibians, *Ranid herpesvirus 1* and *2* (Davison et al., 2006), and are likely the cause of disease in at least 13 other species of fish (the pathogens appear to be herpesviruses by electron microscopy, but have not been genetically characterized) (Waltzek et al., 2009a).

9.2 CATFISH ALLOHERPESVIRUSES

There are two characterized alloherpesviruses that cause substantial losses in catfish aquaculture, *Ictalurid herpesvirus 1* (IcHV1, channel catfish virus (CCV)) and *Ictalurid herpesvirus 2* (IcHV2, black bullhead virus). In a diagnostic setting, distinguishing between the two viruses is important because IcHV2 has not been detected in North America and may be more virulent to channel catfish than IcHV1 (Hedrick et al., 2003). The two virus species are phylogenetically close and are within the genus *Ictalurivirus,* with IcHV1 being the type species (Waltzek et al., 2009a).

9.2.1 *Ictalurid herpesvirus 1*

Ictalurid herpesvirus 1 was first isolated in 1968 from populations of juvenile channel catfish in Alabama, Arkansas and Kentucky suffering massive mortalities due to a severe hemorrhagic disease termed channel catfish virus disease (CCVD) (Fijan et al., 1970). This was the first intensively studied of the alloherpesviruses. It was first characterized in 1971 (Wolf and Darlington, 1971).

Ictalurid herpesvirus 2 was first identified in aquacultured adult black bullheads in Italy in 1994 (Alborali et al., 1996). This virus caused severe disease outbreaks that had a part in devastating the small black bullhead aquaculture industry in Italy (Roncarati et al., 2014).

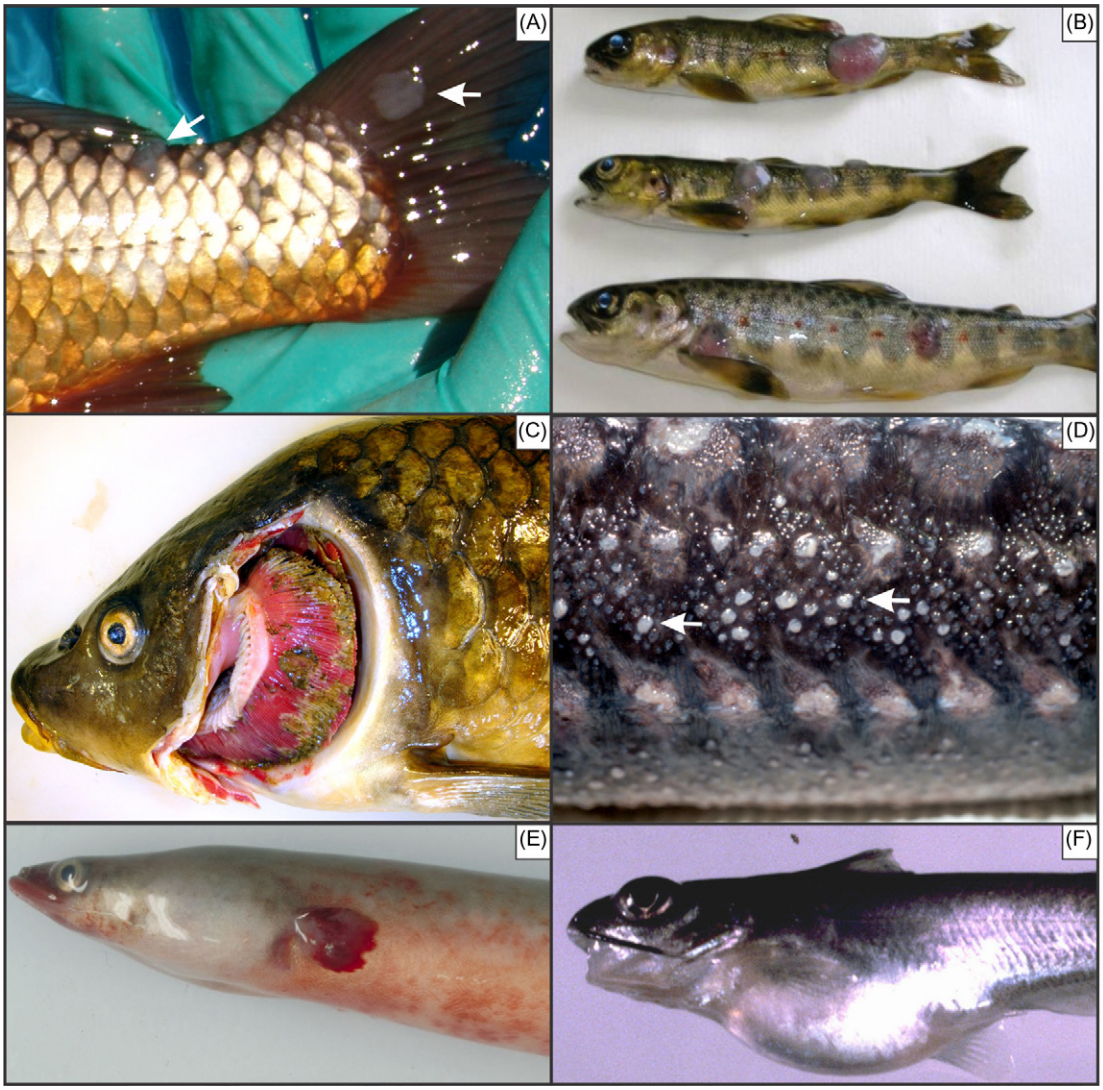

FIGURE 9.1 Images displaying external gross pathology induced by several alloherpesviruses that negatively impact aquaculture. (A) A common carp infected with *Cyprinid herpesvirus 1* displaying foci of epidermal hyperplasia (*arrows*). (B) Atlantic salmon infected with *Salmonid herpesvirus 4* displaying Atlantic salmon papillomatosis lesions. (C) Common carp infected with *Cyprinid herpesvirus 3* displaying necrotic gill lesions associated with KHVD. (D) Skin of white sturgeon infected with *Acipenserid herpesvirus 2* displaying white multifocal epithelial hyperplasia on skin (*arrows*). (E) European eel infected *with Anguillid herpesvirus 1* displaying petechial hemorrhages on the skin. (F) Channel catfish fingerling infected with *Ictalurid herpesvirus 1* displaying exophthalmia and coelomic distension associated with channel catfish virus disease. *Image B from Doszpoly, A., Karaseva, T.A., Waltzek, T.B., Kalabekov, I.M., Shchelkunov, I.S., 2013. Atlantic salmon papillomatosis in Russia and molecular characterization of the associated herpesvirus. Dis. Aquat. Organ. 107, 121–127 by permission from Disease of Aquatic Organisms. Image C provided by Mark Okihiro. Image E from Haenen, O., Dijkstra, S., van Tulden, P., Davidse, A., van Nieuwstadt, A., Wagenaar, F., et al., 2002. Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, Anguilla anguilla in the Netherlands since 1996. Bull. Eur. Assoc. Fish Pathol. 22, 247–257; CVI-NL no. 519348.*

CCVD can cause high mortality, reduced growth and a predisposition to bacterial diseases (Plumb, 1978). Severe outbreaks of CCVD are often associated with environmental stress and crowding. Almost all CCVD cases occur in heavily stocked channel catfish fingerling ponds during warm summer months. The optimum temperature for disease progression is 27°C or higher (Plumb, 1973a). Although older fish can be affected (Hedrick et al., 1987), natural outbreaks almost exclusively occur in catfish that are less than 1 year of age. In experimental challenge trials, very young fish are more resistant to the virus and become more sensitive as they age to 60-days posthatch. Furthermore the resistance was correlated to the maternal parent's CCV neutralizing antibody titer, suggesting that maternal transfer of antibodies may influence early susceptibility (Hanson et al., 2004). In the catfish producing region of the United States, the optimum temperatures for CCVD occur from July through September when fingerlings are less than 4 months of age. Under these conditions, more than 90%

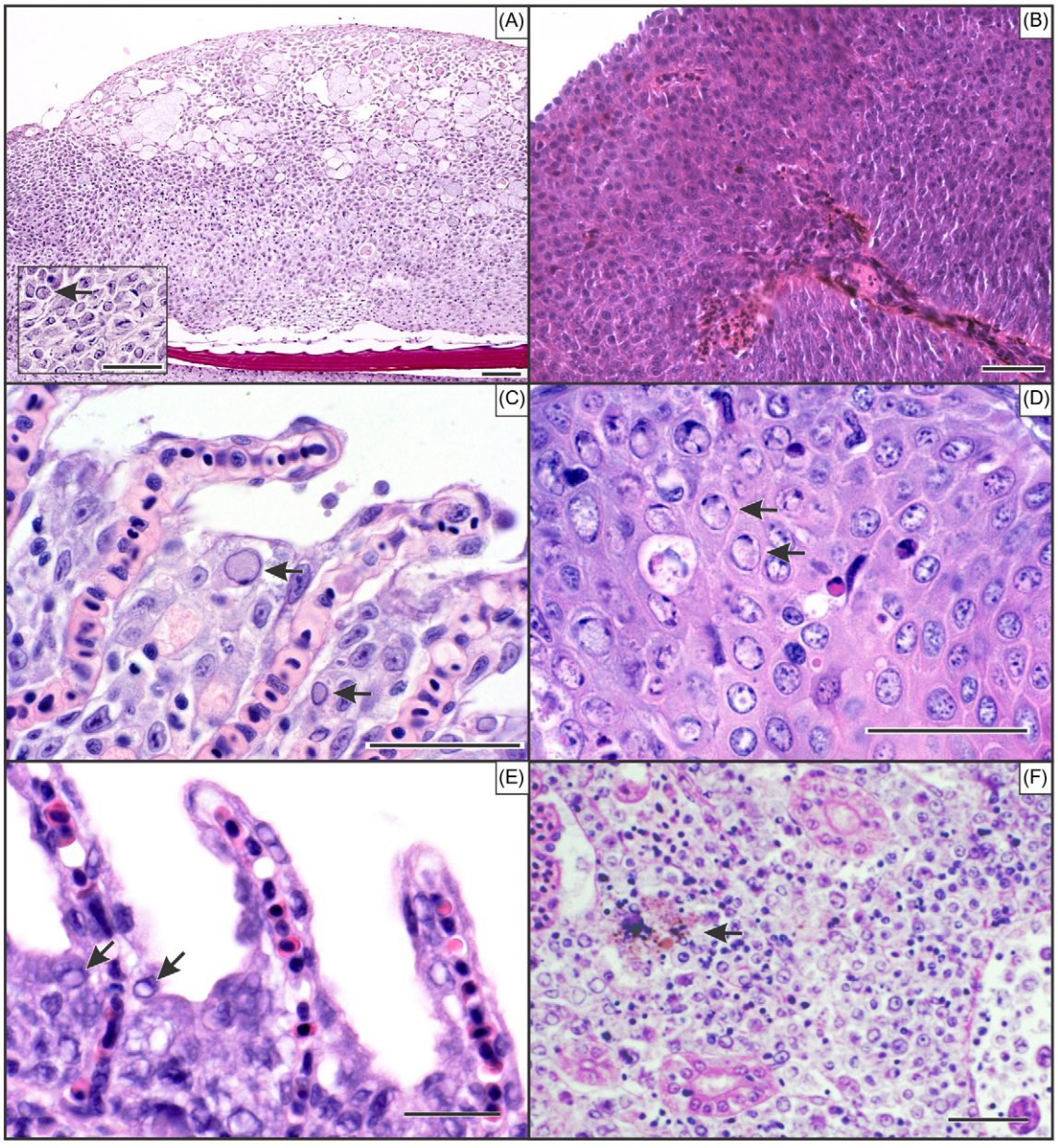

FIGURE 9.2 Histopathologic findings of alloherpesvirus infections in cultivated finfishes (hematoxylin and eosin, H&E, stain). (A) Koi infected with *CyHV1* displaying epidermal hyperplasia of the integument. Inset shows higher magnification of the keratinocytes revealing karyomegaly, margination of chromatin, and pale eosinophilic nuclear inclusions (*black arrow*). (B) Atlantic salmon infected with SalHV4 displaying extensive hyperplasia of the integument. (C) Koi infected with *CyHV3* displaying severe branchitis including hyperplasia and fusion of the secondary lamellae. Gill epithelial cells displaying karyomegaly, margination of chromatin, and pale eosinophilic nuclear inclusions (*black arrow*). (D) White sturgeon infected with AciHV2 resulting in epidermal hyperplasia with keratinocytes displaying karyomegaly, marginated chromatin, and hypochromatic nuclei (*black arrows*). (E) Gill of European eel infected with AngHV1, showing severe hyperplasia of the secondary lamellae and pale eosinophilic nuclear inclusions (*black arrows*) (F) Kidney of catfish with IcHV1 showing severe necrosis (*black arrows*) and lymphocytic inflammation. Scale bars = (A) 50 μm, (B–F) 20 μm. *Images C, D and F provided by Ronald Hedrick. Images B (Doszpoly, A., Karaseva, T.A., Waltzek, T.B., Kalabekov, I.M., Shchelkunov, I.S., 2013. Atlantic salmon papillomatosis in Russia and molecular characterization of the associated herpesvirus. Dis. Aquat. Organ. 107, 121–127) and E (Hangalapura, B.N., Zwart, R., Engelsma, M.Y., Haenen, O.L., 2007. Pathogenesis of Herpesvirus anguillae (HVA) in juvenile European eel Anguilla anguilla after infection by bath immersion. Dis. Aquat. Organ. 78, 13–22) by permission from Disease of Aquatic Organisms.*

of the population may die in less than 2 weeks from the first signs of disease, yet most IcHV1 endemic populations will experience no obvious CCVD. Losses due to CCVD on fingerling production operations can vary substantially from year-to-year without obvious environmental cues or changes in management.

Once catfish become infected with IcHV1, either through vertical or horizontal transmission, they become lifelong carriers. The first evidence of vertical transmission was the finding that CCVD-affected fry in one of the first documented outbreaks were offspring from brood fish that had high IcHV1 neutralizing titers (Plumb, 1973b). Detection and/or experimental induction of IcHV1 recrudescence has been difficult. In one experiment, IcHV1 antigen (but no culturable virus) was detected in ovarian tissue of immunosuppressed adult fish immediately after spawning (Plumb et al., 1981). Later, IcHV1 were cultured from adult catfish during the winter when temperatures were below 8°C and this isolation was enhanced if the fish were immunosuppressed with dexamethazone and the leukocytes cocultured with channel catfish ovary (CCO) cells (Bowser et al., 1985). However, conditions needed for recrudescence appear to be specific because several researchers have tried to induce IcHV1 production in carrier fish using dexamethazone with little success (Arnizaut and Hanson, 2011). The detection of carrier fish requires indirect evaluation (antibody detection) (Amend and McDowell, 1984; Crawford et al., 1999) or the detection of latent viral DNA because there are no infectious virus produced during latency. The detection of IcHV1 DNA in asymptomatic adult channel catfish was first done using Southern blot analysis (Wise et al., 1985). This method was used to demonstrate that offspring from IcHV1 carriers were positive for the IcHV1 genome (Wise et al., 1988). Subsequently, several IcHV1-specific PCR assays were developed and used to evaluate carrier fish (Baek and Boyle, 1996; Boyle and Blackwell, 1991; Gray et al., 1999).

Vertical transmission allows IcHV1 to be effectively maintained in a population. IcHV1-specific PCR on recently hatched fry from five representative fingerling operations demonstrated latent carrier status in 10–20% in each population of the sampled fish (Thompson et al., 2005). These farms represented approximately 20% of the commercial catfish fingerling production. Therefore, it can be assumed that IcHV1 is endemic in most aquaculture populations of channel catfish in the southeastern United States. Furthermore, evaluating incidence of latent IcHV1 in endemic fingerling populations over time showed an increase even though no CCVD was detected. This suggests that horizontal transmission occurs in the populations without overt disease (Thompson et al., 2005). When evaluating the use of PCR to detect and eliminate carrier brooders, we found that numbers of carrier offspring were reduced, but it did not eliminate vertical transmission to the subsequent generation (unpublished data). This suggests that the PCR detection of carrier brood stock was not sensitive enough for effective culling.

Channel catfish with acute CCVD may swim disoriented or hang at the surface or pond bank. These fish often display mild hemorrhaging at the base of the fins, exophthalmia and distended coelomic cavity (Fig. 9.1F). Internally, there is clear or yellow ascites and a swollen posterior kidney. The posterior kidney is the primary target tissue during acute CCVD. Early in infection this organ displays extensive edema, inflammation and necrosis of hematopoietic tissue and tubules. This is followed by focal necrosis, hemorrhage and edema of the liver and gastrointestinal tract and necrosis of pancreatic tissue, congested spleen and focal areas of hemorrhage in musculature (Plumb et al., 1974; Wolf et al., 1972). Occasionally, margination of the chromatin and eosinophilic intranuclear inclusion may be seen but these cellular changes are not as common as with other herpesviruses.

The innate immunity of channel catfish to IcHV1 likely plays an important role in CCVD resistance. Exposure of catfish to poly I:C or catfish reovirus as inducers of the innate immune system make channel catfish resistant to experimental CCVD (Chinchar et al., 1998; Plant et al., 2005). Natural killer-like cells from naïve channel catfish have also been shown to kill IcHV1 infected cells (Hogan et al., 1996).

The adaptive immune response of channel catfish to a productive IcHV1 infection is protective and long-lasting. Once a population of channel catfish recovers from CCVD or an IcHV1 positive population has been through the first summer, they become resistant to experimental infection (Arnizaut and Hanson, 2011; Plumb, 1973b). The protective components of this immunity have not been clearly defined, but the antibody response is important. Passive immunity can be conferred to channel catfish if serum from a CCVD survivor is transferred to a naïve recipient (Hedrick and McDowell, 1987). IcHV1 antibodies can persist for more than 2 years after exposure (Bowser and Munson, 1986; Hedrick et al., 1987). The IcHV1-specific antibodies in the serum of older fish increase during summer months (Bowser and Munson, 1986), and may be boosted by periodic virus recrudescence (Arnizaut and Hanson, 2011). Furthermore, early-life-stage resistance was correlated to neutralizing antibodies in the maternal parent, suggesting that maternal transfer of antibodies may play a role in preventing disease outbreaks (Hanson et al., 2004). The immunity and control of IcHV1 latency in fingerlings that are carriers due to vertical transmission of the virus is not understood. We have found the carrier rate within positive spawns to comprise 40–75% of the fish (Hanson et al., 2004) and these carrier fry are susceptible to CCVD when exposed to exogenous IcHV1 (Hanson et al., 2004; Kancharla and Hanson, 1996). However, these fish do not express culturable virus under experimental conditions, even when exposed to high temperatures and/or injected with dexamethasone (Arnizaut and Hanson, 2011).

9.2.2 Ictalurid herpesvirus 2

Much less has been reported about the biology of IcHV2. Natural outbreaks of acute herpesvirus disease occurred in adult black bullheads in Italy at above 20°C. The disease was similar to CCVD in causing disoriented swimming, scattered hemorrhages and extensive necrosis of the kidney tissue (Alborali et al., 1996). This virus was shown to be antigenically and genetically distinct from IcHV1 and has been experimentally shown to be capable of causing high losses in channel catfish fry and juveniles at relatively lower temperatures (24°C) (Hedrick et al., 2003). Partial genomic sequencing reveals that IcHV2 is closely related to IcHV1 and they are grouped in genus *Ictalurivirus* (Doszpoly et al., 2008, 2011a; Waltzek et al., 2009a).

The most common method for diagnosing CCVD is a combination of clinical signs, pathology and cell culture. IcHV1 produces rapidly developing cytopathic effect (CPE) on cultures of CCO or brown bullhead cell lines. The CPE consists of syncytium formation and subsequent contraction forming cellular radiating spindles to attachments on the plate or neighboring cells. For cell cultures, the use of whole fry (up to 2 cm), viscera from small fingerlings (2–5 cm) or posterior kidney from larger juveniles is suggested. When a 0.5 mL inoculum of a 1:10 dilution of tissue from a CCVD affected fish is inoculated into a 12 cm^2 flask of cells, CPE is usually seen within 2 days at 30°C.

Similar clinical signs, pathology and CPE are seen with IcHV2. However, IcHV2 has a broader cell host range; it will produce CPE on bluegill fry cell and the epithelioma papulosum cyprini (EPC) (derived from fathead minnow) cell line; whereas, IcHV1 will not (Alborali et al., 1996).

Molecular means are suggested for confirmation and are required for detecting carriers. Several traditional PCR assays are available to detect IcHV1 (Baek and Boyle, 1996; Boyle and Blackwell, 1991; Gray et al., 1999; Thompson et al., 2005). These methods are highly sensitive and effective in detecting latent virus. Their specificity has been tested against other alloherpesviruses, but not between IcHV1 and IcHV2. Recently, IcHV2 specific hydrolysis probe (TaqMan) PCR was developed and tested against multiple isolates of IcHV1 with no false positives (Goodwin and Marecaux, 2010). Hydrolysis probe assays that are specific for two genotypes of IcHV1 can also be used to distinguish these two species (Hanson, 2016). There is little effort to contain or eliminate IcHV1 because the virus is widespread in the catfish industry. Instead, catfish fingerling producers primarily use management to prevent disease outbreaks. This mainly involves minimizing handling and crowding of fish during warm summer months. If CCVD occurs in the hatchery, the fry are generally depopulated and facility sterilized before using again. Once a CCVD outbreak begins, every effort should be made to contain the infection because in can spread to other ponds. Experimental vaccines include subunit vaccines (Awad et al., 1989), DNA vaccines (Nusbaum et al., 2002) and live attenuated virus vaccines (Noga and Hartmann, 1981; Vanderheijden et al., 2001; Zhang and Hanson, 1995).

The black bullhead aquaculture industry was devastated by IcHV2 and has since retreated to facilities that could rear the fish at below 24°C (Roncarati et al., 2014).

9.3 CARP ALLOHERPESVIRUSES

There are three characterized alloherpesviruses of carp that cause important diseases in aquaculture and the ornamental fish trade. These are *Cyprinid herpesvirus 1* (CyHV1, carp pox herpesvirus), *Cyprinid herpesvirus 2* (CyHV2, goldfish hematopoietic necrosis virus) and *Cyprinid herpesvirus 3* (CyHV3, koi herpesvirus (KHV)). All three are closely related in sequence and genome structure and are members of the genus *Cyprinivirus* (Davison et al., 2013; Waltzek et al., 2005, 2009a,b). The genomes of all three have been sequenced; all are over 290 kb in length, with CyHV3 being the largest known herpesvirus genome at 295 kb (Aoki et al., 2007; Davison et al., 2013). These pathogens are restricted in host range, CyHV1 and CyHV3 are only known to cause disease in common carp strains and CyHV2 is only known to cause disease in goldfish (*Carassius auratus*) and the closely related Prussian carp, *Carassius gibelio* (Danek et al., 2012; Doszpoly et al., 2011b). All three are also relatively recalcitrant to cell culture and when they do replicate, CPE develops slowly.

9.3.1 Cyprinid herpesvirus 1

The cause of a disease known for centuries as carp pox, characterized as a benign papilloma of common carp, was first cultured and shown to be a herpesvirus by Sano et al. (1985a,b). CyHV1 can cause systemic disease with high mortality in fry (Sano et al., 1991). These fish displayed aberrant swimming behavior, distension of coelomic cavity exophthalmia, darkened skin pigmentation and hemorrhages on the operculum and body. Histological changes seen in moribund fry included necrosis of the liver, kidney and intestines with some cells displaying Cowdry type A intranuclear inclusion bodies. Grass carp, crucian carp and willow shiners were refractive to infection. Tracing viral antigen expression using indirect

fluorescent antibody (IFA) staining demonstrated high expression in the viscera early on during the primary infection and a progression to the epidermis late in infection (Sano et al., 1991). There was an obvious effect of temperature on virus replication and pathogenicity. Evaluating virus production at 10, 15, 20, 25 and 30°C in cell culture showed the virus replication was highest at 15°C and 20°C, moderate at 10°C and 25°C and undetectable at 30°C. In challenge studies, highest losses occurred at 15°C, moderate losses occurred at 20°C and no mortality occurred at 25°C. The survivors of the immersion challenge and adults injected with CyHV1 developed a high incidence of papillomas (Fig. 9.1A). Histologically, hyperplastic keratinocytes often display marginated chromatin and pale eosinophilic intranuclear inclusions (Fig. 9.2 A). These epidermal proliferations developed months after primary exposure and expressed culturable virus and were positive for viral antigens by IFA staining (Sano et al., 1991). In experimentally challenged fish, the papillomas developed and persisted at 15°C, and developed and began to regress at 20°C and 25°C. When the papillomas were allowed to develop at lower temperatures and then the temperature raised to 30°C, they rapidly regressed (Sano et al., 1993a,b). Often the papillomas would reoccur several months after regression (Sano et al., 1991). In situ hybridization studies demonstrated the presence of latent virus in cranial and spinal nerves and subcutaneous tissue when no viral antigens or infectious virus were present (Sano et al., 1993a,b). Whole genome sequencing revealed CyHV1 encodes the oncogene JUNB, which may be partially responsible for tumor development (Davison et al., 2013). The disease has a worldwide distribution and its impact on commercial aquaculture is difficult to assess. The tumors are unsightly and affect marketability of ornamental fish and fish sold live or whole for food, but high mortality due to primary infection has not been reported as a major concern in aquaculture systems. Presumptive diagnosis of CyHV1 infection can be done by observation of papillomas on common carp that regress at elevated temperatures. The diagnosis can be confirmed by testing infected tissues with IFA (Sano et al., 1991) or by PCR (Viadanna et al., in submission). The virus is recalcitrant to culture but it has been isolated on Koi Fin (KF-1), EPC or FHM cell lines at 20°C, which develop CPE at approximately 5 days postinoculation, consisting of vacuolation and rounding cells (Dixon, 2008; Sano et al., 1991).

9.3.2 Cyprinid herpesvirus 2

Jung and Miyazaki (1995) first described a severe disease of cultured goldfish designated as herpesviral hematopoietic necrosis (HVHN) from cases in Japan in 1992 and 1993. The outbreaks occurred in the spring and fall in goldfish of all ages with high mortality. Affected fish were lethargic, but showed no external lesions other than occasional pale patches in the gills. Internally, the swollen and pale kidney and splenomegaly were the most notable signs. Histopathology consisted primarily of necrosis of the hematopoietic tissues of the anterior and posterior kidney and the splenic pulp. Other lesions included focal necrosis and inflammation of the lamina propria and submucosa of the intestine and occasional pancreatic necrosis (Jung and Miyazaki, 1995). Filtrates from spleen and kidney produced CPE on FHM and EPC cells at 20°C, consisting of vacuolation and cell rounding, but the virus disappeared after 3–4 passages. Other outbreaks were reported in the United States (Goodwin et al., 2006a; Groff et al., 1998; Nieger et al., 2015), Australia (Stephens et al., 2004), the United Kingdom (Jeffery et al., 2007), Hungary (Doszpoly et al., 2011a) and China. These studies found similar pathology with the additional observation of diffuse branchial hyperplasia, leukocyte infiltration and necrosis. In addition, others were unsuccessful in culturing the virus except Jeffery et al. (2007), who were able to demonstrate CPE on the Koi Fin (KF) cell line at 20°C. A goldfish cell line (Li and Fukuda, 2003) has been used to propagate CyHV2, allowing for additional challenge studies (Hedrick et al., 2006) and the ability to sequence the genome (Davison et al., 2013).

The disease appears restricted to goldfish and closely related species, such as Prussian carp. Common carp (Hedrick et al., 2006; Jung and Miyazaki, 1995) and koi × goldfish hybrids are resistant to HVHN (Hedrick et al., 2006). Prussian carp are very sensitive, with several outbreaks occurring in aquacultured and wild populations (Danek et al., 2012; Doszpoly et al., 2011b; Luo et al., 2013).

Given the importance of HVHN to goldfish production and the ornamental fish trade and the recalcitrant nature of CyHV2 to cell culture, two PCR assays have been established and validated. These include conventional PCR (Waltzek et al., 2009b) and hydrolysis probe (TaqMan) quantitative PCR (Goodwin et al., 2006b). The sensitive qPCR was used to demonstrate viral genome presence in healthy adult goldfish, suggesting latency (Goodwin et al., 2006b).

The options for HVHN disease prevention are limited to avoidance, and possibly the use of resistant fish. The qPCR assay detected latent CyHV2 in nondiseased fish (Goodwin et al., 2006b), so the use of this method in screening populations during broodfish selection would likely reduce the potential for HVHN. In a problematic environment, the use of resistant fish may also be warranted, such as the Koi × goldfish hybrid (Hedrick et al., 2006). However, whether fish become carriers and shed virus after CyHV2 exposure has not been evaluated. Ito and Maeno (2015) developed a formalin-inactivated vaccine that was effective against CyHV2 in goldfish; but future research is needed to determine the length of protection.

9.3.3 Cyprinid herpesvirus 3

Since 1997, disease causing extremely high mortality in all ages of common carp (many of the first cases occurred in Koi carp) has been identified in the United States, Europe and Asia (Bretzinger et al., 1999; Hedrick et al., 1999). The disease was recognized by severe gill hyperplasia and necrosis. Hedrick et al. (2000) were able to isolate the causative agent in cell culture and reproduce the disease experimentally. Based on TEM of the isolated virus, infected tissues and infected cell cultures the virus was classified as a herpesvirus and designated as KHV with the disease designated KHV disease (KHVD) (Hedrick et al., 2000). Based on partial genome sequence data it was apparent that KHV was closely related to CyHV1 and CyHV2, and therefore was renamed *Cyprinid herpesvirus 3* (Waltzek et al., 2005). Genome sequencing of CyHV3 and later CyHV2 and CyHV1 confirmed that these viruses were very closely related, supporting the designation of the three in the *Cyprinivirus* genus (Aoki et al., 2007; Davison et al., 2013).

The disease is highly transmissible and causes high mortality in all strains of common carp, both in the wild and in aquaculture, when the temperature is between 18°C and 28°C, with the optimum being 22–26°C. Affected fish are lethargic, and display endophthamia, and white patches on the skin and gills due to epithelial hyperplasia and necrosis (Fig. 9.1C). The gills filaments often will be visibly swollen, with necrosis and sloughing occurring at the tips. Secondary bacterial infection, such as *Flavobacterium columnare*, is common in affected fish. The fish usually have swollen kidneys and splenomegaly. Histopathology demonstrates epithelial hyperplasia, hypertrophy and necrosis in the affected gill and epidermal tissue and occasionally epithelial cells within the affected areas will have enlarged nuclei with marginated chromatin surrounded a lighter-staining central region (Fig. 9.2C). There is also necrosis and inflammation in the hematopoietic tissues of the kidneys, the spleen, liver and lamina propria of the intestine (Hedrick et al., 2000).

The disease is restricted to *Cyprinus carpio* and Koi × crucian carp (*Carasius carasius*) hybrids with goldfish × common carp hybrids being somewhat susceptible (Bergmann et al., 2010b; Hedrick et al., 2006). However, other species may become infected and shed KHV and thus, may function as unapparent carriers on aquaculture facilities (El-Matbouli et al., 2007; El-Matbouli and Soliman, 2011; Fabian et al., 2013; Kempter and Bergmann, 2007; Sadler et al., 2008).

Although CyHV3 can grow on several common carp cell lines and produce vacuolating CPE after 4–6 days, this is not considered reliable. Instead, it is recommended that a presumptive diagnosis of KHVD be made based on clinical signs and pathology with confirmation done using CyHV3 PCR (OIE, 2015). There are several PCR methods published; the OIE manual suggests the use of the assay developed by Bercovier et al. (2005) or that of Yuasa et al. (2005) or the hydrolysis probe-based qPCR developed by Gilad et al. (2004). Bergmann et al. (2010a) compared several molecular assays and found that the Gilad qPCR assay was by far the most sensitive. In addition, they found that sequence variation could result in some false negatives in case samples. Engelsma et al. (2013) developed a nested PCR that detects all CyHV1-3, including newly discovered divergent strains of CyHV3.

Current KHVD management is largely based on either avoidance or the use of vaccines. If possible, avoidance is generally the best option, but requires vigilant screening of fish stocks, thorough disinfection of infected facilities and the use of a noninfected source of water. Other sources of the virus including residual virus present in invertebrates should also be considered (Fabian et al., 2013; Kempter and Bergmann, 2007; Kielpinski et al., 2010). If KHVD does occur, elevating the temperature will reduce losses, but survivors will carry the virus and subsequent shedding will likely result in new KHVD outbreaks in any naïve fish that are added to the facility. The use of vaccines is also promising; one vaccine (KoVax) is a natural attenuated live vaccine that is commercially produced (Perelberg et al., 2005; Ronen et al., 2003; Weber et al., 2014). Another vaccine is a designed recombinant CyHV3 vaccine developed using BAC technology (Boutier et al., 2015).

9.4 EEL ALLOHERPESVIRUS—*ANGUILLID HERPESVIRUS 1*

In 1985, Japanese eels (*Anguilla japonica*) and European eels (*Anguilla anguilla*) farmed in recirculation systems in Japan suffered from increased mortality rates (Sano et al., 1990). Using a cell line derived from the kidneys of an eel (EK-1) (Chen et al., 1982), a virus was isolated from the affected eels (Sano et al., 1990). By TEM, typical herpesvirus-like virions were observed, and the virus was tentatively named *Herpesvirus anguillae*. At the same time, a herpesvirus was also reported in eels in Europe (Békési et al., 1986), but isolation was only successful after the importation of the susceptible EK-1 cell line more than a decade later (Davidse et al., 1999). Serologically and genetically, herpesvirus isolates from eels from Europe and Asia are highly similar, and are regarded as a single virus species, *Anguillid herpesvirus 1* (AngHV1), that belongs to the genus *Cyprinivirus* (Chang et al., 2002; ICTV, 2014; Rijsewijk et al., 2005; van Beurden et al., 2010; Waltzek et al., 2009a,b).

Since its first discovery in the mid-1980s, AngHV1 has been reported repeatedly in Japanese and European eels in warm-water culture systems in East Asia, and in cultured and wild European eels in Europe (reviewed in Van Beurden et al., 2012b).

As such, AngHV1 has been suggested to play a contributory role in the decline of the endangered wild European eel stocks (Haenen et al., 2012). In addition, AngHV1 has been detected by PCR in another freshwater eel species, namely the American eel (*Anguilla rostrata*) cultured in Taiwan and imported from China to Poland (Kempter et al., 2014; Shih, 2004). Upon experimental infection with eel herpesvirus in Formosa, Ueno et al. (1992) reported high mortality of common carp, but this observation has not been confirmed by others. Thus far, the host range of AngHV1 seems to be restricted to freshwater eels of the genus *Anguillidae*, in particular the Japanese and European eel, and putatively the American eel. Eels of all ages are susceptible, although smaller eels were used for most experimental infections.

In high-density intensive recirculation production systems, AngHV1 outbreaks have been associated with stress factors, such as poor water quality or eel sorting (Haenen et al., 2002; van Beurden et al., 2012b). Progress of the disease shows a clear temperature dependency in culture systems, with clinical symptoms being most severe at 25–26°C. Depending on the circumstances, morbidity can be high, resulting in decreased growth rates, whereas mortality rates are usually no more than 10% (Davidse et al., 1999; Haenen et al., 2002; Sano et al., 1990). In case of complicating factors, such as secondary infections, mortality rates can be considerably higher (Chang et al., 2002). Clinical symptoms may vary among and within outbreaks, and include apathy, varying degrees of fin and skin hemorrhages (particularly in the head, beak, operculum and caudal abdominal surface) (Fig. 9.1E) and congestion of the gills (van Beurden et al., 2012a,b). Gross pathological findings may range from a clinically normal to pale spleen, a pale and hemorrhagic liver, a distended gall bladder, swollen kidneys and ascites (Chang et al., 2002; Davidse et al., 1999; Haenen et al., 2002; Lee et al., 1999; Sano et al., 1990; Ueno et al., 1992). In general, experimental infections demonstrated comparable symptoms, although to a more limited degree and generally without mortality.

Diagnosis of AngHV1 should not be solely based on clinical symptoms and gross pathology because skin and fin hemorrhages are rather nonspecific in eels (Haenen et al., 2010), and two pathogenic RNA viruses, namely, Eel virus European (EVE) and Eel virus European X (EVEX), may cause a similar hemorrhagic disease (van Beurden et al., 2012b). Descriptions of histopathological changes induced by AngHV1 infection are limited, but include skin necrosis, gill hyperplasia, inflammation, necrosis and lamellar fusion (Fig. 9.2 E), and liver inflammation and necrosis (Armitage et al., 2014; Hangalapura et al., 2007; Lee et al., 1999; Sano et al., 1990). An immunohistochemistry assay and an in situ hybridization assay have been developed for the detection of AngHV1 in histological slides (Chang et al., 2002; Hangalapura et al., 2007; Shih et al., 2003). Virus isolation by inoculation of suspensions of the gills and a pool of internal organs (including liver, kidney and spleen) on a susceptible cell line remains the gold standard. AngHV1 can successfully be isolated and propagated in several cell lines of eel origin, but the most widely used is the EK-1 cell line (Chen et al., 1982). The optimal virus growth temperature for AngHV1 in EK-1 cells is approximately 25°C (Sano et al., 1990). Upon the observation of CPE, characterized by giant cell formation, immunological assays can be used to identify the causative virus as AngHV1 (Davidse et al., 1999; Varvarigos et al., 2011). More recently, molecular assays have been developed to demonstrate the presence of AngHV1 genetic material in positive cell cultures or directly in organs of affected eels. These include three conventional PCR assays targeting the DNA polymerase gene (Armitage et al., 2014; Rijsewijk et al., 2005; Shih, 2004), and a qPCR targeting a conserved capsid protein gene (van Beurden et al., 2016).

No vaccines or antivirals against AngHV1 are commercially available. In addition, experimental AngHV1 infection studies have suggested the possibility of stress-dependent reactivation of latent virus (van Nieuwstadt et al., 2001). Losses due to AngHV1 infection in eel farms can therefore only be reduced by management practices (ie, quarantine measures, stress reduction and water temperature adjustment) (Haenen et al., 2002). AngHV1 infection proceeds horizontally, thus hygienic measures between infected and noninfected culture units should be taken in order to avoid cross-contamination. Stress factors play an important role in the development of AngHV1-associated disease, and should be minimalized in case of an outbreak. These include avoidance of handling infected eels, optimization of water quality and treatment of secondary parasitic or bacterial infections. Finally, at eel farms with a regulated water temperature, lowering the water temperature to below 22°C may reduce the mortality rates and clinical symptoms due to AngHV1 infection (Haenen et al., 2002).

9.5 STURGEON ALLOHERPESVIRUSES

Two alloherpesviruses, Acipenserid herpesvirus 1 (AciHV1) and *Acipenserid herpesvirus 2* (AciHV2), have been characterized from wild and farm-reared sturgeon in North America and Europe. Although AciHV1 has occasionally been associated with disease in farm-reared sturgeon, AciHV2 has repeatedly caused significant epizootics in both North American and European sturgeon farms (Doszpoly and Shchelkunov, 2010; Hedrick et al., 1991; Kelley et al., 2005; Kurobe et al., 2008; Watson et al., 1995). Serologic and phylogenetic evidence suggests that these sturgeon alloherpesviruses are only distantly related (Kelley et al., 2005; Kurobe et al., 2008; Waltzek et al., 2009a,b; Watson et al., 1995). Phylogenomic analyses have revealed that AciHV2 is most closely related to IcHV1 and IcHV2 in the genus *Ictalurivirus* (Doszpoly et al., 2008, 2011a; Kelley et al., 2005; Waltzek et al., 2009a,b).

9.5.1 Acipenserid herpesvirus 1

Acipenserid herpesvirus 1 was isolated from juvenile white sturgeon (*Acipenser transmontanus*) experiencing increased mortality on a farm in California (Hedrick et al., 1991). Years later, AciHV1 was again isolated from farmed white sturgeon in California and for the first and only time in Europe on an Italian farm (Kelley et al., 2005; Kurobe et al., 2008). Experimental infection trials resulted in 35% cumulative mortality among juvenile white sturgeon (Hedrick et al., 1991). Few gross external or internal signs were observed; however, microscopic examination of hematoxylin and eosin-stained tissues revealed a diffuse hyperplastic dermatitis. Affected keratinocytes appeared swollen with slightly enlarged nuclei, displaying marginated chromatin and occasional nuclear inclusions (Hedrick et al., 1991).

Diagnosis of AciHV1 is best accomplished through cell culture followed by ancillary testing. Viral replication occurs on white sturgeon skin cells (WSSK-1) at temperatures between 10°C and 20°C with the optimum being 15°C (Hedrick et al., 1991). CPE includes the formation of syncytia 2–4 days postinoculation at 15°C. Analysis of infected tissues or WSSK-1 cells by TEM reveals naked hexagonal nucleocapsids (110 nm in diameter) in the nucleus and enveloped particles surrounded by a coarse electron-dense tegument layer (230 nm in diameter) within cytoplasmic vacuoles (Hedrick et al., 1991). Although AciHV1-specific molecular diagnostic tests have not been developed, PCR amplification and sequencing of the viral DNA polymerase or terminase genes have been used to confirm the presence of AciHV1 (Kelley et al., 2005; Kurobe et al., 2008; Waltzek et al., 2009a). To date, no vaccines or antiviral drugs have been tested against AciHV1.

9.5.2 Acipenserid herpesvirus 2

Acipenserid herpesvirus 2 was first detected in adult white sturgeon in California during routine sampling. The virus was isolated on the white sturgeon spleen (WSS-2) cell line from the ovarian fluid of a healthy adult sturgeon (Watson et al., 1995). Shortly thereafter, the virus was again isolated from the skin of a subadult white sturgeon from a farm-reared population experiencing erosive skin lesions associated with elevated morbidity and mortality. Other North American detections of AciHV2 include wild white sturgeon from Idaho and Oregon (Kelley et al., 2005; Kurobe et al., 2008), farm-reared shortnose sturgeon (*Acipenser brevirostrum*) from northeastern Canada (Kelley et al., 2005; Kurobe et al., 2008; LaPatra et al., 2014) and managed lake sturgeon (*Acipenser fulvescens*) from Wisconsin (Waltzek and Doszpoly, unpublished). Experimental bath infection trials resulted in up to 80% cumulative mortality among juvenile white sturgeon after 6 weeks (Watson et al., 1995). Affected juveniles displayed erratic swimming behavior, lethargy, raised spherical mucoid skin lesions on the head and pectoral fins (Fig. 9.1D) and hyperemia of the ventral scutes, mouth and anus (Watson et al., 1995). Lower dosage challenge studies failed to induce clinical signs and mortality; however, subclinical infections were detected 70 days postinfection (Watson et al., 1995). Microscopic examination of hematoxylin and eosin-stained tissues from moribund juveniles revealed diffuse hyperplastic dermatitis with marked secondary erosions of the integument, oropharyngeal mucosa and respiratory mucosa. Affected keratinocytes displayed karyomegaly, marginated chromatin and hypochromatic nuclei (Fig. 9.2D) (Watson et al., 1995).

The first detection of AciHV2 in Europe was a severe epizootic that occurred on a Russian farm rearing Siberian sturgeon (*Acipenser baeri*) fingerlings (Shchelkunov et al., 2009). The disease predominantly affected Siberian sturgeon, but was also isolated from Bester (*Acipenser ruthenus* × *Huso huso* hybrid) juveniles. Similar to AciHV2 epizootics in North American sturgeon (LaPatra et al., 2014; Watson et al., 1995), the Siberian sturgeon herpesvirus displayed a clear tropism for the integument, resulting in focal hyperplastic and hemorrhagic lesions. Siberian sturgeon fingerlings experienced 100% cumulative mortality by 14 days following bath exposure. Two-year-old Siberian sturgeon experienced a less severe (36% cumulative mortality) and protracted disease course (40 days post inoculation).

Diagnosis of AciHV2 is best accomplished through cell culture followed by ancillary testing. Strains of AciHV2 have been isolated on cell lines derived from white sturgeon (skin; WSSK-1, spleen; WSS-2, liver; WSLV, gonad; WSGO) and Siberian sturgeon (organ, SSO-2; fin; SSF-2) cell lines at 15°C (Shchelkunov et al., 2009; Watson et al., 1995). CPE involves the focal destruction of the monolayer with grape-like clusters of cells surrounding infective foci (day 3–7) and progresses to the complete destruction of the monolayer by 2 weeks at 15°C (Shchelkunov et al., 2009; Watson et al., 1995). Analysis of infected tissues or WSSK-1 cells by TEM reveals naked hexagonal nucleocapsids (95–110 nm in diameter) in the nucleus and enveloped particles surrounded by a coarse electron-dense tegument layer (176–250 nm in diameter) within the cytoplasm (LaPatra et al., 2014; Shchelkunov et al., 2009; Watson et al., 1995). Although AciHV2-specific molecular diagnostic tests have not been developed, PCR amplification and sequencing of the viral DNA polymerase or terminase genes have been used to confirm the presence of AciHV2 (Doszpoly et al., 2008, 2011a; Doszpoly and Shchelkunov, 2010; Kelley et al., 2005; Kurobe et al., 2008; Waltzek et al., 2009a). To date, no vaccines or antiviral drugs have been tested against AciHV2.

9.6 SALMONID ALLOHERPESVIRUSES

There are four characterized alloherpesviruses in trout and salmon; all appear phylogenetically related and have been designated *Salmonid herpesvirus 1*, *2*, *3* and 4 (SalHV1-4). With SalHV1-3 being in the genus *Salmonivirus*. Three of these viruses are associated with significant losses in aquaculture: SalHV2 causes *Oncorhynchus masou* virus disease in pacific salmon species in Japan; SalHV3 causes epizootic epitheliotropic disease in lake trout (*Salvelinus namaycush*) in the Great Lakes region in North America and SalHV4 causes Atlantic salmon papillomatosis (ASP) in Atlantic salmon (*Salmo salar*) in northern Europe.

9.6.1 Salmonid herpesvirus 1

Salmonid herpesvirus 1 was first detected in 1971–75 in spawning rainbow trout (*Oncorhynchus mykiss*) at the Washington state Winthrop fish hatchery (Wolf and Taylor, 1975). The virus was characterized and designated as a *Herpesvirus salmonis* (Wolf et al., 1978). The virus was again isolated in California 10 years later in steelhead trout (Hedrick et al., 1986). This isolate designated Steelhead herpesvirus was shown to be a strain of SalHV1 by serology and DNA hybridization (Eaton et al., 1991; Hedrick et al., 1987). This virus was the first salmonid herpesvirus characterized at the genomic level (Davison, 1998) and later analysis showed genetic grouping of salmonid herpesviruses (Waltzek et al., 2009a) and SalHV1 was designated the type species for genus *Salmonivirus* (ICTV, 2014).

SalHV1 has only been associated with mild disease in natural outbreaks and thus, is not of great concern to aquaculture. The clinical signs include general edema of the visceral organs and hyperemia of the liver and adipose tissue (Eaton and Hedrick, 1994). In experimental infections of rainbow trout fry by parenteral administrations of the virus, the fish developed darkened pigmentation, exophthalmia, coelomic distension and shed fecal casts (Wolf and Smith, 1981). The fish also displayed hemorrhages in the fins and pale gills. Internal gross pathology included yellow and blood-tinged ascites. Histology revealed edema of cardiac and skeletal tissue, extensive congestion and necrosis in the liver and kidneys and syncytia was seen in pancreatic tissue. Highest virus concentrations were detected in the kidney tissue. A study using the Steelhead isolate in challenges showed much more limited pathology but did report on the syncytia of hepatocytes (Eaton et al., 1989).

SalHV1 is most closely related to SalHV2, which is much more pathogenic. SalHV1 and SalHV2 have similar characteristics in cell culture and must be distinguished from each other using serology or molecular methods. The presumptive diagnosis of SalHV1 can be done using traditional cell culture. It replicates on the common salmonid cells lines Chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) at 10°C. The virus produces a disseminating syncytium in the cells, but it may take 10 days to 2 weeks to develop and blind passage may be needed to detect low-level infection. Poor replication occurs at higher temperatures and this helps distinguish it from SalHV2, which replicates at 10–20°C. Confirmation requires the use of neutralizing antibodies (Eaton and Hedrick, 1994) or PCR. The PCR method of Aso et al. (2001) produces a ~800-bp band for SalHV1 and a 439-bp band for SalHV2.

There has been minimal research conducted on controlling SalHV1, but avoidance appears to be effective. Carrier brood fish shed virus in ovarian and seminal fluid during spawning and these can be used to detect carriers. In the original cases the virus was isolated from the ovarian fluid of postspawning adults that had higher than expected losses. In this hatchery, the brood fish were replaced and the virus was no longer found.

9.6.2 Salmonid herpesvirus 2

Salmonid herpesvirus 2 is an important pathogen to farmed pacific salmon and rainbow trout in Japan. This pathogen has several common names. including *O. masou* virus (OMV), Nerka virus Towada Lake Akita and Amori Prefecture (NeVTA), yamame tumor virus (YTV). After serological analysis demonstrated serum cross reaction with these isolates and they were serologically distinct from SalHV1 and molecular analysis confirmed, these isolates were designated as a single species (Eaton et al., 1991; Hayashi et al., 1993; Hedrick et al., 1987; Waltzek et al., 2009a,b; Yoshimizu et al., 1995). This virus causes disease in masu salmon (*O. masou*), coho salmon (*Oncorhynchus kisutch*) and kokanee salmon (*Oncorhynchus nerka*), rainbow trout (*O. mykiss*), yearling rainbow trout and Chum salmon (*Oncorhynchus keta*).

There are two recognized forms of disease caused by SalHV2: an acute form caused by primary virus infection and a tumor form caused by recrudescent infection. The acute form is a disseminated viremia and can cause mortality that exceeds 80%. In this form, affected fish appear darkened, display skin ulcers, and become apathetic and anorexic. Internally the liver may have white patches and the intestine is often hemorrhagic. Some fish may display focal areas of epithelial proliferation on the mouth and body (Yoshimizu, 2012). Chronically infected fish may develop papillomas most commonly around the mouth. This develops months after the primary infection. In an experimental challenge of juvenile chum salmon,

tumors began to develop 4 months postinfection and persisted for more than 1 year. Tumor occurrence after experimental challenge was 40–60% in chum salmon, 12% for rainbow trout and almost 100% for masu salmon (Yoshimizu et al., 1987).

Presumptive diagnosis of SalHV2 in diseased fish can be done using clinical signs and cell culture on RTG-2 or CHSE-214 cells at 15°C. For diagnosis of the acute infection liver, kidney and spleen are processed for cell culture. In the tumor form of the disease, it is best to cocultivate tumor tissue with RTG-2 cells (Yoshimizu, 2012). Carrier fish can be detected by screening ovarian fluid and tissues from postspawning adults. Confirmation can be done using serum neutralization assays using SalHV2-specific antisera or by SalHV2-specific PCR (Aso et al., 2001).

Management to control SalHV2 is a combination of sanitation of facilities and equipment, disinfection of incoming water and prevention of vertical transmission, using surveillance of broodstock, and iodine disinfection of eggs. The use of injectable formalin-killed vaccine in broodstock also helps reduce the vertical transmission of the virus (Yoshimizu, 2009; Yoshimizu and Kasai, 2011).

9.6.3 Salmonid herpesvirus 3

Salmonid herpesvirus 3 was characterized by partial sequencing and shown to be a member of the genus *Salmonivirus* (Waltzek et al., 2009a). This virus is recalcitrant to cell culture, but was identified as the cause of epidermal proliferative disease in lake trout fingerlings designated epizootic epitheliotropic disease (EED) or lake trout epidermal hyperplasia. This disease has caused high losses of lake trout in state and federal hatcheries in the Great Lakes region of North America since 1983. The herpesvirus cause of EED was determined by observation of herpesvirus particles in infected tissues, isolation of the virions using differential centrifugation and experimentally reproducing the disease in lake trout using purified virus or tissue filtrates (Bradley et al., 1989; McAllister and Herman, 1989). After detailed ultrastructural analysis, the designation *Salmonid herpesvirus 3* was proposed (Bradley et al., 1989). This virus causes disease in lake trout and lake trout hybrids. Cohabitation and immersion challenge trials using filtrates from infected lake trout show that rainbow trout, brown trout, brook trout and Atlantic salmon are resistant to disease (Bradley et al., 1989; McAllister and Herman, 1989).

Outbreaks of EED have only been documented in aquaculture facilities and usually occur in the spring when temperatures are near 8–10°C and can often be correlated with a predisposing stressing event, such as fin clipping. The mortality pattern can be protracted, often lasting more than several months and cumulative losses may approach 100% (McAllister, 1993). Fish with EED are lethargic, swim near the surface and may display periods of hyperexcitability with disoriented or spiral swimming patterns. Lower portions of the fishes' eyes and mouth may display hemorrhages and gray-to-white mucoid patches are seen on the skin and fins. Fins may also be frayed and secondary *Saprolegnia* infections are common. Internally the fish appear normal except for enlarged spleens and swollen kidneys. Histopathology reveals patches of hypertrophy, hyperplasia and necrosis of keratinocytes with an associated lymphocytic infiltration. Some affected epithelial cells display eosinophilic intranuclear inclusions. Other lesions include edema in the gills, and the presence of macrophages laden with cellular debris in kidneys and liver (Bradley et al., 1989; McAllister and Herman, 1989).

Diagnosis of EED has traditionally relied on clinical signs and histopathology with the use visualization of herpesvirus particles in TEM for confirmation. However, genomic sequence data obtained from infected tissues (Waltzek et al., 2009a), provided the information needed for highly sensitive PCR-based diagnostics (Kurobe et al., 2009) and highly sensitive qPCR (Glenney et al., 2016a). These methods have been used effectively to diagnose EED and SalHV3 carriers.

Management to reduce EED has focused on reducing stress, reducing fish density and on minimizing recirculation of hatchery water. No vaccines have been developed or studied for this virus. Some hatcheries that experience high losses during the initial outbreaks depopulated and disinfected their facilities before obtaining eggs from Wyoming (an area with no history of disease) (Kurobe et al., 2009). The SalHV3-specific PCR can be used to detect carrier brood fish and to allow culling of eggs and fry that may have received the infections by vertical transmission (Kurobe et al., 2009; Glenney et al., 2016a).

9.6.4 Salmonid herpesvirus 4

Salmonid herpesvirus 4 is an alloherpesvirus that causes Atlantic salmon papillomatosis (ASP). As the name implies, ASP is characterized by proliferative skin lesions in Atlantic salmon. This disease has been recognized as a problem in salmon culture facilities since the 1950s and is found in Scotland, the Scandinavian countries and northwestern Russia. In early studies, a herpesvirus was shown to be associated with the proliferative lesions using TEM, but all attempts to culture the virus were unsuccessful (Carlisle, 1977; Shchelkunov et al., 1992). DNA analysis of the tumor tissue using degenerate PCR targeted to conserved alloherpesvirus sequences demonstrated the presence of a unique alloherpesvirus most closely related to SalHV3 (Doszpoly et al., 2013). With this sequence information, they proposed the virus be designated Salmonid herpesvirus 4 and its placement in the *Salmonivirus* genus.

Outbreaks of ASP occur primarily in older Atlantic salmon juveniles (more than 2 years of age) during summer months when water temperatures are 10–16°C, especially during times of smoltification (Bylund et al., 1980; Doszpoly et al., 2013). When temperatures drop below 10°C, the papillomas degenerate and ASP-associated mortality peaks due to secondary infections. High incidences of ASP (sometimes exceeding 85% of some stocks of fish) are problematic in certain aquaculture facilities and losses exceeding 50% due to secondary infections have been documented (Bylund et al., 1980). The disease is seen primarily in aquaculture facilities, but has been documented in wild, natural-spawned stocks of juvenile Atlantic salmon in Finland (Bylund et al., 1980). The clinical signs are well-demarcated elevated epidermal proliferative growths that may exceed 1 cm in diameter and 5 mm in height (Fig. 9.1B). These lesions can occur anywhere on the epidermis, but predominantly on the caudal peduncle. Histologically, these lesions display epithelial cell hyperplasia and karyomegaly of affected cells (Fig. 9.2B). These proliferative lesions also display a loss of goblet cells, loss of the basement membrane and deformation and/or loss of the scales (Doszpoly et al., 2013).

Diagnosis of ASP has relied on histopathology, but the availability of genomic sequence data molecular detection of the presence of SalHV4 is replacing this. Recent work by Glenney et al. (2016a) with SalHV3 qPCR assays based on the terminase gene had demonstrated cross-reactivity with SalHV4 and the presence of another closely related herpesvirus in lake trout (tentatively designated Salmonid herpesvirus 5 SalHV5, Glenney et al., 2016b). They subsequently developed SalHV3-, SalHV4- and SalHV5-specific qPCR assays. Management by avoidance will rely on the development and use of sensitive molecular methods. Prevalence appears to be related to parental source, which suggests possible vertical transmission (Carlisle and Roberts, 1977; Doszpoly et al., 2013). Reported management methods include the use of prophylactic salt and formalin treatments on alternating weeks, minimizing stress and treating secondary infections when the papilloma regression and sloughing occurs (Bylund et al., 1980). Hatcheries in the Kola Peninsula of Russia have shortened the rearing period to one year before fish are released into the rivers because the disease occurs predominantly in older juveniles (Doszpoly et al., 2013).

9.7 CONCLUSIONS

Alloherpesviruses of fish are responsible for many important diseases in aquaculture. They persist in affected populations as inapparent latent infections and can spread to naïve hosts by recrudescence and horizontal transmission or to offspring by vertical transmission. Diseases caused by these viruses are often closely tied to specific temperatures and stress. Management of these diseases generally relies on avoiding carrier sources of fish, minimizing stress and minimizing crowding during the most susceptible ages and temperatures. The development of efficient methods of producing recombinant attenuated viruses using BACs will facilitate vaccine development and the use of affordable live vaccines may become more common management tools in the future. The latent form is difficult to detect and many alloherpesviruses are recalcitrant to isolation in cell culture, making detecting them a challenge for diagnostic laboratories. The identification of conserved genes and the use of degenerate PCR followed by sequencing have allowed the molecular characterization and development of diagnostic tools for uncultivable members of this group.

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Chapter 10

Adenoviruses of Fish

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10.1 INTRODUCTION

Adenovirus was first isolated from human adenoid tissues in 1953 (Rowe et al., 1953), thus the name of this group of unique viruses; the Greek *aden*, *adenos* means "gland." Since then, adenoviruses have been isolated from humans and a wide range of animal species, including cattle (Reddy et al., 1998), pigs (Nagy et al., 2001), chickens (Griffin and Nagy, 2011; Ojkic and Nagy, 2000), dogs (Morrison et al., 1997), bats (Li et al., 2010), horses (Cavanagh et al., 2012), titi monkeys (genus *Callicebus*) (Chen et al., 2011), lizards (Wellehan et al., 2004) and sea lions (Cortés-Hinojosa et al., 2015). Due to sophisticated diagnostic methods and the rapid nature of current genome sequencing techniques, it is anticipated that more will be discovered. However, infection of fish with adenoviruses is rather rare and there is only one known disease caused by fish adenovirus, which is in white sturgeon (Hedrick et al., 1985; Benkö et al., 2002). Three other cases, incidents that were reported to be associated with adenoviruses together with their hosts, are given in Table 10.1. These include epidermal hyperplasia in Atlantic cod and dab (Jensen and Bloch, 1980; Bloch et al., 1986), and lympholeukemia in Japanese red sea bream (Miyazaki et al., 2000). In all three cases, adenoviruses were identified only by electron microscopy.

10.2 STURGEON WASTING DISEASE

Although the infection was first described in 1985 (Hedrick et al., 1985), it seems that it was named "sturgeon wasting disease" by Noga (2010). The name refers to the chronic nature of the infection in cultured white sturgeon (*Acipenser transmontanus*). The virus associated with this infection is white sturgeon adenovirus 1 (WSAdV-1) (Harrach et al., 2011).

10.2.1 Structure and Composition of Virus

Adenoviruses are nonenveloped, their size is about 70–100 nm in diameter and have an icosahedral symmetry with a triangulation number of 25. The capsid consists of 240 nonvertex capsomers (hexons) and 12 vertex capsomers (penton bases). Adenoviruses have a unique morphological feature, the antenna-like fibers that protrude from the vertices. The penton base and fiber make up the penton. Most adenoviruses have one fiber per vertex, but there are exceptions. The aviadenoviruses have two fibers, and, depending on the viral species and serotype, there are one or two fiber genes (Grgic et al., 2011; Griffin and Nagy, 2011). The human adenoviruses 40 and 41 have two fibers with different length (Kidd et al., 1993). For a lizard adenovirus, two fiber genes were identified and virions with either one short or three long fibers per penton were reported (Pénzes et al., 2014). In addition to the hexon, penton base and fiber proteins there are 10 more proteins in the capsid. Some of the proteins are unique to a given genus; for example, proteins V and IX are present only in mastadenoviruses. The buoyant density of the mastadenoviruses in CsCl is 1.31–1.36 g/cm^3. Adenoviruses survive for long periods of time in the environment; no data are available for a fish virus, but some aviadenovirus strains survive at 60°C for 30 min.

The genome of adenoviruses is a single, double-stranded linear DNA ranging from 26,163 to 48,395 bp; hemorrhagic enteritis virus of turkey virus has the shortest genome size, and the white sturgeon virus was reported to have the longest genome (Pitcovski et al., 1998; Harrach et al., 2011). A terminal protein is covalently linked to the 5′ end of each DNA strand. Adenoviruses have a variable length of inverted terminal repeats. The G+C content is variable among viruses from 33.6% to 66.9%. The central part of the genome is well conserved among adenoviruses and codes for the structural proteins of the virus, while the ends show variations among genera containing the genus-specific, open-reading frames (Davison et al., 2003).

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00010-3
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TABLE 10.1 Diseases Associated With Adenoviruses

Disease	Host	Occurrence	References	Comments
Sturgeon wasting disease	White sturgeon	United States, California	Hedrick et al. (1985)	
	Acipenser transmontanus		Benkö et al. (2002)	
Atlantic cod epidermal hyperplasia	Atlantic cod	Baltic sea	Jensen and Bloch (1980)	Adenovirus-like particles by EM
	Gadus morhua			
Dab epidermal hyperplasia	Dab	North Sea	Bloch et al. (1986)	Adenovirus-like particles by EM
	Limanda limanda (L.)	Denmark		
Lympholeukemia	Japanese red sea bream	Japan	Miyazaki et al. (2000)	Adenovirus-like particles by EM
	Pagrus major			

The establishment of the fifth adenovirus genus was proposed in 2002 (Benkö et al., 2002) when it was found through phylogenetic analysis that the DNA polymerase gene of WSAdV-1 represents a separate cluster (GenBank accession number AY082701). Further work and sequence analysis of the middle portion of the genome, mainly the hexon and protease genes showed the distinctness of WSAdV-1 (Kovács et al., 2003).

Based on the International Committee on Taxonomy of Viruses (ICTV) report (Harrach et al., 2011), the sturgeon adenovirus 1 is the type species of the genus *Ichtadenovirus*, and remains the sole member of that genus, and the only confirmed fish adenovirus. The genome is 48,395 bp with 42.7% G+ C content and it is the longest adenovirus genome. The authors claim that the fiber gene was not found in its usual position at the right end of the genome; however, homologues were identified at the left end of the genome. Unfortunately, Harrach et al. have not yet reported the full genome sequence, gene organization and the analysis. The GenBank accession number (AJ495768) given (Harrach et al., 2011) provides sequences of only the hexon gene and protease gene sequences that were identified earlier.

10.2.2 Classification and Virus Replication

Adenoviruses are in the family *Adenoviridae.* Up to this point, there are more than 44 species identified in this family and they are classified into five genera (Harrach et al., 2011). The genus *Mastadenovirus* includes human and mammalian animal viruses, the best studied ones are human adenovirus 2 (HAdV-2) and human adenovirus 5 (HAdV-5). Members of the genus *Aviadenovirus* infect birds. Viruses in the genus *Atadenovirus* are found in a broad range of hosts, such as sheep, cattle, duck, possum and snake and atadenoviruses have been named because of the high A+ T content of their genomes. Viruses in the genus *Siadenovirus* are named due to a putative gene at the left end of the genome, which encodes a protein homolog to sialidases; siadenoviruses were identified in frog, turkey and raptor. There is only a single virus, white sturgeon adenovirus, in the genus *Ichtadenovirus.* The designation of the genus refers to the Greek word, *ichthys*, which means fish. This genus has a single species *Sturgeon ichtadenovirus A*.

Similar to other viruses, adenoviruses follow a characteristic pathway during their replication cycle, which is best studied for mastadenoviruses. Attachment occurs via the knob region of the fiber protein and receptors on the surface of susceptible cells. The Coxsackievirus and adenovirus receptor was identified for HAdV-2 and HAdV-5 (Bergelson et al., 1997), while other HAdVs use CD46 (species *Human adenovirus B*) (Sirena et al., 2004) or sialic acid as a receptor. Internalization is done through receptor-mediated endocytosis, depending on the interaction between Arg-Gly-Asp (RGD) motif of the penton base protein and cell-surface integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Wickham et al., 1993). The virions are released from the endosomes and transported to the nuclear pores. After uncoating, the core is delivered to the nucleus where DNA replication, transcription and virus assembly occur. The adenovirus replication cycle is divided into early and late phases, separated by viral DNA replication. There are five early transcription units, the early genes code for early proteins needed for regulation of gene expression and DNA synthesis. The late unit generates five families of mRNAs, the late transcription unit is characterized by extensive splicing resulting in a wide array of late mRNAs to the late, mostly structural proteins (Leppard, 1998). In addition, there are two delayed units. After DNA replication, late genes are transcribed and viral late mRNAs are preferentially transported to the cytoplasm where the structural proteins are synthesized and transported into the nucleus. Virus assembly occurs in the nucleus and viral DNA is encapsidated into the empty capsid via the packaging signal located near the left terminus of the genome (Berk, 2013). The mature virions are released by cell lysis.

10.2.3 Epidemiology

Not much is known about the epidemiology of WSAdV-1, and there are no data on its molecular epidemiology. The infection was first described in California in the mid-1980s in juvenile-cultured white sturgeons. Hedrick et al. (1985) reported a chronic condition when a cumulative mortality was approximately 50% over a 4-month period. LaPatra et al. (1993) also cited adenovirus as a contributor to mortality in young sturgeon in California. No recent reports about disease and/or from other parts of the world have been found.

10.2.4 Pathology and Immunity

There are different opinions in the barely existing literature about the pathogenicity of WSAdV-1. Dr. Noga in his book entitled *Fish Disease: diagnosis and treatment* lists sturgeon wasting disease as a clinically important disease, while on the other hand in the ICTV report (Harrach et al., 2011) one can read that "the virus seems to be nonpathogenic."

Sturgeon wasting disease is a chronic disease of cultured white sturgeon, it was first noted by growers who received stocks from Sacramento River, CA, in 1984. The case was subsequently described and published by Hedrick et al. (1985). There were severe mortalities, fry were lethargic, anorexic and emaciated (Wolf, 1988). The liver was pale and there was no food in the intestinal tract. Histological examination demonstrated that the nuclei of intestinal and spiral valve epithelial cells were enlarged and eventually erupted into the lumen of the gut. Similar lesions were seen in liver cells of one specimen. Electron-dense hexagonal virions in the size range of adenoviruses were seen by electron microscopy in the nuclei of cells of affected tissues, indicating the presence of adenovirus. Experimentally, the virus could be transmitted to juvenile sturgeons by intraperitoneal injection.

No data are available on immunity against fish adenoviruses.

10.2.5 Diagnostic Methods

In general, the rule-out diagnosis starts with the combination of history, clinical signs and pathology. Specific viral diagnosis is done by virus isolation and identification, in this day and age that would mean genomic detection by polymerase chain reaction (PCR). To isolate the virus, cell lines from fresh water fish, BF-2 from blue gill (*Lepomis macrochirus*), BB from brown bullhead (*Ictalurus nebulosus*), FHM from fathead minnow (*Pimephales promelas*), CCO from channel catfish (*Ictalurus punctatus*) and CHSE-214 from chinook salmon (*Oncorhynchus tshawytscha*) were inoculated with tissue homogenates of diseased fish, but the cultivation was unsuccessful with no cytopathic effect being seen (Hedrick et al., 1985). The WSAdV-1 isolate was successfully propagated on white sturgeon spleen (WSS-2) cell line and was cytopathic, although the replication efficiency was low (Benkö et al., 2002; Hedrick et al., 1991).

PCR primers targeting the conserved region of the DNA polymerase were described for the primary detection of WSAdV-1 (Benkö et al., 2002); potentially these primers could be used for the detection of adenoviruses in diagnostic laboratories. In summary, it seems that electron microscopy remains the only method for the detection and identification of fish adenoviruses.

10.2.6 Prevention and Control

No specific recommendations are known for the prevention and control of this infection. As for disinfection: adenoviruses are not sensitive to lipid solvents and 50% alcohol in general, they are relatively heat stable and resistant to variations in pH between 3 and 9. They can be inactivated by 1:1000 concentration of formaldehyde.

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Chapter 11

Papillomaviruses and Polyomaviruses in Fish

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11.1 INTRODUCTION

In the past, both papillomaviruses and polyomaviruses have been categorized as belonging to the family *Papovaviridae*, but at present, the distinct families of *Papillomaviridae* and *Polyomavirdae* have been established. The *Papillomaviridae* family currently contains over 16 genera (eg, *Alphapapillomavirus, Betapapillomavirus* and *Gammapapillomavirus*). Papillomaviruses possess circular, double-stranded DNA that are 6.8–8.4 kbp in length. The natural hosts of papillomaviruses are a wide range of vertebrates, and human papillomaviruses sometimes lead to the formation of malignant tumors. However, the viral genome is rarely found in the host's chromosome. Conversely, polyomaviruses have circular, double-stranded DNA of approximately 5 kbp in length, encoding more than five proteins, with mammals and birds as the main natural hosts. The family has 1 genus *Polyomavirus*, and includes the Simian virus 40 (SV-40), BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), Merkel cell polyomavirus, Budgerigar fledgling disease polyomavirus (BFPyV) and bovine polyomavirus (BPyV). Viral proteins, such as the large T antigen of SV-40, sometimes lead to malignant tumors and the polyomavirus genome is rarely integrated into the host chromosome. The mammalian polyomaviruses are nonpathogenic in natural hosts, whereas the avian polyomavirus (Budgerigar fledgling disease virus) is pathogenic in parakeets.

There have been only a few reports of papillomavirus, polyomavirus and papovavirus infections in fish (Table 11.1). In the 1960s and 1970s, there were three studies regarding papovavirus-like viruses discovered in fish, including eels (Schwanz-Pfitzner, 1976; Deys, 1969; Kollinger et al., 1979). Emerson et al. (1985) detected papovavirus-like viruses in papillomatosis-type lesions in winter flounder (*Pseudopleuronectes americanus* (Walbaum)) using electron microscopy. The detected particles were approximately 30 nm in diameter and were sensitive to the deoxyribonuclease enzyme, suggesting that they were small DNA virions. However, no information concerning nucleic acid sequences or pathogenicity is available from these studies. Recently, two novel polyomavirus-like viruses were found in sea bass and eel, and the full genome sequences of both viruses have been determined. As described next, the virus detected in eel was found to have particularly high pathogenicity in that species.

11.2 FISH DISEASES

11.2.1 Japanese Eel Endothelial Cell-infecting Virus

11.2.1.1 History of Discovery

The Japanese eel *Anguilla japonica* is an important food fish in Japanese cuisine, but since 2013, it has been listed by the Japanese Ministry of the Environment as an endangered species. Nevertheless, 110,000 tons of eel are consumed annually. Viral endothelial cell necrosis of eel (VECNE) affecting *A. japonica* has caused serious problems in the Japanese eel aquaculture industry. In 2008, the total production of Japanese eels in Shizuoka Prefecture, where eel is cultivated on a large scale, was approximately 1700 tons (valued at 4 billion yen). However, 107 tons of eels died due to illness, with VECNE in particular estimated to have killed 31 tons. Although economic losses due to VECNE are a serious problem, the causative agent of the disease had gone undetermined for least 30 years.

Egusa et al. (1989) found histopathologically that accumulation of blood to the center of the gill filament is caused by the central venous sinus filling with blood and the venous sinus subsequently becoming extended. Using electron microscopy, Ono and Nagai (1989) found adenovirus-like particles of approximately 75 nm in diameter in the endothelial

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00011-5
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TABLE 11.1 Fish Papillomavirus and Polyomavirus

Virus Family	Virus Name	Host	References
Papovaviridae	Papovaviruslike virus	Winter flounder (*Pseudopleuronectes americanus*)	Emerson et al. (1985)
Polyomaviridae	Japanese eel endothelial cell-infecting virus	Japanese eel (*Anguilla japonica*)	Mizutani et al. (2011)
Polyomaviridae	Black sea bass–associated polyomavirus 1	Black sea bass (*Centropristis striata*)	Peretti et al. (2015)

cells of eel gill filaments. Tanaka and Egusa (1992) also indicated the possible viral nature of the pathogen via filtration experiments. After inoculating healthy eels with extracted samples from VECNE eels, Inouye et al. (1994) and Ono and Nagai (1997) found congestion and hemorrhage characterized by necrosis in the glomerular capillaries of the gill, liver and kidney, as well as in the endothelial cells of the endocardium. They also found adenovirus-like particles in the nuclei of endothelial cells in the experimentally inoculated eels. Therefore, VECNE is thought to be caused by cytopathic effects (CPEs) and necrosis in target organs with blood vessel dysfunction, caused by an adenovirus-like virus infection. Although Kusuda et al. (1989) and Masuda and Ono (1999) found novel birnaviruses in VECNE, their pathogenicity is still unknown.

Researchers believed that VECNE was caused by an adenovirus-like virus and tried to detect this virus using polymerase chain reaction (PCR) with adenovirus primers, but no primers resulted in successful amplification. Ono et al. (2007) established a cell culture system from Japanese eel vascular endothelial (JEE) cells to isolate the causative virus of VECNE. When a filtered sample of homogenized VECNE-positive gills was inoculated into JEE cells, CPEs were observed. Particles of 75 nm in diameter were also observed in the cell nuclei. Although Ono et al.'s results suggested that the causative agent of VECNE was an adenovirus, and they characterized the virus in their report, there was no definitive evidence that adenovirus was in fact the infectious agent. Mizutani et al. (2011) successfully determined the full genome sequence of the causative virus from the supernatant that caused CPE using rapid determination system of viral nucleic acid sequences (Mizutani et al., 2007) and next-generation sequencing. The elucidated virus was named *Japanese eel endothelial cell-infecting virus (JEECV)* and was found not to belong to Adenoviridae, but rather to Polyomaviridae.

11.2.1.2 Pathology of VECNE

As described previously and shown in Fig. 11.1, VECNE is characterized by reddening of the fins with swollen abdomen and intense congestion, as well as hemorrhage and destruction of blood vessels in the gills, liver, kidney and intestine. Histopathologically, VECNE is characterized by intense congestion in the central venous sinus of gill filaments. Degeneration of endothelial cell nuclei is observed in blood vessels.

11.2.1.3 Characterization of JEECV

JEECV is approximately 75 nm in diameter (Fig. 11.2), and it can tolerate chloroform, pH 3.0 and incubation at ≤42°C (Ono et al., 2007). It reaches 10^7 copies in JEE cells and 10^5 copies in the supernatant (Mizutani et al., 2011). Analysis of 70th-passage JEECV in JEE cells indicated a genome length of 15,131 nucleotides with circular, double-stranded DNA (GenBank accession number AB543063). More than 15 open reading frames are predicted in both strands. In the open reading frames, a large T antigen-like protein of SV-40 was found. Other predicted proteins were not homologous to any virus genes. Interestingly, while the coding region of the large T-like antigen of JEECV had no introns, polyomaviruses usually have one splice donor and two accepting sites in the large T-antigen coding region. The large T-like antigen of JEECV contains motifs conserved among polyomaviruses, such as DNAJ motif, canonical nuclear localization signal, zinc finger motif and conserved region 1. However, the biological function of this JEECV protein is not yet clear.

11.2.1.4 Molecular Epidemiology

There have been no studies detecting JEECV in natural habitats. Okazaki et al. (2015) reported the detection of JEECV in yellow Japanese eels collected from two distant rivers in Japan in 2012, which seemed to be healthy. Their study used conventional sensitive PCR for detection of JEECV and a total of four eels (20%) were found to be JEECV-positive. Furthermore, some changes in nucleic acids and amino acids in this region were found, compared with prototype JEECV. Furthermore, Okazaki et al. reported JEECV infects to elvers caught from the sea near Japan and mature eels from their spawning area (in press).

FIGURE 11.1 Eel infected by JEECV (Dr. Ono).

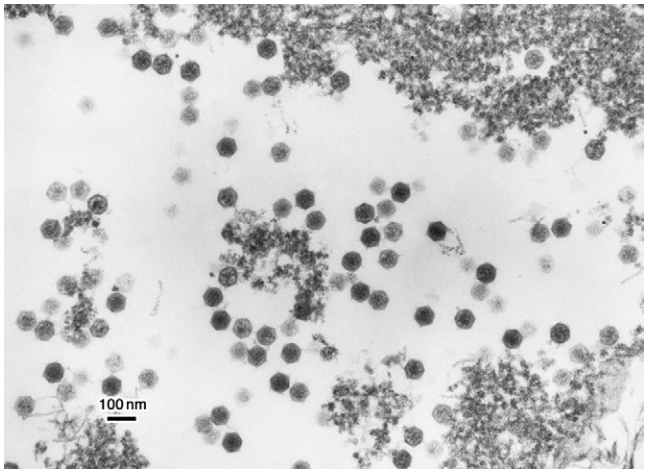

FIGURE 11.2 Electron micrograph of JEECV (Dr. Ono).

After maturation, freshwater eels metamorphose to the silver stage and begin a long-distance (3000–6000 km) migration to spawn. In the case of European eels, it is thought that infection by the European-X eel rhabdovirus prevents this long-distance migration (van Ginneken et al., 2005).

11.2.1.5 Diagnostic Methods

A serological detection method has not been established. Conventional PCR and the more sensitive TaqMan real-time PCR are used for detection of viral genomic DNA (Mizutani et al., 2011). The primers and probe are designed for the large T antigen-like region; the primers are 025-1 (5′-TTGCCGCACCTGCTTCAG-3′) and 025-2 (5′-CGAACACCGTAATTGGAATAAAGC-3′) for a 120-bp PCR product, and the probe is 025P (5′-FAM-ACACGCTGCTCAAATTGCTGCTGCCT-TAMRA-3′). The thermal cycling profile of the assay comprises the following steps: 2 min at 50°C, 10 min at 95°C, and then 45 cycles of PCR at 95°C with denaturing for 15 s and at 60°C for 1 min with annealing/extension for 1 min.

11.2.1.6 Prevention and Control

A vaccine for JEECV is not available, but treatment of eels infected experimentally with JEECV under nonfeeding conditions at 35°C for more than 3 days is an effective control method for VECNE (Tanaka et al., 2008).

11.2.2 Black Sea Bass–Associated Polyomavirus 1

Recently, Peretti et al. (2015) reported the complete genome sequence of novel polyomavirus from the black sea bass (*Centropristis striata*). In a previous report (Pastrana et al., 2013), they found a divergent variant of St. Louis polyomavirus (STLPyV) in a sanitized human skin wart specimen using random-primed rolling circle amplification (RCA) combined with deep sequencing, as Polyomaviridae has circular DNA. To find novel polyomavirus in fish, they used the same technique. First, scale, skin, lateral muscle, mouth, eye, anogenital opening, swim bladder and liver tissue were combined and treated with detergents and nucleases. Virions were purified using ultracentrifugation. Extracted DNA was subjected to RCA and deep sequencing. The genome of black sea bass–associated polyomavirus 1 (BassPyV1) was found to be

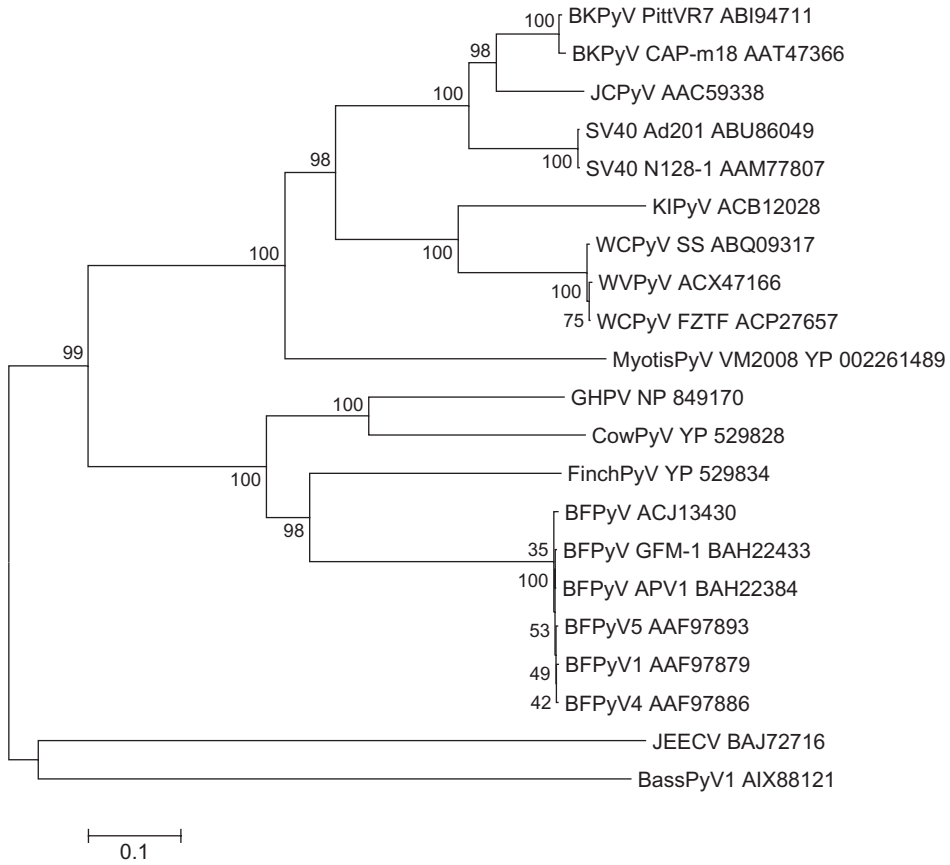

FIGURE 11.3 Phylogenetic tree analysis of the large T antigen region in polyomaviruses. The phylogenetic tree was obtained using the neighbor-joining method with 1000 bootstrap replicates, and the branch length is indicated at each branch node. The horizontal scale indicates 0.1 amino acid substitutions per site. *GHPV*, goose polyomavirus; *BFPyV*, budgerigar fledgling disease polyomavirus; *Finch PyV*, finch polyomavirus; *Cow PyV*, crow polyomavirus; *BKPyV*, BK polyomavirus; *SV-40*, simian virus 40; *WCPyV*, WU polyomavirus; *Myosis PyV*, myosis polyomavirus; *JCPyV*, JC polyomavirus; *WUPyV*, WU polyomavirus; *KIPyV*, KI polyomavirus; *JEECV*, Japanese eel endothelial cell-infecting virus; *BassPyV1*, black sea bass–associated polyomavirus 1.

circular and 7.4 kb in length. The predicted large T antigen for this virus has homology to JEECV (Fig. 11.3). Interestingly, BassPyV1 lacks the small t antigen. The predicted VP1 major capsid protein has a typical length, while the predicted VP2 minor capsid protein is longer than the typical VP2. Because BassPyV1 sequences are extremely divergent from terrestrial polyomaviruses, Pastrana et al. (2013) discussed the possibility that BassPyV1 evolved separately from terrestrial polyomaviruses over a period of more than 350 million years. There is no information regarding the pathogenicity of this virus and diagnostic methods, and the virus has not been isolated using cell culture.

ACKNOWLEDGMENTS

I thank Drs. Ono (Tokai Univ.) and Nagai (Tokyo Univ. of Agriculture and Technology) for allowing the use of their figures. I also thank Ms. Okazaki, Mr. Naoi, and Dr. Omatsu (Tokyo Univ. of Agriculture and Technology) for their assistance.

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Chapter 12

Circoviruses of Fish

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12.1 INTRODUCTION

Circoviruses are small (12–27 nm), nonenveloped viruses with an icosahedral capsid containing a short single-stranded, covalently closed circular DNA genome of 1.7–2.3 kilobases. After the description of the first circovirus (porcine circovirus, PCV) in a porcine cell culture (Tischer et al., 1974, 1982), a number of viruses with similar characteristics were discovered and the family *Circoviridae* was established to accommodate them. *Circoviridae* is a relatively new, but rapidly growing family of viruses as a result of the unprecedented recent evolution of molecular detection techniques. The family is divided into two recognized genera, *Circovirus* and *Gyrovirus* (Fauquet et al., 2005; Biagini et al., 2011), but a further tentative genus, *Cyclovirus* was proposed (Li et al., 2010; Biagini et al., 2013) for genomes that share basic characteristics of circoviruses, but can be phylogenetically distinguished from those already in the accepted genera. The genomes of viruses in the three genera exhibit several common features, but the significant divergence detected in the type species of *Gyrovirus* genus, namely chicken anemia virus, led to a proposal (Hino and Prasetyo, 2009) to transfer *Gyrovirus* into the family *Anelloviridae*.

Except for the negative-sense genome organization of gyroviruses, circovirus genomes are ambisense and contain two major genes (*rep* and *cap*), encoding the replication-associated (Rep) and the capsid (Cap) proteins. Rep is involved in the replication of the virus whereas Cap subunits serve as building blocks of the entire capsid of the virion and are also the main antigenic targets of the immune response.

The viruses replicate in the nucleus of the cell according to the rolling circle replication model (Mankertz et al., 1998; Cheung, 2004) with the aid of circular, double stranded (ds) replicative DNA intermediates. Cellular DNA polymerases are used for the generation of the ds intermediates during the S phase of cell division. A complex replication process was described for PCVs (Cheung, 2003) during which 9–12 *rep* transcripts are produced, and it is likely that a similar strategy is followed by other circoviruses.

Although the first circovirus identified was of porcine origin, the majority of the subsequently described circoviruses were from avian species; except for a pathogenic variant of the porcine circovirus (PCV2, Allan et al., 1998) and a canine circovirus (Kapoor et al., 2012). The first fish circovirus was discovered in 2010 (Lőrincz et al., 2011), broadening the host range of the family.

Using viral metagenomics and polymerase chain reaction (PCR), new circoviruses and closely or distantly related circovirus-like genomes were detected in reclaimed water and marine samples (Rosario et al., 2009a,b), in cases of sea turtle fibropapilloma (Ng et al., 2009), in human and chimpanzee stools (Li et al., 2010) and Blinkova et al. (2010) described circular viral genomes suggesting an evolutionary link among circoviruses, plant geminiviruses and nanoviruses. The improvement of detection methods led to the discovery of a number of further circoviruses and circo-like viruses (Delwart and Li, 2012), showing that these viruses were more widespread than believed only a few years before.

The genetic variability of circoviruses is surprisingly high, equaling that of some RNA viruses; the rate of nucleotide substitution for PCV was estimated to be of 1.2×10^{-3} substitutions/site/year (Firth et al., 2009). In addition to mutations participating in their rapid evolution, the important role of recombination events was also proven for PCV (Cadar et al., 2012) and columbid circoviruses (Cságola et al., 2012).

Circoviruses are considered to be host-specific, although PCV2 infection of other species, such as rodents (Lőrincz et al., 2010) and cattle (Nayar et al., 1999; Kappe et al., 2010), had also been reported and it is therefore likely that they

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Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00012-7
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have a narrow host range. The viruses are mainly transmitted through the fecal–oral route, but vertical transmission of PCV and some avian viruses is also known to occur.

The ability of fish circoviruses to induce diseases in infected fishes is not known yet (see later), but avian and PCVs share several pathogenic characteristics that may help extrapolate and understand how these viruses affect an organism. As a result of the simplistic design of the genome (two major open reading frames, ORFs) and the virion (a single, repetitive Cap protein), the virus, regardless of the species it can infect, has to rely on the replication machinery of the infected cell and also must be able to handle the humoral and cellular immune reactions of the host targeting mainly the Cap protein. Circoviruses replicate in dividing cells, therefore young animals are more likely to develop a disease. Dividing cells are always available in the activated immune system, which is just one of the reasons why these viruses are considered to be strongly immunosuppressive (Todd, 2000). Pathogenesis of circoviral diseases and the general outcome of the infection are also very similar in birds and pigs, suggesting that fish infections might have the same result because of these similarities. For example, PCVs are involved in a wide variety of diseases, mostly known as porcine circovirus-associated diseases (PCVD), manifesting in several different clinical venues (Segalés, 2012), depending on the age of the animals, the virus load and factors that affect the immune system or the general health of the infected pig. The infection may remain subclinical or develop into systemic or organ-specific diseases. All PCVDs, including the subclinical form, are characterized by lymphocyte depletion and immune suppression. It is well established that cofactors are usually needed for the development of a PCVD. The diseases, when and if they develop, are characterized in both pigs and avians not only by the circovirus infection, but also by those secondary infections that were given the opportunity by the malfunction of the immune system and contribute to the clinical manifestation of the disease (Allan et al., 1998; Allan and Ellis, 2000; Todd, 2004).

12.2 SPECIFIC MAIN FISH DISEASE

As stated previously, the ability of fish circoviruses to induce disease is not yet known. There are, however, data indicating that these viruses may affect fish in similar ways as nonfish viruses do in their respective hosts. The detection of the first fish circovirus (barbel circovirus, BaCV, Lőrincz et al., 2011) was from cases when increased mortality of barbel fry (*Barbus barbus*) was observed and testing for other known infectious agents (viruses, bacteria and parasites) or toxic substances yielded negative results. Fish fry hatched and reared using BaCV infected eggs died shortly after hatching and the virus was consequently detected in them. Later in 2011, a number of fully developed European catfish (*Silurus glanis*) of 6–50 kg body weight were found dead in Hungary's Lake Balaton and routine testing for causative agents remained negative. A second fish circovirus (catfish circovirus, CfCV) was discovered in these fish (Lőrincz et al., 2012) and was suggested as the cause of the observed death cases. There is no indisputable evidence proving that these novel viruses were responsible for the disease of barbel or catfish, but both cases correspond well with what is seen in pigs or birds where young age or exhaustion are among the most important predisposing factors leading to the development of circovirus-induced diseases.

The connection between virus and disease is even less evident in case of the third fish circovirus (Eel circovirus, EeCV). EeCV was described in European eel (*Anguilla anguilla*) that was reported (Doszpoly et al., 2014) as a result of a routine limnological survey when an adult eel with cauliflower-like growths on the mouth was captured and necropsied.

Shortly after the first report of a fish circovirus (Lőrincz et al., 2011), surveys for other fish and amphibian circoviruses were initiated. As a result of those studies, partial DNA sequences of new fish circoviruses were amplified by PCR in roach (*Rutilus rutilus*), common bream (*Abramis brama*), asp (*Aspius aspius*) and round goby (*Neogobius melanostomus*), indicating that circoviruses may be common in fish (Tarján et al., 2014), but the study could not show any connection between the presence of these viruses and diseases. Using PCR further circovirus *rep* sequences were shown in cyprinid fish (*Labeo rohita* and *Catla catla*), but interestingly these sequences could not be extended into full genomic copies and the authors found evidence that they were integrated parts of the host genome.

12.3 STRUCTURE AND COMPOSITION OF VIRUS

Our present knowledge about fish circoviruses is based mainly on genetic studies. Complete genomic copies of barbel, catfish and eel circoviruses, and partial sequences from the other mentioned fish viruses were produced by PCR and sequenced. By comparing these sequences with each other and known circoviruses of other species, it can be postulated that virion structures of fish viruses are characteristic to circoviruses in general, a single-stranded circular genome surrounded by a single repetitive Cap protein (Biagini et al., 2011). The putative structures of several circoviral Cap proteins were examined and compared with the crystallographic PCV2 Cap (Patterson et al., 2012) and in addition to antigenic differences, a high degree of conservation was also observed, revealing key functional regions. Using molecular replacement models and the known PCV2 capsid structure, it was shown that BaCV of all known circoviruses shared the closest similarity to PCV2 Cap

and CfCV represented the opposite end of the spectrum. The structural organization of the Caps revealed differences in the regions corresponding to epitopes of PCV2 Cap, but the overall structure of every circovirus Cap showed a high degree of similarity, the beta-sheet core configuration indicated that fish circoviruses, like others, had icosahedral structures.

12.4 CLASSIFICATION AND VIRUS REPLICATION

During a study in 2010 (Lőrincz et al., 2011), an attempt to show the presence of circoviruses in freshwater fishes including barbel (*B. barbus*), pike perch (*Sander lucioperca*), European catfish (*Silurus glanis*), European eel (*Anguilla anguilla*) and cyprinids as common carp (*Cyprinus carpio*), Prussian carp (*Carassius gibelio*) or bream (*Abramis brama*) failed to produce positive results of the tested fish except for barbels. Full genomic sequences of two barbel circoviruses (BaCV1 and BaCV2) originating from different fish collected at different locations were determined. The genome size of both BaCVs was 1957 bases organized in a single-stranded DNA (ssDNA) molecule, forming a covalently closed circular structure. The nucleotide sequences of the two viruses were 94.7% identical, showing a low level of divergence. The genome organization of all three established fish circovirus genomes (BaCV, CfCV and EeCV) are shown in Fig. 12.1. Two major ORFs arranged in opposite directions, characteristic for viruses of the *Circovirus* genus were identified in BaCV. The longer ORF (ORF1) is located on the positive strand between 48 and 1005 nucleotide (nt) positions, starting with a standard ATG but with a potential alternative initiation at nt 9 (CTG) encoding 319 or 332 amino acids (aa). A TTG initiation codon was identified for the reversely oriented ORF2, starting at nt 1905 and extending to nt 1264, with a coding capacity of 214 aa. When comparing predicted amino acid sequences of BaCV1 and BaCV2, ORF1 showed 97.5% similarity whereas ORF2 showed 95.3%.

Blast search (Altschul et al., 1997) of ORF1 and two amino acid sequences indicated that they were both similar to proteins of members of the *Circoviridae* family and also to cycloviruses (Li et al., 2010) and circular genomes from environmental samples (Rosario et al., 2009a). The closest relative of the putative ORF1 protein (51% identity) was the replication-associated protein (Rep) of the human stool-associated circular virus (NG13) described by Li et al. (2010), but porcine and avian circovirus Reps also had 44–50% identity. According to the Blast and Pfam database search (Finn et al., 2008), BaCV ORF1 is a member of the viral Rep family, with an RNA helicase domain that is present in some, but not all, known circovirus Reps. A number of capsid protein sequences of the Circo-capsid superfamily was retrieved throughout the ORF2 Blast search with similarity results between 31% and 35%, the closest relatives being waterfowl circoviruses. Two additional but smaller, oppositely oriented ORFs (ORF3, 115 aa; ORF4, 98 aa) were also identified in the genomes of BaCV1 and BaCV2. Blast search of these did not indicate any homology with known viral proteins.

Like in barbel, two different circoviruses (CfCV) were detected in catfish by PCR (Lőrincz et al., 2012), the size of both genomes was 1966 nt, somewhat bigger than that of BaCVs. The two CfCV sequences were 99.4% identical and also encoded two major ORFs in opposite orientations. ORF1 is located between 251 and 1193 nt with a potential coding capacity of 314 aa, whereas ORF2 is present between 1934 and 1253 nt encoding 227 aa. Unlike BaCV, the CfCV ORF2 had an ATG initiation codon. Two minor ORFs, ORF3 (157 aa) and ORF4 (136 aa) with unknown function or similarity to known viral genes were identified in the genome.

Analysis of CfCV ORF1 indicated homology with other circovirus Reps, showing that the putative protein was a member of the circovirus Rep family. The closest relative of CfCV based on ORF1 aa Blast comparisons was NG13 with 55% identity, whereas the rest of known circovirus Reps including BaCV showed lower similarities (49–52%). The Cap aa comparison in Blast also indicated NG13 (35% similarity) to be the closest relative of CfCV. BaCV Cap was only 28% similar, which is still higher than any of the other known circovirus Caps.

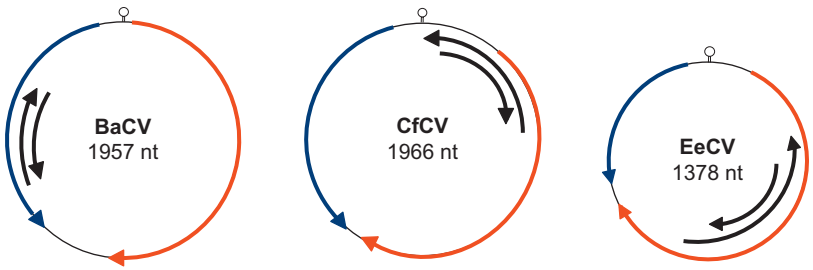

FIGURE 12.1 Genome organization of known fish circoviruses. Names and genome size are indicated in the middle. *Blue* (dark gray in print versions) and *red* (light gray in print versions) *arrows* indicate the relative positions and lengths of *cap* and *rep* genes respectively. Minor ORFs are represented by *black arrows*. The upper small circles with the thin lines show the site of the stem loop.

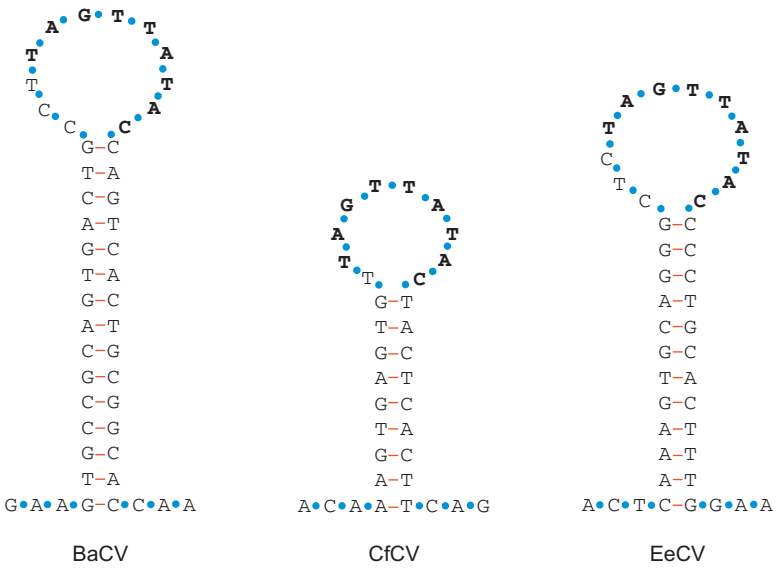

FIGURE 12.2 Stem-loop structures of the known fish circoviruses.

The third and, up to now, the last known complete fish circovirus (eel circovirus, EeCV) genome was reported in 2014 (Doszpoly et al., 2014). Although they share several characteristic features, EeCV is significantly different from BaCV and CfCV. The most obvious difference is in the size of the genome, EeCV is only 1378 nt in size, but forms a ssDNA circle for other circoviruses. Another difference is in the genome organization where the putative Cap coding ORF3 is significantly shorter than in other circoviruses, including CfCV or BaCV. This virus also carries two major ORFs in opposite orientation with coding capacities of 114 aa of the *cap* gene and 286 aa of the *rep*, the latter containing the conserved RNA helicase domain as in CfCV and BaCV. The closest relative of EeCV based on ORF1 was CfCV with 51% aa identity. The predicted Cap aa sequence did not show convincing homology to any protein in GenBank, but it carried the arginine-rich amino terminal stretch typical for circoviruses (see later). Further ORFs (ORF2 and ORF4) were also detected in overlap with ORF1. ORF2 is rather long and the deduced aa sequence showed homology to hypothetical gene products of pigeon and duck circoviruses (Mankertz et al., 2000; Chen et al., 2006). The ORF4 putative protein shares homology with another circovirus (beak and feather disease virus) protein (Niagro et al., 1998).

Samples from 34 different fish species were collected and tested for circovirus *rep* sequences during a survey in Hungary (Tarján et al., 2014). Full viral genomes were not amplified, but partial sequences were obtained from 6 out of 236 samples. Two of the positive cases turned out to be PCV contaminants, but three previously unknown fish viruses were also detected in roach (*R. rutilus*), asp (*Aspius aspius*) and round goby (*N. melanostomus*). One *rep* sequence from common bream (*Abramis brama*) was highly similar to BaCV *rep*.

Analysis of the complete fish circovirus genomes (BaCV, CfCV and EeCV) and their putative ORFs revealed several common features of these viruses. All three circular genomes encode a *cap* and a *rep* gene, oriented in opposite directions to each other and separated by intergenic regions of varying length: BaCV: 100 and 259 nt, CfCV: 282 and 60 nt, EeCV: 107 and 65 nt at the 5′ and the 3′ ends, respectively. All three virus genomes carry a conserved nonanucleotide 5′-TAGTATTAC-3′ sequence, like most members of the *Circoviridae* family. This stretch is part of the stem-loop structure (Fig. 12.2) of circoviruses in the 5′ intergenic region between *rep* and *cap*. The stem-loop element is an essential part of the genome, playing a role in replication initiation. The length of the double-stranded stem is 15 and 12 bp for BaCV and EeCV, respectively, but it is only 9 bp for CfCV, shorter than the stem of most circoviruses, but similar to reported cyclovirus stems (Li et al., 2010). The nonanucleotide sequences were compared with those of other members of the family (Table 12.1) Most known circoviruses share the sequences obtained for fish viruses, although for example, PCV2 differs in the first nt, where A is present instead of T. It is important to state that all of the known circoviruses from *Anseriformes* have a G/T substitution at position 3 compared with fish circoviruses, excluding the possibility of common origin.

Another common feature of the fish viruses and other circoviruses is the N-terminal arginine-rich stretch of the putative Cap proteins, homologous to other circovirus Cap nuclear localization signals. BaCV, 31 aa: RRRAR RRAGR RRRTR GWRRR KYVRR QRHRG R; CfCV, 31 aa: RRRTF RRPIR RRMHR RTRGR RMIRR RSRRS R; EeCV, 32 aa: RRRVY RRKSN RRPIR NCQRR YRRPI RRERN NR.

TABLE 12.1 Stem-Loop Nonanucleotide Sequences of Different Circoviruses[a]

Nonanucleotide Sequence	Virus Species
TAGTATTAC	*Fish circoviruses* (BaCV, CfCV, EeCV)
	Porcine circovirus type 1, Columbid circovirus, Gull circovirus, Finch circovirus
AAGTATTAC	Porcine circovirus type 2
TA**T**TATTAC	Circoviruses of *Anseriformes*
CAGTATTAC	
GAGTATTAC	Circoviruses of nonanseriform birds
TA**A**TA**C**TA**T**	Cycloviruses

[a]*Differences are in bold and underlined.*

12.5 EPIDEMIOLOGY

Partial and complete fish circovirus genomes were subjected to thorough phylogenetic analyses using entire genomes, *cap* or *rep* genes separately and also the predicted protein sequences encoded by these. According to these results, BaCV, CfCV and EeCV are members of the *Circovirus* genus, the closest relative to the barbel and the catfish viruses is NG13, a circular DNA genome originally detected in stool samples of children and described as an outlier to the proposed *Cyclovirus* genus (Li et al., 2011). Although the similarity of NG13 to CfCV and BaCV was low, it was detected not only for the entire genome, but also for Cap aa sequences, indicating a close relation among these viruses. The authors hypothesized that the similarity of CfCV and BaCV to NG13 may be the result of a food-borne NG13 contamination from a fish source or that these viruses may have a common, but thus far unknown origin (Lőrincz et al., 2012). The common origin of these viruses was confirmed by the nonamer sequence of the stem-loop of NG13 that is identical to viruses of the *Circovirus* genus, but its genome organization is similar to *Cyclovirus* genomes (Delwart and Li, 2012). Although the closest evolutionary relative of EeCV is CfCV, the eel circovirus did not form a monophyletic group with CfCV or BaCV (Doszpoly et al., 2014). According to the genome organization, EeCV belongs to the *Circovirus* genus as confirmed by the presence of the conserved nonamer in the stem-loop and by the homology of ORF2 and 4 to hypothetical genes of pigeon and duck circoviruses or the beak and feather disease virus (Mankertz et al., 2000; Chen et al., 2006; Niagro et al., 1998).

Of all the putative fish circoviruses where only the complete *rep* was amplified, only the one from common bream showed characteristics of the members of *Circovirus* genus, the rest of these sequences grouped with circular ssDNA genomes mostly of untreated sewage origin (Tarján et al., 2014). Phylogenetic analyses of partial integrated rep-like sequences in fish genomes showed that they were closely related to BaCV and CfCV (Fehér et al., 2013). Several point mutations and indel mutations were observed in the coding region, resulting in frameshifts and multiple termination sites. The authors suggested that integration of these viral elements might have progressed together with, or following the diversification of cyprinid fish.

12.6 PATHOLOGY AND IMMUNITY

There is very little known about pathogenicity of these viruses, therefore pathology or immunology of fish circoviruses can only be predicted and discussed based on knowledge about avian and PCVs and based on the observations made when virus DNA was detected in fish. The lack of experimental infections and the data directly linking pathological or histopathological lesions to fish circovirus infections make all of these predictions uncertain. As discussed previously, circoviruses are generally considered strongly immunosuppressive, have a preference for attacking the activated immune system and clinical signs are more prominent in young animals or animals with a variety of coinfections. It is also known that circoviruses are mostly present in animals in the form of subclinical infections. The first fish circoviruses discovered (Lőrincz et al., 2011) originated from cases when an increased (up to 100%) mortality of barbel fries of 4–6 days of age was observed, and also when fries were hatched from BaCV-infected eggs under laboratory conditions. Researchers excluded every known pathogenic or toxic cause that might have been responsible for the phenomenon. Once the first BaCV sequences were available, a limited search for these viruses was initiated during which barbels of different geographic origin were tested.

Eight of the 19 collected fish, regardless of the age or the site of collection were positive for the virus. The virus was simultaneously present in several organs, but without causing any pathological lesions. The liver and the spleen were the most frequently affected organs, but occasional infection of the eggs was also detected. The increased mortality of fry and the symptomless infection of adults in good physical condition is in accordance with what can be expected considering the characteristics of circovirus infections of nonfish species.

The appearance of circovirus-associated clinical signs is not unusual when there is a deterioration of the physical condition, such as in the case of European catfish that showed increased mortality during the exhaustive spawning season of 2011 (Lőrincz et al., 2012) when more than 2000 specimens of moribund and dead large-sized sexually matured catfish were found in Hungary's Lake Balaton. No clear link was established between the CfCV infections and the mortality, but again no other causative agent could be detected in these fish. Pathology and histopathology revealed skin lesions, vascular dilations in the skin, inflammation of the gastrointestinal tract and nuclear fragmentation in the hematopoietic cells, which raised the suspicion of some kind of unidentified toxicosis that was also excluded through laboratory tests. CfCV DNA was detected mostly in the liver, spleen, gills, kidneys and the gonads.

The connection of EeCV to any disease is unlikely. A single adult eel with cauliflower-like growths near the mouth was caught during a routine survey (Doszpoly et al., 2014). Gross pathology and microscopic analysis were both performed, showing cell proliferation at the oral area characteristic of benign papilloma, a benign myoma on the epicardial surface extending into the myocardium, vacuolization in the hepatocytes and multifocal hemosiderin deposition in the red pulp of the spleen. Attempts to isolate the virus failed. PCR and qPCR resulted in specific products when testing gills, liver, spleen, kidney and the labial proliferative tissue. According to qPCR, the highest virus load was present in the liver and the spleen with more than 100-times higher concentration than in the proliferative tissue area.

12.7 DIAGNOSTIC METHODS

Thus far, every known reliable detection method of fish circoviruses is based on nucleic acid amplification. Sequence-independent techniques were used successfully for identifying novel circo- and cycloviruses, but for a practical diagnostic approach, PCR methods are available. All of the fish circoviruses known to date were discovered using a similar PCR approach. The consensus nested system developed by Halami et al. (2008) is suitable for the amplification of a wide range of circovirus sequences. The primers target a conserved region within the *rep* gene. Once the sequence of the detected circovirus *rep* fragment is established, primers are designed facing outward from the already known area in order to amplify the rest of the genome (Table 12.2). This method was successfully utilized when CfCV, BaCV and EeCV complete genomic sequences were determined, but for the rest of the cases, the designed outward PCR failed to amplify full genomes, resulting in only partial integrated and nonintegrated circoviral *rep* sequences. In order to facilitate the amplification of less abundant circovirus genomes, multiple primed rolling circle amplification using exonuclease resistant random primers is a suitable method to pre-amplify the genomic copies (Johne et al., 2006) before the actual PCR.

For PCR sample preparation, according to the reports several tissue and organ samples or, depending on the size of the fish, entire bodies were used. The most commonly used tissues (Lőrincz et al., 2011, 2012; Fehér et al., 2013; Doszpoly et al., 2014; Tarján et al., 2014) were liver, spleen, gills, kidneys, gonads, intestine, brain and blood. In each case, approximately 0.1 g or less of these were homogenized and clarified by low-speed centrifugation, using the supernatant for DNA extraction by commercially available nucleic acid extraction kits. Depending on the species and the age of the fish, some variety was observed in the presence of fish circovirus DNA in these samples, but no clear tissue or organ tropism could be measured, except in eel where a significantly higher virus load was detected in spleen and liver than in the rest of the tested organs.

Diagnostic options are practically limited to molecular detection of viral DNA; no pathological or histopathological lesions could so far be directly linked to fish circovirus infections. Virus isolations also failed. Doszpoly et al. (2014) used an eel kidney cell line for this purpose, but cytopathic effect (rounding and detachment of cells) was only observed after the first EeCV inoculation, further culturing of the virus through passages did not succeed. Propagation of BaCV in different established fish cell lines (Lőrincz et al., unpublished) also failed, after two-to-three passages viruses faded out from the cultures.

12.8 PREVENTION AND CONTROL

No information is currently available in terms of prevention and control regarding fish circoviruses. This is due to the lack of knowledge about the pathogenicity and spread of these viruses, and analogies with terrestrial circovirus infections could be misleading in the case of aquatic viruses.

TABLE 12.2 Primers Used for PCR Detection of Fish Circoviruses

Name of Primer	Sequence	Orientation	Species	Reference
CVS	5′-AGAGGTGGGTCTTCACNHTBAAYAA-3′	oF	Multiple, including fish	Halami et al. (2008)
CVaS	5′-AAGGCAGCCACCCRTARAARTCRTC-3′	oR		
CNS	5′-AGCAAGGAACCCCTCAYYTBCARGG-3′	iF		
CNaS	5′-ACGATGACTTCNGTCTTSMARTCACG-3′	iR		
MTL	5′-GTGATTACGCCAACGTGATG-3′	F	Barbel	Lőrincz et al. (2011)
MTR	5′-GCCCTTTGTGCAGTATTCGT-3′	R		
BaCVF	5′-AGGCGATAGACGTTGTGAAG-3′	F		
BaCVR	5′-CTTGTCCATCGTATCCATCC-3′	R		
HTF	5′-CAGACCATGCTTCCGGTACT-3′	F	European catfish	Lőrincz et al. (2012)
HTR	5′-GGGCTTCCTCGAAGGTTATC-3′	R		
	5′-GCG CTT GAG GAT TCT CAT TC-3′	oF	European eel	Doszpoly et al. (2014)
	5′-CAG ATC GTT CCT CTT CCC TT-3′	oR		
	5′-GAC TTT GGA TGG AAG AAG CC-3′	iF		
	5′-CCT TGT TAT GCT GGT CGT TG-3′	iR		
	5′-AGG CAA CGA CCA GCA TAA CA-3′	F[a]	European eel	Doszpoly et al. (2014)
	5′-AGT CGT CGA TGC AGG CCA AG-3′	R[a]		

F, Forward; iF, internal forward; oF, outer forward; R, reverse; iR, internal reverse; oR, outer reverse. Primers with the prefix "o" or "i" are for nested PCR.
[a]*Primers designed for quantitative PCR.*

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Part III

RNA Viruses of Fish

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Chapter 13

Retroviruses of Fish

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13.1 INTRODUCTION

The study of retroviruses has provided a wealth of information toward our understanding of cell growth and differentiation and regulation of gene expression. The first retroviruses to be studied were those that caused disease in chickens. Subsequently, several retroviruses known to induce proliferative diseases in animals, which include primates, mice, rats, cats, cows, sheep, goats, koalas and fish, have been discovered.

Proliferative skin lesions with suspected viral etiologies have been found in white sucker (*Castostomus commersoni*), walleye (*Sander vitreus*), yellow perch (*Perca flavescens*), hooknose (*Agonus cataphractus*) and European smelt (*Osmerus eperlanus*). Retroviruses have also been linked to the development of lymphoma in northern pike (*Esox lucius*), leukemia in Chinook salmon (*Oncorhynchus tshawytscha*) and leiomyosarcoma of the swim bladder in Atlantic salmon (*Salmo salar*). The etiologic relationship of retroviruses with these diseases is primarily based on the observation of type C retrovirus-like particles ranging in size from 80 to 150 nm in diameter, and the presence of reverse transcriptase activity in homogenates prepared from neoplastic tissues. Experimental transmission has been achieved with some of these diseases.

Retroviruses are enveloped viruses that acquire a lipid bilayer from the host cell during the budding process. Embedded in the lipid bilayer are the virus envelope surface (SU) and transmembrane (TM) glycoproteins. The virus envelope surrounds the capsid core, which comprises the structural proteins, capsid (CA), matrix (MA) and nucleocapsid (NC) encoded by the *gag* gene. The viral enzymes, reverse transcriptase (RT), integrase (IN) and protease (PR), encoded by the *pro-pol* genes, are packaged in the virion. The retrovirus genome is a dimer of single-stranded, positive-sense RNA. Also packaged in the virion is a cellular transfer RNA (tRNA), which serves as the primer for initiation of minus-strand DNA synthesis during reverse transcription. Entry of retroviruses into the host cell is triggered upon binding of the viral envelope glycoprotein to receptors on the host cell. The virion core is released into the cytoplasm of the cell after fusion of the viral envelope with cellular membranes. Reverse transcription of the viral RNA genome into double-stranded linear DNA occurs within the viral ribonucleoprotein core in the cytoplasm of the host cell. As DNA synthesis nears completion, the preintegration complex enters the nucleus where the viral complementary DNA integrates into the host cell chromosome. Once integrated, the host cell transcription machinery is responsible for synthesis of spliced and unspliced viral RNAs that are transported to the cytoplasm for translation of viral proteins and assembly into new progeny virions. The newly assembled virus buds from the host cell and, as the virus particle buds or after release from the cell, undergoes final processing, producing the mature virion.

The *Retroviridae* family comprises two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae*. The *Orthoretrovirinae* are further classified into six genera, one of which, the *Epsilonretrovirus*, includes the fish retroviruses, walleye dermal sarcoma virus (WDSV) and walleye hyperplasia viruses (WEHVs). To date, complete or partial viral sequences have been obtained for only five exogenous fish retroviruses and one endogenous retrovirus of zebrafish (zebrafish endogenous retrovirus (ZFERV)) from *Danio rerio* (Frerichs et al., 1991; Holzschu et al., 1995; LaPierre et al., 1999; Paul et al., 2006; Shen and Steiner, 2004). ZFERV and the exogenous retroviruses, snakehead retrovirus (SnRV) and salmon swim bladder sarcoma virus (SSSV) have not been assigned to a specific genus. The molecular mechanisms that direct the pathogenesis of retroviral infections in fish are not well understood. WDSV is a *complex retrovirus* and the type-specific member of the *Epsilonretrovirus* genus in the family *Retroviridae*. The different fish retroviruses and the diseases associated with them are summarized in Table 13.1.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00013-9
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TABLE 13.1 Diseases Associated With Retroviruses in Fish

Virus	Disease	Fish Host
Exogenous Retrovirus		
Walleye dermal sarcoma virus	Dermal sarcoma	*Sander vitreus*
Walleye epidermal hyperplasia virus	Discrete epidermal hyperplasia	*Sander vitreus*
Swim bladder sarcoma virus	Leiomyosarcoma of the swim bladder	*Salmo salar*
Perch epidermal hyperplasia virus	Epidermal hyperplasia	*Perca flavescens*
Snakehead retrovirus	No known disease	*Ophicephalus striatus*
Endogenous Retrovirus		
Zebrafish endogenous retrovirus	No known disease	*Danio rerio*
Suspect Retroviruses		
Salmon leukemia virus	Plasmacytoid leukemia	*Oncorhynchus tshawytscha*
Northern pike virus	Lymphosarcoma	*Esox lucius* *Esocid masquinongy*

13.2 DERMAL SARCOMA

Dermal sarcoma in walleye was first identified by Walker (1969) and has subsequently been found elsewhere in North America (Yamamoto et al., 1976). Walleye dermal sarcoma (WDS) is a seasonal disease and is observed on 20–30% of walleye in Oneida Lake in New York during the spring spawning run. WDS has been reported to occur in 5–10% of walleye in lakes in Canada (Walker, 1969; Yamamoto et al., 1976, 1985a). In late fall, cutaneous tumors become apparent and remain on the fish throughout the winter. During the following spring spawning period, the lesions regress (Bowser et al., 1988; Bowser and Wooster, 1991). Tumors are rarely observed during the summer.

Dermal sarcoma in walleye is a cutaneous mesenchymal neoplasm that arises within the dermal stratum spongiosum (Fig. 13.1) (Coffee et al., 2013; Martineau et al., 1990,b; Poulet et al., 1994; Walker, 1969; Yamamoto et al., 1985b). The tumors are single to multifocal randomly distributed nodules that can coalesce and range in size from 0.2 to 1 cm in diameter. Histologically, the tumors are well defined, nonencapsulated masses comprised of spindle cells organized in bundles that whorl around scales (Coffee et al., 2013; Martineau et al., 1990a,b; Poulet et al., 1994; Walker, 1969; Yamamoto et al., 1985b). Mitotic figures are rare. Regressing tumors are accompanied by mononuclear cell infiltration and epidermal ulceration. WDS is rarely invasive, although invasive tumors have been identified in adult walleye with naturally occurring disease and in some experimentally infected walleye fingerlings (Bowser et al., 1998, 2002a,b; Earnest-Koons et al., 1996). Invasive tumors observed during experimental transmission studies developed on the head of the fish near the operculum and ranged in size from 2 to 3.5 cm in diameter and invaded the underlying musculature and bone of the operculum.

Evidence for retroviral infection in WDS was first noted by electron microscopic examination of tumor tissue and observation of type C retrovirallike particles. Purification of viral particles from regressing tumors using sucrose density gradient centrifugation revealed particles with prominent spikes that were 90–100 nm in diameter (Martineau et al., 1990a,b, 1991a,b; Rovnak et al., 2007). The viral particles were found to sediment at 1.17–1.18 g/mL and exhibit Mn^{+2}-dependent RT activity (Martineau et al., 1990a,b, 1991a,b; Rovnak et al., 2007). A viral etiology for WDS was demonstrated by experimental transmission of WDS to walleye fingerling using cell-free tumor homogenates prepared from regressing tumors (Martineau et al., 1990a,b). Walleye fingerlings that were 12 weeks of age were inoculated intramuscularly, and 87% of fish developed tumors by 14 weeks after infection. The tumors were grossly and histologically identical to those observed on naturally affected fish. Disease is also efficiently transmitted by topical application and oral gavage of the tumor filtrates (Bowser et al., 1997). In contrast to the efficient transmission of WDS with virus prepared from regressing tumors, homogenates from developing tumors were unable to transmit disease (Bowser et al., 1996).

Infection of walleye with WDSV likely occurs through direct contact with infected fish or contact with virus in water during the spring spawning period (Bowser et al., 1999). Experimental transmission of disease by waterborne exposure was documented in a study in which tumor-free walleye were placed in a hatchery raceway that harbored tumor-positive fish.

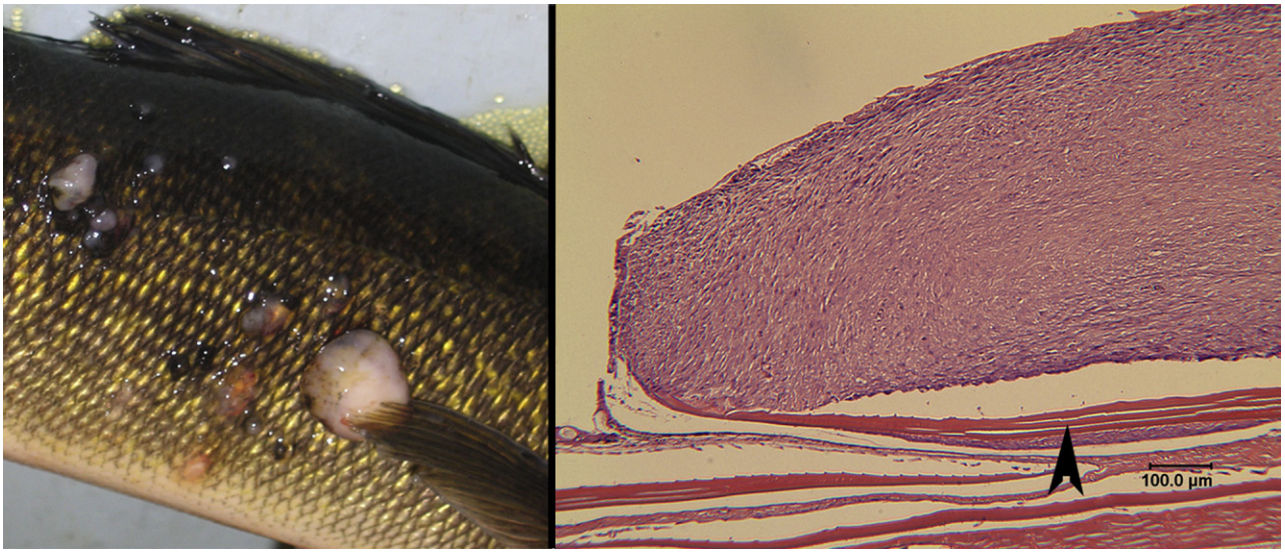

FIGURE 13.1 Walleye dermal sarcoma. (Left) Multifocal dermal nodules on the body of a walleye. (Right) Photomicrograph of walleye dermal sarcoma. Scales are indicated with an *arrow*.

A screen barrier to prevent direct contact was installed in a raceway to separate the two populations of fish. Up to 89% of the tumor-free walleyes developed WDS, suggesting that horizontal transmission of disease is likely to occur when fish are temporally housed in hatcheries. A study conducted from 1995 through 2003 at the Oneida Lake Fish Cultural Station provided evidence that tumor-positive walleye do not return with tumors during following spring spawning seasons, suggesting that fish develop resistance to disease and implicating a role for the immune response (Getchell et al., 2000, 2004). There is very limited information available on the role of the immune response in the pathogenesis of WDS and for other retroviral infections in fish, which highlights the need for additional research. These results and those from experimental transmission studies indicate that most walleye are likely to develop WDS during their lifetime and that there is a higher incidence in younger sexually mature adults (Bowser et al., 1997, 1999; Getchell et al., 2000, 2004).

The potential for an expanded host range of WDSV is suggested by the experimental transmission of dermal sarcoma to yellow perch and sauger (*Sander canadensis*), a closely related species that cohabits with walleye (Bowser et al., 2001; Holzschu et al., 1998). A report of dermal sarcoma in a wild-caught yellow perch subsequently held in a display tank has also been documented (Bowser et al., 2005).

The retrovirus WDSV was cloned from viral DNA isolated from a regressing tumor and found to be 12.7 kb in length (Holzschu et al., 1995; Martineau et al., 1991a,b, 1992). Sequence analysis showed that the virus was a complex retrovirus with three open reading frames, in addition to *gag*, *pro*, *pol* and *env*, which are designated *orf a (rv-cyclin)*, *orf b* and *orf c* (Fig. 13.2) (Holzschu et al., 1995). The *orf a* and *orf b* genes are located in the 3′ proximal region of the genome, and *orf c* is located between the 5′ LTR and *gag*. During reverse transcription, WDSV utilizes a histidyl-tRNA as a primer for minus-stranded DNA synthesis. Cloning and sequence analysis of WDSV allowed for the generation of reagents to investigate virus expression in tumors. A significant finding was the observed differences in viral gene expression in developing and regressing tumors. Developing tumors contain 1 copy of viral DNA per cell, whereas cells of regressing tumors have 10–50 copies of viral DNA, most of which are unintegrated (Bowser et al., 1996; Martineau et al., 1991a,b, 1992). Northern blot analysis revealed that developing tumors express only the *rv-cyclin and orf b* subgenomic transcripts, whereas regressing tumors express subgenomic transcripts, spliced *env* transcript and genomic RNA (Quackenbush et al., 1997). The paucity of genomic viral RNA and lack of infectious virus in developing tumors explains the inability to transmit disease (Bowser et al., 1996).

WDSV encodes three accessory proteins, Retroviral Cyclin (RV-cyclin), Orf B and Orf C (Daniels et al., 2008; LaPierre et al., 1998a; Nudson et al., 2003; Quackenbush et al., 2009; Rovnak et al., 2001, 2007; Rovnak and Quackenbush, 2010). Expression and localization of the three accessory proteins has been demonstrated in cells from naturally infected fish and in cell culture by expression of recombinant proteins (Daniels et al., 2008; Nudson et al., 2003; Rovnak et al., 2001, 2007; Rovnak and Quackenbush, 2010). The RV-cyclin and Orf B proteins are the only proteins expressed during tumor development, which leads to the hypothesis that these proteins contribute to oncogenesis. RV-cyclin expression in a variety of cells promotes cell growth and is able to rescue yeast that are deficient in G1 cyclins from growth arrest (Brewster et al., 2011;

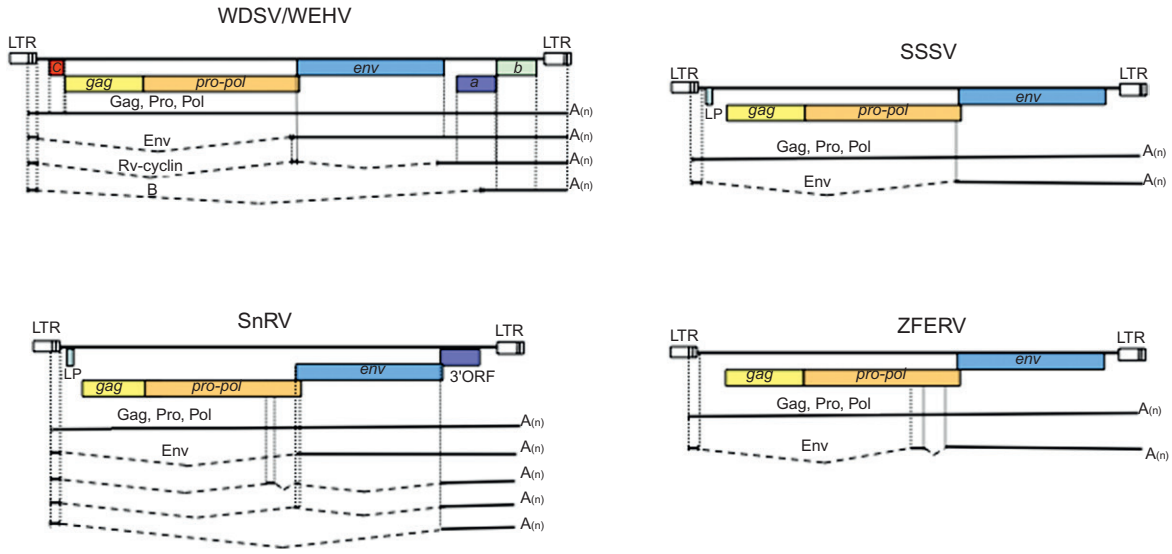

FIGURE 13.2 Genomic organization of fish retroviruses. The diagram depicts the structure of WDSV, WEHV, SnRV and ZFERV provirus, open-reading frames and the major viral transcripts. Vertical lines denote common termini of transcripts, exons and reading frames. The V-shaped dashed lines show positions of introns. *WDSV*, walleye dermal sarcoma virus; *WEHV*, walleye hyperplasia virus; *SnRV*, snakehead retrovirus; *ZFERV*, zebrafish endogenous retrovirus.

LaPierre et al., 1998a,b; Rovnak et al., 2007; Rovnak and Quackenbush, 2010). Development of hyperplastic skin lesions in transgenic mice that expressed RV-cyclin from a keratin promoter was observed after tail-clipping (Lairmore et al., 2000). In contrast, expression of RV-cyclin in transgenic zebrafish did not result in tumor formation, nor was there an increase in tumor formation after *N*-ethyl *N*-nitrosourea treatment or following amputation of the tail fin (Paul et al., 2010). These data suggest that RV-cyclin alone is not responsible for tumor development and that secondary events, which are not completely defined, are required. WDSV RV-cyclin is a nuclear protein that colocalizes and copurifies with RNA Polymerase II (RNA Pol II) and other components of host cell transcription machinery (Brewster et al., 2011; Rovnak et al., 2005; Rovnak and Quackenbush, 2002, 2006). The outcome of the physical association of RV-cyclin with host cell transcription machinery affects the regulation of virus and host gene expression in response to a variety of stimuli. RV-cyclin negatively regulates transcription from the WDSV promoter and NF-kB-dependent and interferon promoters (Hronek et al., 2004; Quackenbush et al., 2009; Rovnak et al., 2005; Rovnak and Quackenbush, 2002, 2006). RV-cyclin also enhances the expression of several host genes (Birkenheuer et al., 2015; Brewster et al., 2011). RV-cyclin interacts with host cell proteins through two domains: a transcription activation domain and a cyclin box motif (LaPierre et al., 1998a,b; Rovnak et al., 2005; Rovnak and Quackenbush, 2002). The transcription activation domain of RV-cyclin binds to TATA binding protein-associated factor 9 (TAF9) (Rovnak and Quackenbush, 2006). The cyclin box domain directly interacts with cyclin-dependent kinases 3 and 8 (CDK3 and CDK8), and a subdomain within the cyclin box interacts with the Aα subunit of protein phosphatase 2A (PP2A) (Brewster et al., 2011; Rovnak et al., 2012). Binding of RV-cyclin to CDK8 results in the displacement of cyclin C. CDK8 and cyclin C are components of a submodule of the Mediator complex, which is a coregulator of transcription. CDK8 is one of three cyclin-dependent kinases that regulate RNA Pol II transcription initiation, elongation and reinitiation by phosphorylating the carboxy terminal domain (CTD) of RNA Pol II. CDK8 phosphorylates other regulators of transcription, which results in alteration of the epigenetic structure of chromatin to enhance or repress RNA Pol II transcription initiation and elongation. CDK8 is an oncogene in colon cancer and melanoma and promotes efficient transcription elongation of hypoxia-induced and the serum-response genes (Donner et al., 2010; Firestein et al., 2008, 2010; Galbraith et al., 2013; Kapoor et al., 2010). The serum-response genes are also known as immediate early genes, and they include the known oncogenes *FOS*, *EGR1* and *JUN*. Binding of RV-cyclin to CDK8 results in increased CDK8 kinase activity that is directed toward the RNA Pol II CTD, resulting in increased expression of *FOS*, *EGR1* and *JUN* messenger RNA (mRNA) levels due to enhanced transcription elongation (Birkenheuer et al., 2015). Increased expression of these genes suggests a role for RV-cyclin during tumor development.

WDSV Orf B is expressed during tumor development and localizes to the plasma membrane in structures consistent with focal adhesions and in the cytoplasm with actin stress fibers (Rovnak et al., 2007). Orf B interacts with the scaffold protein Receptor for Activated C Kinase (RACK1) (Daniels et al., 2008). RACK1 was first identified

as a PKC binding protein that stabilizes the active conformation of PKC. Since then, additional binding partners of RACK1 have been identified supporting a role for RACK1 in a variety of biologic functions (Adams et al., 2011; Li and Xie, 2015). Expressed Orf B is found in a complex with membrane-associated, constitutively activated PKCα. The AKT signaling pathway is also activated in Orf B-expressing cells (Daniels and Quackenbush, unpublished). Activation of the PKC and AKT signaling pathways results in the ability of Orf B-expressing cells to proliferate in the absence of serum and to form colonies in a semisolid medium. These data implicate WDSV Orf B as a key protein in the transformation of cells.

An association of apoptosis with tumor regression was demonstrated by terminal deoxynucleotidyl transferase biotin-UTP nick end labeling (TUNEL) of sections from regressing tumors (Rovnak and Quackenbush, 2010). WDSV Orf C has been detected in regressing tumors by Western blot and immunofluorescence (Nudson et al., 2003; Rovnak et al., 2007; Rovnak and Quackenbush, 2010). Orf C is found to colocalize with cytochrome c in cells transiently transfected with an Orf C expression plasmid (Nudson et al., 2003). Orf C localization to mitochondria is associated with perinuclear clustering and loss of mitochondrial membrane potential, which leads to apoptosis.

Taken together, investigation of the WDSV accessory proteins suggests that RV-cyclin and Orf B work in concert to drive proliferation and transformation of WDSV-infected cells. RV-cyclin also constrains virus replication early after infection. At the time of spawning, there is a change in viral gene expression through mechanisms that are not clearly understood but which may include endocrine stimulation, immune responsiveness or exposure to ultraviolet light. The changes result in the production of an infectious virus and subsequent tumor regression. Orf C is likely produced at this time to promote apoptosis of tumor cells and release of infectious viruses. Gross and histopathology are routinely used to diagnose WDS. The presence of WDSV in tumors can be confirmed by polymerase chain reaction (PCR). Vaccines are not available. Little is known about the host response during the WDSV life cycle, but is likely to play an important role during tumor regression and protection from tumor development in subsequent years.

13.3 DISCRETE EPIDERMAL HYPERPLASIA

Epidermal hyperplasia in walleye and yellow perch was first identified by Walker in Oneida Lake in New York State in 1969 (Walker, 1969). Two forms of epidermal hyperplasia occur on walleye and are associated with viruses from the *Herpesviridae* and *Retroviridae* families (Kelly et al., 1983; LaPierre et al., 1998a,b; Yamamoto et al., 1985a). *Percid herpesvirus 1* has been isolated from walleye with diffuse epidermal hyperplasia, a skin lesion characterized by epidermal plaques with indistinct borders. Discrete epidermal hyperplasia in walleye is the result of a retrovirus infection. Discrete epidermal hyperplasia in yellow perch is also associated with a retrovirus (Coffee et al., 2013). Two distinct types of epidermal hyperplasia have also been observed on northern pike (*Escocid lucius*) and on muskellunge (*Esocid masquinongy*) (Graham et al., 2004; Yamamoto et al., 1983). The hyperplasia known as "blue spot disease" is associated with *Esocid herpesvirus-1* (Graham et al., 2004; Yamamoto et al., 1983). A retroviral etiology is suspected in the second type of hyperplasia, which is described as a smooth translucent epidermal hyperplasia with slightly elevated plaques (Coffee et al., 2013; Yamamoto et al., 1983). Type C retrovirallike particles have been observed by electron microscopy; however, attempts to propagate virus were not successful (Sonstegard, 1976; Wingvist et al., 1968; Yamamoto et al., 1983).

Walleye epidermal hyperplasia (WEH) is characterized as broad, translucent flat plaques with distinct margins (Fig. 13.3) (Coffee et al., 2013; Poulet et al., 1994; Yamamoto et al., 1985a). Lesions are found on the skin of the head, trunk and fins and can range in size from 2 to 3 mm up to 50 mm in diameter. Microscopically, the epithelium is greatly expanded in thickness and consists of proliferating Malpighian cells with ample mitotic figures. The basement membrane and dermis are not affected. Goblet cell hyperplasia is observed at the junction with normal dermis. Epidermal hyperplasia in yellow perch is grossly and microscopically identical to the disease in walleye. Similar to WDS, WEH exhibits a seasonal cycle, although the incidence of disease is less than that observed for WDS (Bowser et al., 1988; Getchell et al., 2004). Getchell et al. (2004) evaluated the prevalence of WEH on walleye in Oneida Lake, New York, and found that the incidence of WEH in walleye incrementally increases with the age of the fish such that 20% of fish 8+ years of age were affected. In contrast to what has been documented with WDS, walleyes with WEH in the spring do not appear to return without lesions the following year (Getchell et al., 2000, 2004).

Two independent retroviruses were identified in discrete epidermal hyperplasia lesions in walleye (LaPierre et al., 1998a,b, 1999). These viruses were cloned directly from purified virions or RNA isolated from hyperplasia lesions and have been designated as WEH virus type 1 (WEHV-1) and WEH virus type 2 (WEHV-2). Sequence analysis of WEHV-1 and WEHV-2 determined that the proviruses are 12,999 and 13,125 bp in length, respectively (Fig. 13.2) (LaPierre et al., 1999). The genome organization of WEHV-1 and WEHV-2 is similar to that of WDSV. In addition to *gag*, *pro*, *pol* and *env*, WEHV-1 and WEHV-2 also contain three accessory genes designated *orf a*, *orf b* and *orf c* in the same relative positions in

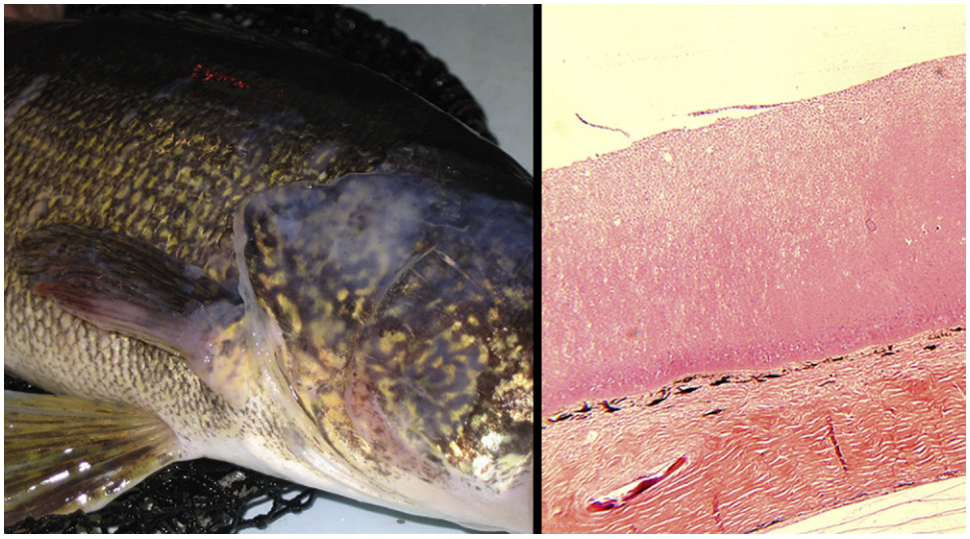

FIGURE 13.3 Walleye discrete epidermal hyperplasia. (Left) Epidermal plaques on the head and trunk. (Right) Photomicrograph of WEH.

the genome. The retroviral cyclin encoded by WEHV *orf a* has a cyclin box motif but does not include the TAF9-binding domain found in WDSV RV-cyclin (Rovnak and Quackenbush, 2010).

Hyperplasia lesions from walleye contain one to three copies of integrated WEHV-1 or WEHV-2 proviral DNA (LaPierre et al., 1998a,b). Northern blot analysis of lesions collected in the fall and spring indicate that there are temporal differences in viral gene expression (LaPierre et al., 1998a,b, 1999). Low levels of the viral *orf a* mRNA are detected in lesions collected in the fall. Lesions collected during the spring spawning season exhibit abundant levels of full-length genomic RNA and *env*, *orf a* and *orf b* spliced mRNAs. Information regarding the function of the WEHV accessory genes is limited.

Experimental transmission of WEH to walleye fingerlings has been documented (Bowser et al., 1998). A total of 97% of fish inoculated with cell-free material prepared from lesions collected in the spring developed WEH. These lesions contained WEHV DNA and were grossly and microscopically consistent with epidermal hyperplasia in naturally affected adult animals.

Two retroviruses have also been identified in hyperplasia lesions from yellow perch and are designated as perch epidermal hyperplasia virus type 1 (PEHV-1) and perch epidermal hyperplasia virus type 2 (PEHV-2) (Quackenbush, Bowser and Casey, unpublished; Quackenbush et al., 2001). Based on partial sequence information, the perch retroviruses are distinct from the walleye retroviruses, but the genome organization of the perch retroviruses is expected to be similar to those of WEHV-1, WEHV-2 and WDSV. As with WDSV, the walleye and perch hyperplasia viruses utilize a histidyl-tRNA as a primer for initiation of minus strand DNA synthesis. The WEHVs are members of the *Epsilonretrovirus* genus in the family *Retroviridae*. It is expected that the perch hyperplasia retroviruses will also be classified in the *Epsilonretrovirus* genus when the complete viral sequences are available.

13.4 SWIM BLADDER LEIOMYOSARCOMA

Leiomyosarcoma of the swim bladder in Atlantic salmon (*Salmo salar*) was first recognized in pen-reared salmon at a commercial fish farm in Scotland in 1976 (Duncan, 1978; McKnight, 1978). The disease was noted in 4.6% of the fish that were put in a sea cage the prior year. These fish were in poor physical condition and had multiple nodular masses on the surface of the swim bladder (McKnight, 1978). A second outbreak of disease occurred in 1996 in Atlantic salmon collected from the Pleasant River in Maine that were housed at a fish hatchery in Massachusetts and used as brood stock for the Atlantic salmon recovery program (Bowser et al., 2012; Paul et al., 2006). Mortality among the affected salmon from the Pleasant River peaked at 35% in the spring of 1998. Affected fish exhibited signs of lethargy, hemorrhages on the fins and body and swollen abdomens that contain firm nodular masses on the external and internal surfaces of the swim bladder. The swim bladder of affected salmon was expanded by the presence of these masses.

Histologically, the tumors are comprised of spindle cells organized into interlacing bundles divided by bands of collagen (Fig. 13.4) (Coffee et al., 2013; Duncan, 1978; McKnight, 1978). Neoplastic cells exhibit moderate anisocytosis, variable

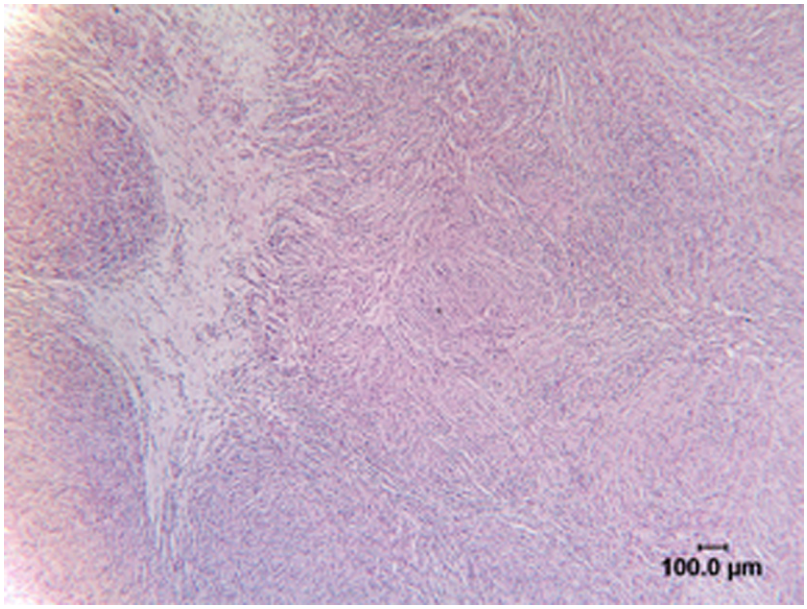

FIGURE 13.4 Photomicrograph of Atlantic salmon swim bladder leiomyosarcoma.

nuclear pleomorphism, frequent pyknotic nuclei and abundant mitotic figures. Tumor cells stain positive for desmin and weakly positive for smooth muscle actin, consistent with the diagnosis of leiomyosarcoma.

Electron microscopy was used to examine neoplastic tissue collected from Atlantic salmon during the first outbreak of disease in 1976, which revealed the presence of budding retroviruslike particles (Duncan, 1978). These observations led investigators to pursue a molecular approach to identify possible retrovirus sequences in swim bladder leiomyosarcomas collected from the second outbreak. Degenerate PCR primers that target conserved sequences in the RT genes of retroviruses were used to amplify a tumor-associated retroviral sequence (Fig. 13.2) (Bowser et al., 2012; Paul et al., 2006). The complete sequence of Atlantic salmon swim bladder sarcoma virus (SSSV) was obtained and found to be 10.9 kb in length. SSSV contains *gag, pro*, *pol* and *env* genes and utilizes a methionine-tRNA as a primer for replication. Full-length viral transcripts and singly spliced *env* transcripts are detected in tumors (Paul et al., 2006). The virus has not been propagated in cell culture.

SSSV is an exogenous retrovirus that does not contain viral accessory genes, which distinguishes it from the complex fish retroviruses WDSV and WEHV. Phylogenetic analysis places SSSV between the *Gammaretrovirus* and *Epsilonretrovirus* genera. Unlike mammalian and avian simple retroviruses, related endogenous copies of SSSV are not present in the Atlantic salmon genome (Paul et al., 2006). SSSV-associated tumors contain high proviral copy numbers (≥30 copies per cell) and a polyclonal integration pattern (Paul et al., 2006). The mechanisms that lead to the accumulation of proviruses in tumors are not known.

Gross and microscopic features are used to confirm the presence of swim bladder leiomyosarcoma. The presence of SSSV can be detected in blood of salmon by PCR (Bowser et al., 2002a,b). Salmon can be screened for infection with SSSV using viral-specific PCR primers. There are no vaccines available.

13.5 PLASMACYTOID LEUKEMIA

A previously unrecognized disease was reported in 1988 at net pen farms that rear Chinook salmon (*O. tshawytscha*) in British Columbia, Canada (Kent et al., 1997, 1990). The disease is known as *marine anemia* or *plasmacytoid leukemia (PL)* and is responsible for significant mortality of pen-reared fish. The disease has been reported to occur in wild salmon, in fresh water-reared salmon in California and Washington and farm-reared salmon in Chile (Eaton et al., 1994a; Kent et al., 1997). The mortality rate of salmon with PL ranges from 2.5% up to 50%.

PL in Chinook salmon is clinically recognized by pale gills, lethargy and by occasional bilateral exophthalmos. Fish exhibit splenomegaly, enlargement of renal tissues and serosanguious ascites. Petechial hemorrhages are observed in the liver, pancreas, heart and skeletal muscle. Microscopically, there is an infiltration of immature plasmablasts in most organs (eg, renal interstitium, spleen, lamina propria of the intestine, pancreas, liver, pericardium, retrobulbar tissue). The

plasmablasts are slightly ovoid and 10–20 µm in diameter with basophilic cytoplasm and abundant mitotic cells (Coffee et al., 2013; Eaton et al., 1994a).

PL is often detected in Chinook salmon after epizootics of bacterial kidney disease, a common bacterial infection caused by *Renibacterium salmoninarum*. A microsporidian parasite, *Enterocytozoon salmonis*, is also frequently found in salmon with PL. An epidemiological study of marine anemia determined that disease is widely distributed in salmon farming regions and was more commonly diagnosed in late summer. This peak in marine anemia also occurs during the peak of other infectious diseases (Stephen et al., 1996). The source of the PL infectious agent is not clear. Disease often occurs in fish that have recently been moved to seawater pens suggesting that either the fish have been previously infected when they arrive or are infected soon after arrival to the seawater pens.

Two infectious agents have been implicated in the development of PL: salmon leukemia virus (SLV) and the microsporidium parasite *Enterocytozoon salmonis*, although *Enterocytozoon salmonis* is more likely a cofactor in PL (Kent et al., 1997). Electron microscopic examination of tissues collected from PL-positive chinook salmon identified enveloped virus particles that were 110 nm in diameter (Eaton et al., 1994a; Eaton and Kent, 1992). Sucrose density centrifugation was used to purify virus particles from tissue homogenates prepared from kidney and spleen. Fractions with a buoyant density of 1.16–1.18 g/mL were pooled and found to contain RT activity that was Mn^{+2}-dependent and displayed a temperature optimum of 15–20°C. Polyacrylamide gel electrophoresis of virus purified from tumor tissue revealed the presence of several polypeptides some of which are recognized by antisera from PL-positive fish (Eaton et al., 1994a,b). To date, no sequence of this virus is available.

Attempts to propagate SLV in fish cell lines have not been successful. However, two cell lines established from kidney and eye tissues from PL fish were found to harbor the virus (Eaton et al., 1993). After 8 weeks in culture, the cells developed vacuoles and syncytia. This same cytopathic effect (CPE) develops 3–4 weeks after subsequent passage. Electron microscopic examination of cells exhibiting CPE revealed the presence of enveloped virus particles with a diameter of 110 nm. RT activity was detected in the culture supernatants, indicating the presence of extracellular virus, and fractionation of virus particles from these cells on sucrose gradients also identified a peak of Mn^{+2}-dependent RT activity at 1.16–1.18 g/mL buoyant density and contained polypeptides with molecular masses similar to those found in virus particles from tumors.

The amount of RT activity detected in PL tissues is substantially lower than that found in WDS samples (Kent et al., 1997), suggesting large differences in viral expression in these two diseases. Based on the virus particle morphology and the presence of RT activity from tissue of naturally and experimentally infected salmon and cell culture, SLV is likely a retrovirus. However, until viral sequences are available, the virus cannot be classified within the *Retroviridae*.

Experimental transmission of PL readily occurs when Chinook salmon are injected with tissue homogenates and 0.22-µm filtrates prepared from kidney and spleen collected from PL-positive fish (Kent and Dawe, 1990, 1993). Transmission of PL to sockeye salmon and Atlantic salmon has also been achieved (Kent and Dawe, 1990). To address a role for *Enterocytozoon salmonis* as an etiologic agent in PL, experimental transmission studies were conducted with 0.22-µm filtered inocula (Kent and Dawe, 1993). These fish were also treated with Fumagillin DCH, an antiprotozoal compound used to treat *Enterocytozoon salmonis*. Treatment with Fumagillin did not have any effect on the development of PL in the experimentally treated fish. Salmon infected with *R. salmoninarum* alone did not have histologic evidence consistent with PL. These studies provide strong evidence for a viral etiology of PL in Chinook salmon. A role for other agents, such as *Enterocytozoon salmonis*, as cofactors for development of PL should be considered, because most of the severe field cases of PL are associated with *Enterocytozoon salmonis* infection.

Diagnosis of PL has relied primarily on histologic examination. However, histology has not been adequate for diagnosis of PL in fish with concurrent *R. salmoninarum* (Newbound et al., 1995). Therefore, an indirect immunofluorescent antibody diagnostic assay (Saksida et al., 1999) for PL in affected fish was developed (Newbound et al., 1993, 1995). This assay utilizes monoclonal antibodies that react with tissue from PL-positive animals. These monoclonal antibodies recognize a 75-kDa protein in tumor tissue but do not react with protein preparations from *R. salmoninarum* or from sucrose gradient-purified SLV proteins (Newbound et al., 1995). The sensitivity of the indirect fluorescent antibody diagnostic was significantly higher than that of histology in detecting PL in experimentally infected fish; however, it was found to be unreliable when tested in the field (Newbound et al., 1995; Saksida et al., 1999).

13.6 LYMPHOSARCOMA

The first reports of lymphosarcoma on northern pike and muskellunge date to the turn of the 20th century. It is a hematopoietic neoplasm that has been observed on adult fish and is suspected of having a retroviral etiology. Lymphosarcoma of northern pike and muskellunge is also a seasonal disease; there is a low incidence of disease during the summer months, followed by an increase in prevalence in the fall. It is thought that disease is horizontally transmitted by contact during the spring spawning season. During epizootics, the frequency of disease has been reported to be as high as 20%.

FIGURE 13.5 Lymphosarcoma on the body of a northern pike.

These neoplasms are primarily cutaneous with soft, irregular masses that can reach up to several centimeters in diameter. These lesions can become ulcerated and can infiltrate adjacent skeletal muscle (Fig. 13.5) (Coffee et al., 2013). Histologically, the dermis and epidermis are infiltrated by pleomorphic round cells that are organized into sheets in the dermis and replace the epidermal architecture. The neoplastic cells have varying amounts of eosinophilic cytoplasm with nuclei that may be 10–12 μm in diameter. Anisocytosis and anisokaryosis along with a moderate number of mitotic figures may be present. Lymphoid infiltration is known to occur in other tissues (eg, kidney, spleen and liver) (Sonstegard, 1976, 1977). Immunohistochemistry and ultrastructural studies indicate that the tumors are T lymphocytic or histiomonocytic in origin.

Similar to that described for the walleye and salmon retroviruses, a suspect retrovirus has been implicated due to the observation of C-type retroviral particles in tumors by electron microscopy and the presence of RT activity (Papas et al., 1976, 1977; Sonstegard and Chen, 1986; Wingvist et al., 1968). When tumor homogenates were fractionated on sucrose density gradients, those fractions with a density of 1.15–1.17 g/mL exhibited RT activity with a temperature optimum of 20°C. Attempts to propagate the virus associated with lymphosarcoma have not been successful. DNA isolated from tumors obtained from feral northern pike will transform NIH3T3 cells (Van Beneden et al., 1990).

Experimental transmission of lymphosarcoma to northern pike and muskellunge has been achieved by transplantation of tumor tissue and by inoculation with tumor cells and with cell-free filtrates (Brown et al., 1976; Ljungberg, 1976; Mulcahy and O'Leary, 1970; Sonstegard, 1976). Previously, this disease was observed only in adult fish; however, in 2002, lymphosarcoma was reported in hatchery-reared yearling tiger muskellunge (*Escocid lucius*× *Esocid masquinongy*) (Bowser et al., 2002a,b). The fish were obtained from a different hatchery and did not have evidence of lesions prior to their arrival. These fish were found to have tumors that were consistent with those observed in adult fish. The original source of the infection was not identified. To investigate the infectious nature of these lesions, young-of-the-year tiger muskellunge obtained from a different hatchery were inoculated with cell-free filtrate prepared from the yearling fish tumors. By 32 weeks after infection, 4 of the 20 inoculated fish developed lymphosarcoma that was consistent with that described previously in adult fish (Bowser et al., 2002a,b). The discovery of this disease in young fish reared in a fish hatchery raises concerns and highlights the need for fish health management practices that include monitoring of broodstock and importation of new fish to limit transmission.

13.7 SNAKEHEAD RETROVIRUS

The exogenous SnRV was identified during characterization of cell lines established from three Southeast Asian fish: striped snakehead (*Ophicephalus striatus*), climbing perch (*Anabas testudineus*) and snakeskin gourami (*Trichogaster pectoralis*) (Frerichs et al., 1991). Electron microscopy of the cell lines revealed the presence of type C virus particles. The culture supernatants contained high levels of RT activity and induced cytolytic infection of the blue gill fry BF-2 cell line (*Lepomis machrochirus*). SnRV was cloned by RT-PCR from virions purified from the SSN-1 cell line (Hart et al., 1996). The viral genome is 10.2 kb in length and includes an open reading frame between *env* and the 3′LTR (Fig. 13.2). An arginine tRNA primer binding site distinguishes SnRV from other retroviruses. Endogenous copies of SnRV were not identified in the snakehead fish genome. The presence of SnRV in wild populations of fish has not been extensively investigated. The fish were healthy at the time the cultures were established and there is no known association of SnRV with disease.

13.8 ZEBRAFISH ENDOGENOUS RETROVIRUS

Endogenous retroviruses have been identified in a wide range of vertebrates. These retroviruses are integrated into germ line cells and in most cases have acquired an accumulation of mutations and genetic deletions over time to become transcriptionally inactive. However, some endogenous retroviruses are expressed and can produce infectious virus and reinfect the host germ line resulting in multiple copies of the endogenous retroviral genome in the germ line. ZFERV was identified as a transcriptionally active virus during a screen for genes expressed in the thymus of zebrafish (Shen and Steiner, 2004). The virus was cloned and sequenced and found to be 11.2 kb in length with genes encoding *gag*, *pol* and *env* (Fig. 13.2). Expression is highest in larval and adult thymocytes. A recent study identified amplification of ZFERV in zebrafish T-cell malignancies (Frazer et al., 2012). However, this was not unique to malignant T cells; increased copy numbers of ZERV were also observed in normal T cells. At this time, the role of ZFERV in T-cell oncogenesis is unknown and will require further investigation to elucidate its overall function.

ACKNOWLEDGMENTS

The authors thank Paul R. Bowser and Rodman G. Getchell, Cornell University, for providing gross and histopathology photographs. Research in the Quackenbush laboratory was supported in part by a National Institutes of Health grant RO1CA95056 and the American Cancer Society grant RPG-00313-01-MBC.

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Chapter 14

Reoviruses of Aquatic Organisms

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14.1 INTRODUCTION

The name of the family *Reoviridae* is derived from *reo*: sigla from "*r*espiratory *e*nteric *o*rphan," due to the early recognition that the viruses are isolated from respiratory and gastrointestinal tracts, but were not associated with any known disease state; thus they were considered "orphan" viruses (ie, infect respiratory and gastrointestinal tracts, usually without disease symptoms) (Sabin, 1959). In this sense, the family is well-known for containing mammalian reoviruses, genus *Orthoreovirus*, that can be recovered from gastrointestinal and upper respiratory tracts of healthy humans and animals and whose etiological role is difficult to evaluate. However, the family *Reoviridae* currently contains 15 recognized genera (Attoui et al., 2012), making it one of the most complex in all virology, and the ability to cause disease is variable in the different genera in this family, and in some cases, it depends on the virus strain and host species. This family is the largest and the best studied of all the double-stranded RNA (dsRNA) virus families (Mertens, 2004), and holds a unique position in cell biology: the 5′-terminal cap structure of mRNA was discovered in 1974 on reovirus mRNAs (Miura et al., 1974; Banerjee, 1980). Moreover, interest in reoviruses has resurged in the past decade because of their oncolytic properties (Strong et al., 1998) and potential use in cancer therapy (Gollamudi et al., 2010). This family contains four distinct groups of aquatic reoviruses, two of which infect fish: those in the well-established genus *Aquareovirus* with 11 genome segments (Winton et al., 1987; Lupiani et al., 1995; Attoui et al., 2012), and isolates of the newly discovered piscine orthoreovirus (PRV) with 10 genome segments (Palacios et al., 2010; Kibenge et al., 2013) in the genus *Orthoreovirus* (Rimstad and Wessel, 2015); and two groups of isolates from diseased crabs that make up the genera *Cardoreovirus* (Zhang et al., 2004) and *Crabreovirus* (Weng et al., 2007; Deng et al., 2012) with 12 segments. Additional crustacean reoviruses, such as *Eriocheir sinensis* reovirus (*Es*RV816) with 10 segments (Zhang and Bonami, 2012), *Macrobrachium nipponense* reovirus (*Mn*RV) with 12 segments (Zhang et al., 2015) and *Scylla serrata* reovirus (*Ss*RV) with 13 segments (Chen et al., 2011) have not yet been assigned to a genus.

14.1.1 Reovirus Classification and Virion Properties

The current classification of the *Reoviridae* family is detailed in the Ninth Report of the International Committee on Taxonomy of Viruses (ICTV) (Attoui et al., 2012). The *Reoviridae* family has 15 recognized genera divided into two subfamilies: subfamily *Spinareovirinae* (with virions having 12 "spikes" (turrets) protruding from the core through the outer capsid, hence "turreted" viruses) and subfamily *Sedoreovirinae* (with virions having a smooth, unspiked appearance and three capsid shells, hence "nonturreted" viruses) (Attoui et al., 2012). In addition to this structural classification, amino acid identities of >30% in the RNA-dependent RNA polymerase (RdRp) sequence and the presence and sequence of conserved terminal nucleotide motifs have been used as criteria for the classification of genera within this family (Auguste et al., 2015). To date, the 15 genera in family *Reoviridae* include seven genera containing animal viruses (*Orthoreovirus*, *Aquareovirus*, *Coltivirus*, *Orbivirus*, *Rotavirus*, *Seadornavirus*, *Cardoreovirus*), three genera containing plant viruses (transmitted by arthropods: *Oryzavirus*, *Fijivirus*, *Phytoreovirus*), three genera containing insect viruses (*Cypovirus*, *Dinovernavirus*, *Idnoreovirus*), one genus of fungus virus (*Mycoreovirus*) and one genus of marine protist (*Mimoreovirus*). The RdRp gene has been used to elucidate the evolutionary relationships among the viruses in the genera of the family *Reoviridae* because the polymerase protein is the most conserved of the viral protein products (Attoui et al., 2000, 2002a). Phylogenetic relationships within the family based on the RdRp gene are illustrated in Fig. 14.1.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00014-0
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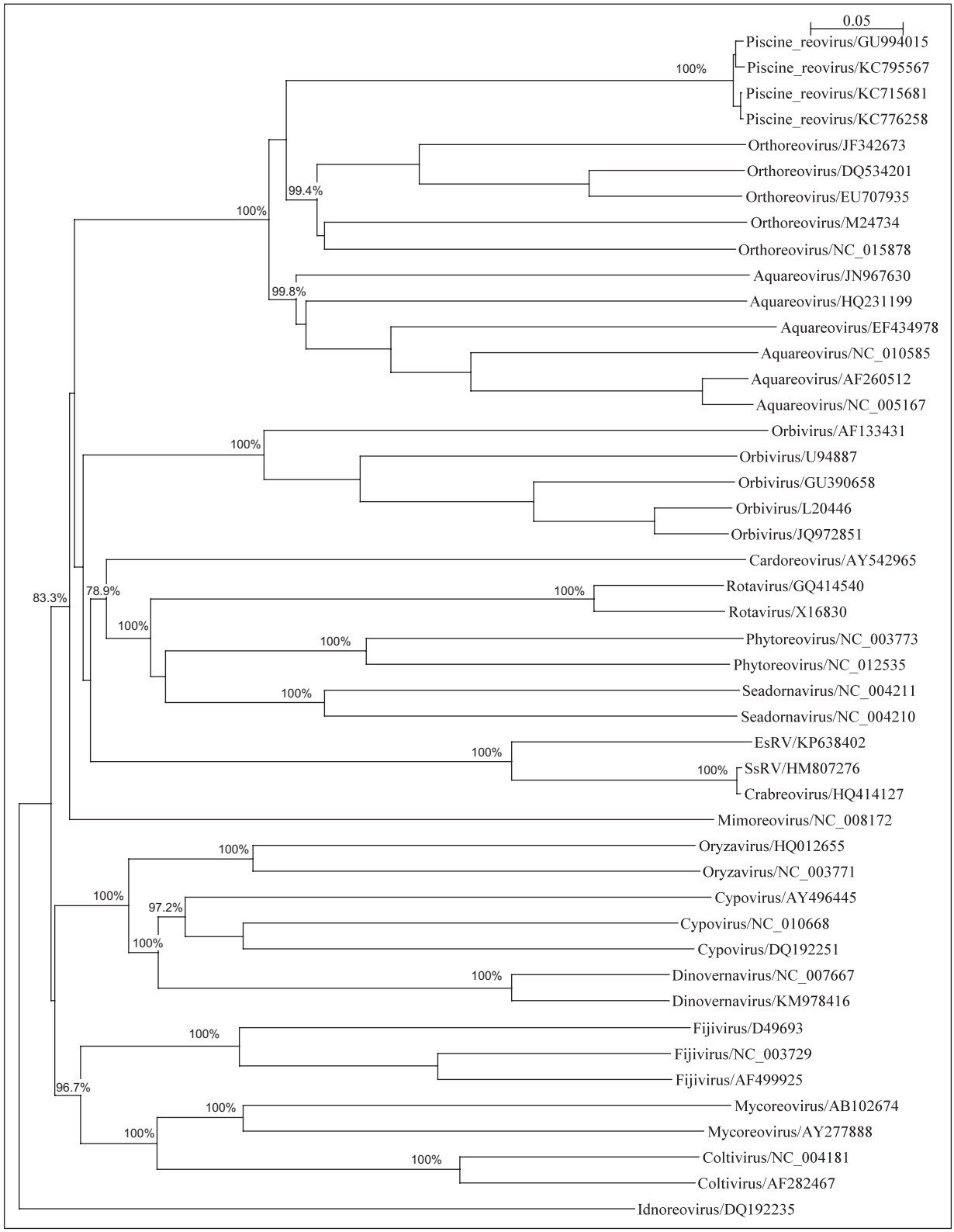

FIGURE 14.1 Phylogenetic tree showing the relationships within family *Reoviridae*. Nucleotide sequences of RdRp gene of selected members in the different genera of family *Reoviridae* were used to generate a phylogenetic tree. The sequences of member viruses used for comparison are identified by the genus name/GenBank accession number (Piscine orthoreovirus/GU994015, KC795567, KC715681, KC776258; *Orthoreovirus*/JF342673, DQ534201, EU707935, M24734, NC_015878; *Aquareovirus*/JN967630, HQ231199, EF434978, NC_010585, AF260512, NC_005167; *Mimoreovirus*/NC_008172; *Orbivirus*/AF133431, U94887, JQ972851, L20446, GU390658; *Cardoreovirus*/AY542965; *Phytoreovirus*/NC_003773, NC_012535; *Seadornavirus*/NC_004211, NC_004210; *Fijivirus*/D49693, NC_003729, AF499925; *Rotavirus*/GQ414540, X16830; *Idnoreovirus*/DQ192235; *Oryzavirus*/HQ012655, NC_003771; *Cypovirus*/AY496445, NC_010668, DQ192251; *Dinovernavirus*/NC_007667, KM978416; *Mycoreovirus*/AB102674, AY277888; *Coltivirus*/NC_004181, AF282476. *Ss*RV/HM807276 (Chen et al., 2011) is not yet assigned to a genus, and there is a new proposed genus *Crabreovirus*/HQ414127 (Shen et al., 2015)). Sequences were processed using ClustalX 2.0 (Larkin et al., 2007). The multiple sequence alignment was manually verified and adjusted to reach high-quality alignment. The phylogenetic tree was constructed by maximum likelihood using the neighbor-joining method and Tamura-Nei genetic distances (Saitou and Nei, 1987). Bootstrapping was performed for 1000 times. Bootstrap supports of topology are shown below the nodes, only for nodes between genera. The scale bar represents the number of substitutions per site. *Courtesy of Dr. Yingwei Wang, School of Mathematical and Computational Sciences, University of Prince Edward Island, Canada.*

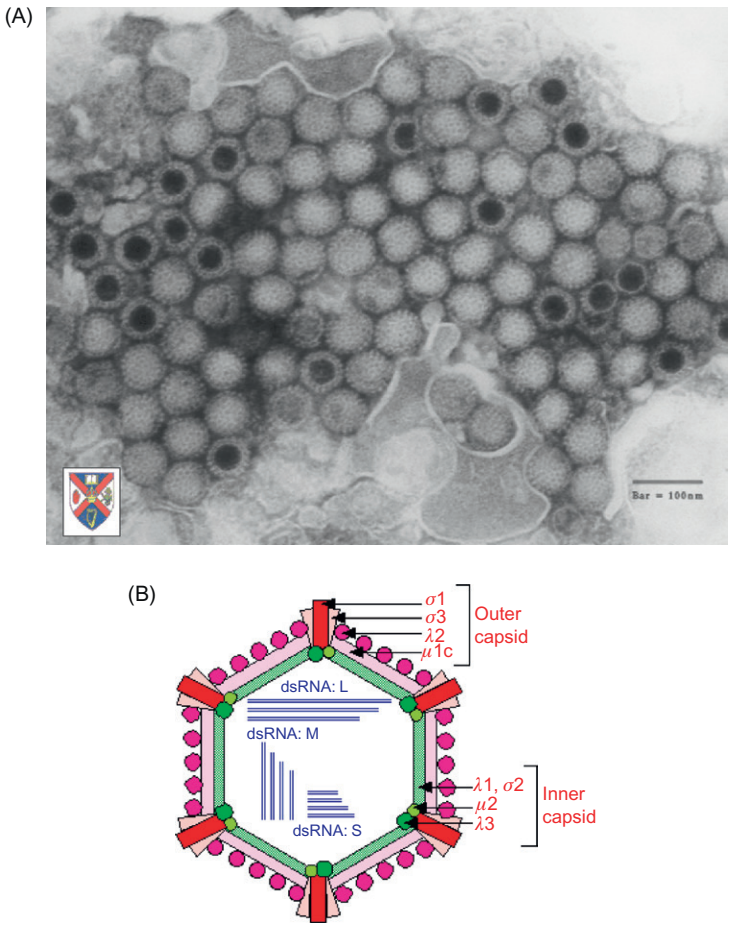

FIGURE 14.2 **Panel (A)** Negatively stained electron micrograph of reovirus particles. **Panel (B)** Schematic representation of the *Orthoreovirus* structure. **Panel (A)** *From* http://www.microbiologybytes.com/virology/3035pics/Reo.gif *Copyright 1 MicrobiologyBytes (2007), with permission* http://creativecommons.org/licenses/by-sa/3.0/ *no changes were made;* **Panel (B)** *From* http://www.microbiologybytes.com/virology/Reoviruses.html *Copyright 1 MicrobiologyBytes (2007), with permission* http://creativecommons.org/licenses/by-sa/3.0/ *no changes were made.*

The reovirus particles are nearly spherical icosahedral, 60–85 nm in diameter, nonenveloped, with one (genera *Cypovirus* and *Dinovernavirus* only), two or three capsid shells (Fig. 14.2) identified as the "subcore," "core" and "outer capsid," respectively; the outer capsid differs in appearance in the two subfamilies (Attoui et al., 2012). The inner capsid layers and proteins are primarily involved in virus assembly and replication, whereas the outer capsid proteins are involved in virus transmission, cell attachment and penetration and show much greater variation reflecting differences in the targeted host species (Mertens, 2004). The viral genome is segmented dsRNA, 19–32 kbp (total) (see Chapter 2: Classification and Identification of Aquatic Animal Viruses, Table 2.3 in this book). The number of segments (9, 10, 11 or 12, copackaged in equimolar proportions, representing one copy of each genome segment per virion) varies with the genus (Table 14.1; Auguste et al., 2015), although the genus *Mycoreovirus* currently contains viruses with both 11 and 12 genome segments (Attoui et al., 2012), and new reovirus isolates from the mud crab *S. serrata* in southern China, which are not yet assigned to a genus, have 13 genome segments (Chen et al., 2011).

The virus particles are very stable, as demonstrated by remaining infectious over long periods of time at room temperature (eg, the *Orbivirus* bluetongue virus has been isolated from blood held at room temp for 25 years). The genera *Orthoreovirus*, *Rotavirus* and *Aquareovirus* are resistant to lipid solvents and a wide pH range, whereas the genera *Orbivirus* and *Coltivirus* have a narrow zone of pH stability (pH 6–8) and lose some infectivity on exposure to ether.

14.1.2 Reovirus Genome Organization

The genome segments of the members of *Reoviridae* are in three size classes: Large (L), Medium (M) and Small (S) (Fig. 14.2B). The gene-coding assignments vary in the different genera (Table 14.1). The positive strands of the dsRNA

TABLE 14.1 Classification and Biological Features of Animal Reoviruses

Subfamily	Genus	Virus[a]	Number of Genome Segments (dsRNA)	Genome Segment Encoding RdRp (see also Fig. 14.2)	Genome Segment Encoding Outer Capsid/Coat Proteins	Host Range	Disease
Spinareovirinae	*Orthoreovirus*	MRV	10	L1 encodes $\lambda 3$	S1 encodes $\sigma 1$	Mammals, birds and reptiles	Fusogenic orthoreoviruses cause significant and often fatal disease; nonfusogenic porcine orthoreovirus (reassortant MRV3) is highly pathogenic to neonatal pigs
					L2 encodes λB or $\lambda 2$		
		ARV			M2 encodes $\mu 1C$		
		NBV			S4 encodes σC or $\sigma 3$		
		BRV					
		RRV					
		PRV[b]	10	L3 encodes xore RdRp	M2 encodes outer shell	Farmed and wild fish species	Associated with HSMI in Atlantic salmon; melanised spots in white skeletal muscle of Atlantic salmon; HSMI-like disease in coho salmon.
					S1 encodes outer clamp		
	Aquareovirus	Aquareovirus A–G	11	Seg2 or L2 encodes VP2	Seg5 encodes VP5	Fish, crustacea	GCRV is highly pathogenic in grass carp
					Seg6 encodes VP4		
		HGDRV (GCRV104)			Seg10 encodes VP7		
		GCRV-GD108					
	Coltivirus	CTFV	12	Seg1 encodes VP1		Mammals, ticks and mosquitoes	Clinical disease in humans and rodents
		Eyach virus					
Sedoreovirinae	*Orbivirus*	BTV	10	Seg1 encodes VP1	Seg2 encodes VP2	Ruminants, arthropods	Clinical disease in respective domestic animals
		AHSV			Seg6 encodes VP5	Equine species, arthropods	
		EEV			Seg7 encodes VP7		
		EHDV					
		Ibaraki virus					
		Palyam virus					
	Rotavirus	Rotavirus A–G	11	Seg1 encodes VP1	Seg4 encodes VP4	Mammals and birds	Disease in young animals
					Seg9 encodes VP7		

	Seadornavirus	Kadipiro virus	12	Seg1 encodes VP1	Seg4 encodes VP4 Seg9 encodes VP9 Seg10 encodes VP10	Humans, mice and mosquitoes	CNS disease
		Banna virus					
		Liao ning virus					
	Cardoreovirus	*Eriocheir sinensis* reovirus 905 (*Es*RV905)	12	Seg1 encodes VP1	Unknown[c]	Chinese mitten crab *E. sinensis*	Caused 30% mortality in experimental infection
	Crabreovirus[d]	Mud crab reovirus (MCRV)	12	Seg1 encodes VP1	Seg6 encodes VP6 (requires further study)	Mud crab *Scylla serrata*	Sleeping disease (SD)
		S. serrata reovirus[e]				Chinese mitten crab *E. sinensis*	Waterclear disease (CD)
		E. sinensis reovirus WX-2012 (*Es*RV)[f]				Blue crab *Callinectes sapidus*	Trembling disease (TD)
						Shore crab *Carcinus mediterraneus*	Disease with high mortality in wild and captive blue crab populations
		C. sapidus reovirus 1 (*Cs*RV1)				Swimming crab *Macropipus depurator*	Virus is pathogenic
		C. mediterraneus W2 virus (*Cc*RV-W2)					Virus is pathogenic
		M. depurator P virus (*Md*RV-P)					
Unassigned	Unassigned	*E. sinensis* reovirus 816 (*Es*RV816)[g]	10	Seg1 encodes VP1	Unknown[c]	Chinese mitten crab *E. sinensis*	Caused 30% mortality in experimental infection
		Macrobrachium nipponense reovirus[h]	10	Seg1 encodes VP1	Unknown	freshwater prawn *M. nipponense*	Disease in hepatopancreas epithelial cells with 15–60% mortality

[a]*Virus abbreviations: MRV, mammalian orthoreovirus; ARV, avian orthoreovirus; NBV, Nelson Bay orthoreovirus; BRV, baboon orthoreovirus; RRV, reptilian orthoreovirus; PRV, piscine orthoreovirus; HGDRV, Hubei grass carp disease reovirus (Fan et al., 2013); GCRV, grass carp reovirus; CTFV, Colorado tick fever virus; BTV, blue tongue virus; AHSV, African horse sickness virus; EEV, equine encephalosis virus; EHDV, epizootic hemorrhagic disease virus of deer.*
[b]*Protein nomenclature for PRV is as used by Key et al. (2013). HSMI, heart and skeletal muscle inflammation.*
[c]*Unknown because only genome segment 1 of E. sinensis reoviruses (EsRV905 and EsRV816) have been sequenced to date.*
[d]*Proposed in 2012 (Deng et al., 2012).*
[e]*Proposed in 2011 (Chen et al., 2011).*
[f]*Proposed in 2015 (Shen et al., 2015).*
[g]*Proposed in 2012 (Zhang and Bonami, 2012).*
[h]*Proposed in 2015 (Zhang et al., 2015).*

have a 5′-terminal dimethylated type 1 Cap structure (^{m7}GpppGm$^{2'OH}$), which was first discovered in the cypoviruses (Miura et al., 1974; Banerjee, 1980). Most of the genome segments are monocistronic, with a single long open reading frame (ORF) and relatively short noncoding regions (Mertens, 2004). Some segments have second functional, in-frame initiation codons or additional protein-coding ORFs (Attoui et al., 2012). For example, for animal reoviruses, in the genus *Orthoreovirus*, S1 segment is a bi- (*Mammalian orthoreovirus* (MRV), *Baboon orthoreovirus* (BRV) and *Reptilian orthoreovirus* (RRV)) or tricistronic (*Avian orthoreovirus* (ARV) and *Nelson Bay virus* (NBV)) gene segment, encoding structurally and functionally heterogeneous proteins in the different virus species (Clarke and Tyler, 2008). In addition, there are differences in the final protein coding potential due to differences in posttranslational cleavage strategies and the use of alternative sites for translation initiation (Benavente and Martínez-Costas, 2007; Coombs, 2008). In PRV, the L2 segment is putatively polycistronic, encoding λ2 protein and at least a small protein p11 (Markussen et al., 2013); S1 segment is bicistronic, encoding outer clamp protein σ3 and a nonstructural p13 protein (Palacios et al., 2010; Kibenge et al., 2013); and S2 segment and is possibly bicistronic, encoding core clamp protein σ2 and a hypothetical p8 protein (Markussen et al., 2013). In genus *Aquareovirus*, Seg11 is bicistronic in *Aquareovirus A* and Hubei grass carp disease reovirus (HGDRV; Fan et al., 2013), whereas in *Aquareovirus C* and *Aquareovirus G*, the bicistronic segment is Seg7. In genus *Coltivirus*, genome segments 1–8 and 10–11 are monocistronic, whereas Seg9 contains a leaky-stop codon and produces two proteins. In genus *Orbivirus*, all genome segments are monocistronic except Seg9 and Seg10, where mRNAs are translated from either of two in-frame AUG codons. In genus *Rotavirus*, Seg9 and Seg11 also have alternate start codons (in-frame for Seg9 and out-of-frame for Seg11) used to initiate protein synthesis. In genus *Seadornavirus*, all 12 genome segments are monocistronic (Attoui et al., 2012).

All reovirus genome segments sequenced thus far have conserved 5′- and 3′-terminal nucleotide sequences of variable length, which are useful for reovirus classification (Chen et al., 2011; Thimmasandra Narayanappa et al., 2015; Flowers et al., 2016). An inverted repeat is positioned next to the terminal conserved sequences. These inverted repeats are unique in each genome segments. The conserved terminal sequences and the inverted repeats play important roles in replication and RNA packaging. The genome segments and functions of the reovirus proteins for genus *Aquareovirus* and for PRV are summarized in Tables 14.3 and 14.5, respectively. The conserved 5′- and 3′-terminal nucleotide sequences of aquatic reoviruses (PRV, *Aquareovirus*, *Cardoreovirus*, *Crabreovirus*, *Ss*RV, *Cs*RV1) and *Orthoreovirus* genome segments are compared in Table 14.4.

14.1.3 Reovirus Replication

The reovirus replication cycle is depicted in Fig. 14.3. Reoviruses replicate in the cytoplasm. Similar to virus families with genomes of negative sense, single-stranded RNA, reoviruses carry their own enzymes for replication because the host cell machinery lacks enzymes that can make mRNA off these genomes. In addition, many host cells contain antiviral defense mechanisms (including induction of apoptosis, interferon production, modification of host cell translation mechanisms, RNA silencing) that specifically recognize and would be activated by naked dsRNA within the host cell cytoplasm (reviewed by Mertens, 2004). Consequently, once in the cytoplasm, the reovirus virion is only uncoated partially by lysosomal hydrolases, retaining its genome within a subcore (or core) particle, such that the transcription of the dsRNA genome by viral polymerase occurs inside the subviral particle. The partial uncoating activates the viral transcriptase and capping enzymes to produce full-length capped mRNAs, using only the minus strand of each viral gene segment as templates (Watanabe et al., 1968; Shatkin and LaFiandra, 1972). In some genera (eg, *Rotavirus* and *Aquareovirus*), the outer capsid can be modified by trypsin treatment to form these infectious or intermediate subviral particles. The mRNAs are immediately translated into proteins by the ribosomes. These mRNAs associate with newly synthesized viral proteins to form new subviral particles and also serve as templates for minus-strand synthesis to generate nascent genomic dsRNA within the particles (Antczak and Joklik, 1992). These in turn serve as templates for transcription of more mRNA (uncapped) that is translated into viral proteins (Skup and Millward, 1980). The selection of viral mRNAs for packaging is highly specific, likely depending on the conserved terminal sequences at both ends of the RNA segments, resulting in the packaging of one copy of each genome segment per particle. Genome segment reassortment occurs readily in cells coinfected with different viruses of the same species, which presumably share the same packaging signals (Attoui et al., 2012). Reovirus replication results in intracytoplasmic inclusion bodies (viroplasms). These inclusion bodies are the sites of viral replication and assembly (Shao et al., 2013).

Virus release is by cell lysis or budding. Some members like *Orbivirus* and *Rotavirus* are initially enveloped when released, but such envelopes are transient. These viruses mature at the endoplasmic reticulum membrane into internal spaces and are eventually released when the cell lyses.

Reoviruses in the genera *Orthoreovirus* and *Aquareovirus* are the only known examples of nonenveloped viruses that induce cell–cell fusion and syncytium formation (ie, are fusogenic) in virus-infected cells. The *Orthoreovirus* genus is divided into nonfusogenic (MRV) and fusogenic orthoreoviruses (NBV, BRV, ARV, RRV, *Broome Orthoreovirus* BroV) (Duncan, 1999; Thalmann et al., 2010) by virtue of possession of a fusion-associated small transmembrane (FAST) protein

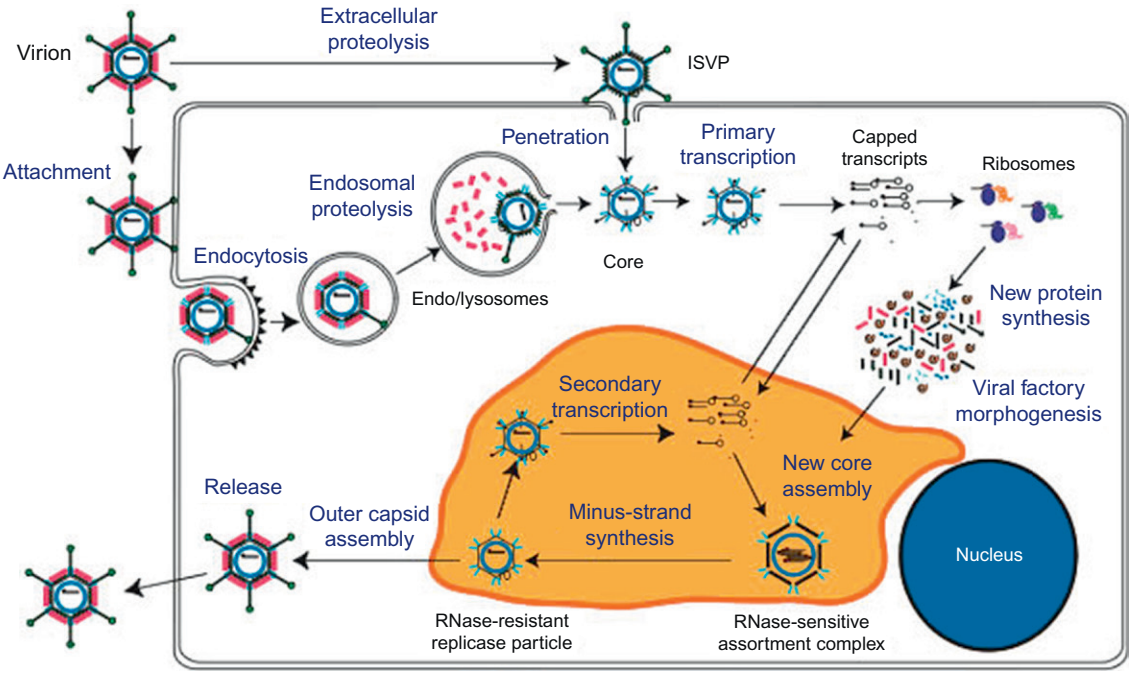

FIGURE 14.3 Schematic of the reovirus replication cycle. Reoviruses replicate in the cytosol of infected cells. Following penetration of the cellular membrane, viral cores begin transcribing the 10 viral genome segments. The 10 viral genome segments encode 12 viral proteins (eight structural and four nonstructural). The nonstructural protein, μNS, forms the matrix of viral factories where new cores assemble and begin secondary rounds of transcription. The viral cores are coated with the outer capsid proteins μ1, σ3 and σ1 to form intact virions that are released following cell lysis. *From* https://courses.cit.cornell.edu/parker_lab/Reovirus.htm. *Reproduced with permission of Dr. John S. L. Parker, James A Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University.*

(also referred to as p10, p13, p14 or p15 in different reoviruses) (Shmulevitz and Duncan, 2000). Syncytia formation commences 10–12 h postinfection, resulting in a more rapid lytic response and enhanced kinetics of virus release (Attoui et al., 2012). Some of the members of the *Aquareovirus* genus also possess a FAST protein and induce syncytium formation. Most recently, PRV has been assigned to the genus *Orthoreovirus* (Rimstad and Wessel, 2015) and its nonstructural protein p13 is not a FAST protein and is therefore nonfusogenic (Key et al., 2013).

14.1.4 General Pathogenesis of Reoviruses

The pathogenicity of reoviruses varies according to genus (Table 14.1). Whereas for the genus *Orthoreovirus,* the nonfusogenic orthoreovirus, MRV, is not clinically significant (Sabin, 1959) except for rodents, bat origin MRV3 in humans in Europe (Chua et al., 2008) and for porcine orthoreoviruses, the fusogenic orthoreoviruses NBV (Chua et al., 2007) and BRV (Leland et al., 2000) that infect primates, ARV (van den Brand et al., 2007) that infects birds and RRV (Lamirande et al., 1999) that infects reptiles have been shown to cause significant and often fatal disease. Porcine orthoreoviruses causing diarrhea have been reported in China (Dai et al., 2012), Korea (Kwon et al., 2012) and the United States; the US isolates are MRV serotype 3 recombinant (S1 segment) of mammalian and bat MRV3 that occur in mixed infection with porcine epidemic diarrhea virus, but are individually highly pathogenic to neonatal pigs, leading to lethal enteric disease (Thimmasandra Narayanappa et al., 2015). Interestingly, the US porcine MRV3, while lacking the FAST protein, induces syncytia in BHK-21 cells and in intestinal villous epithelial cells, but not in Vero cells (Thimmasandra Narayanappa et al., 2015). The genera *Orbivirus* and *Rotavirus* contain several pathogens for domestic animals. The genera *Coltivirus* and *Seadornavirus* are zoonoses (and cause disease in adult hamsters and suckling mice). Among the aquatic reoviruses, genus *Aquareovirus* affects fish (with pathology in some farmed fish species), PRV (genus *Orthoreovirus*) is associated with heart and skeletal muscle inflammation (HSMI) (Palacios et al., 2010), an emerging disease of marine-farmed Atlantic salmon in Europe (Norway, Ireland and Scotland), Chile (Godoy et al., 2016) and Canada-British Columbia (DFO, 2016), and *Cardoreovirus*, *Crabreovirus* and other reoviruses isolated from crabs (Chen et al., 2011; Weng et al., 2007), shrimps and prawns (Zhang et al., 2015) have only been isolated from diseased animals (see also Table 14.2). Proteolytic enzymes (trypsin or chymotrypsin) generally increase infectivity of reoviruses.

TABLE 14.2 Pathogenicity of Aquatic Reoviruses

Aquatic Reovirus Genus or Species	Virus Isolates	Clinical Presentation
Aquareovirus A	Chum salmon reovirus (CSV)	Isolated from asymptomatic Chum salmon *Oncorhynchus keta* in Japan in a disease outbreak associated with low mortality rates (Winton et al., 1981), and from masou salmon *Oncorhynchus masou* fry undergoing large mortalities on island of Hokkaido, Japan (Lupiani et al., 1995)
	Masou salmon reovirus	
	Threadfin reovirus	Isolated from mass mortality (100%) of cultured threadfin *Eleutheronema tetradactylus* fingerlings in an aquaculture farm in Singapore, and 60% mortality in sea bass fingerlings (Seng et al., 2002, 2005b)
	American oyster reovirus 13p2	Isolated from American oysters but caused significant mortality in rainbow trout (Meyers, 1983) and blue gill fingerlings (44% mortality) (Meyers, 1980)
	Angel fish reovirus	Isolated from marine angelfish *Pomacanthus semicirculatus* presenting head and lateral line erosion syndrome (Varner and Lewis, 1991)
	Atlantic salmon reovirus	
	Chinook salmon reovirus DRC	
	Smelt reovirus	Isolated from landlocked Canadian rainbow smelt *Osmerus mordax* suffering a large mortality (Marshall et al., 1990)
	Striped bass reovirus (SBR)	Isolated from a moribund striped bass *Morone saxatilis* that was also infected with bacteria (Samal et al., 1990)
	Scophthalmus maximus reovirus (SMReV)	Isolated from diseased marine turbot *S. maximus* in China (Ke et al., 2011)
	Atlantic halibut reovirus	58–98% mortality with severe multifocal liver necrosis in captive/farmed juvenile Atlantic halibut *Hippoglossus hippoglossus* in Nova Scotia, Canada (Cusack et al., 2001), Scotland (Ferguson et al., 2003) and most recently in Norway (Blindheim et al., 2015)
	Tasmanian Atlantic salmon aquareovirus (TSRV)	Of low pathogenicity; natural infections do not seem to be associated with clinical disease or mortality (Crane et al., 2000). Caused relatively high mortality rates in Atlantic salmon fry (less than 6 months old) during experimental infection (Zainathan, 2012)
Aquareovirus B	Chinook salmon reovirus B	Isolated during routine examination of sexually mature Coho salmon *Oncorhynchus kisutch* in Oregon (Lupiani et al., 1995)
	Chinook salmon reovirus LBS, YRC and ICR	
	Coho salmon reovirus CSR, ELC and SCS	
Aquareovirus C	Golden shiner reovirus (GSV or GSRV)	Low mortality with petechial hemorrhages in ventral surface, eye, muscle, fatty tissue and intestinal mucosa of golden shiner bait fish *Notemigonus crysoleucas* (Plumb et al., 1979), and a significant pathogen of farmed grass carp and fathead minnows and wild "creek chub" in the United States (Goodwin et al., 2006)
	Grass carp reovirus (GCRV) GCRV873	Grass carp hemorrhagic disease with high mortalities (50–95%) in fingerling and yearling grass carp *Ctenopharyngodon idellus* (Chen and Jiang, 1984; Fang et al., 1989), black carp *Mylopharyngodon piceus*, topmouth gudgeon *Pseudorasbora parva* (Li et al., 1997) and rare minnow *Gobiocypris rarus* (Wang et al., 1994)
	White bream reovirus (WBRV) A-127/06	Isolated from white bream with skin and gill damage with varying degrees of fin erosion in the "Old Danube," Vienna, Austria (Schachner et al., 2014)
Aquareovirus D	Channel catfish reovirus (CCRV)	Initially isolated from channel catfish in two farms undergoing chronic mortality (5%) (Amend et al., 1984). Since then, regularly isolated from different locations in California

(Continued)

TABLE 14.2 Pathogenicity of Aquatic Reoviruses (Continued)

Aquatic Reovirus Genus or Species	Virus Isolates	Clinical Presentation
Aquareovirus E	Turbot reovirus (TRV)	Isolated from farmed turbot *S. maximus* in northwestern Spain during disease outbreak with continuous but low mortality (total cumulative loss of 4%) with affected fish showing abdominal distension and hemorrhagic foci in the anus and base of the fins and hemorrhagic ascites (Lupiani et al., 1989; Rivas et al., 1996)
Aquareovirus F	Chum salmon reovirus PSR	
	Coho salmon reovirus SSR (SSRV)	
Aquareovirus G	American grass carp reovirus (AGCRV)	Not strongly associated with disease in fish, but it is often detected by cell culture during routine inspections of healthy cultured golden shiners *N. crysoleucas* and moribund fathead minnow *Pimephales promelas* (Goodwin et al., 2010). It has been reported in association with a single fish kill involving grass carp *Ctenopharyngodon idella* during the winter months (Goodwin et al., 2006; Jaafar et al., 2008). It was also isolated from Golden ide *Leuciscus idus melanotus* with epidermal hyperplasia (Neukirch et al., 1999)
GCRV-HZ08[a]	GCRV-HZ08	Causes 60–80% mortality in experimental infection of grass carp of approximately 10 cm (Zhang et al., 2010)
GCRV-GD108[b]	GCRV-GD108	Isolated from diseased grass carp in Guangdong province, China, in 2009; causes 75–90% fish mortality associated with hemorrhagic symptoms (Ye et al., 2012)
HGDRV[c]	Grass carp reovirus strain 104 (GCRV104)	Isolated from diseased grass carp with severe hemorrhages on the muscle, intestine, fins and gills in Hubei province, China, in 2009; causes 80% fish mortality associated with hemorrhagic symptoms after 1 week (Fan et al., 2013)
Unassigned aquareovirus	Guppy reovirus	Isolated from a moribund guppy in 2001 from a fish farm in Singapore; it caused severe mortalities in infected guppies and is a major threat to the local ornamental fish farming industry (reviewed in Zainathan, 2012)
Orthoreovirus	Piscine orthoreovirus (PRV)	Associated with HSMI in farmed Atlantic salmon (Palacios et al., 2010) and HSMI-like disease in coho salmon (Godoy et al., 2016). A new disease in rainbow trout similar to HSMI and associated with a PRV-related virus was also reported in Norway (Olsen et al., 2015).
		Associated with melanized spots in white skeletal muscle of farmed Atlantic salmon (Bjørgen et al., 2015)
Cardoreovirus	*E. sinensis* reovirus (*Es*RV905)	Isolated from diseased Chinese mitten crab *E. sinensis* with trembling disease (Zhang et al., 2004)
Crabreovirus[d]	Mud crab reovirus (MCRV)	Isolated from mud crab *S. serrata* with sleeping disease in China (Weng et al., 2007)
	S. serrata reovirus (*Ss*RV)	Isolated from mud crab *S. serrata* with waterclear disease in China (Chen et al., 2011)
	C. sapidus reovirus 1 (*Cs*RV1)	Isolated from wild and captive blue crab *C. sapidus* populations in United States with disease and high mortality (Bowers et al., 2010)
Unassigned crabreoviruses	*M. depurator* P virus (DpPv)	Isolated from diseased swimming crab *M. depurator* (Vago, 1966)
	C. mediterraneus W2 virus (*Cc*RV-W2)	Isolated from diseased shore crab *C. mediterraneus* (Mari and Bonami, 1988)

(Continued)

TABLE 14.2 Pathogenicity of Aquatic Reoviruses (Continued)

Aquatic Reovirus Genus or Species	Virus Isolates	Clinical Presentation
Unassigned crustacean reoviruses	*E. sinensis* reovirus (*Es*RV816)	Isolated from diseased Chinese mitten crab *E. sinensis* with trembling disease; experimentally infected crabs had 30% mortality, but showed no typical trembling sign (Zhang and Bonami, 2012)
	M. nipponense reovirus (*Mn*RV)	Associated with red body disease, black gills and heavy mortalities in freshwater prawn *M. nipponense*. Infection signs in hepatopancreas epithelial cells (Zhang et al., 2015)

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[a]GCRV-HZ08, Grass carp reovirus HZ08; shown by phylogenetic analysis to be significantly different from other known aquareoviruses (Zhang et al., 2010).
[b]GCRV-GD108, Grass carp reovirus GD108; shown by phylogenetic analysis to be significantly different from other known aquareoviruses (Ye et al., 2012).
[c]HGDRV, Hubei grass carp disease reovirus; proposed as a new species in the genus Aquareovirus in 2013 (Fan et al., 2013).
[d]Crabreovirus genus proposed in 2012 (Deng et al., 2012).

14.2 GENUS AQUAREOVIRUS

14.2.1 Clinical Significance

The name aquareovirus was coined by Winton et al. (1987) for reoviruses isolated from aquatic (water) animal hosts. These viruses have a broad geographical range and to date, they have been isolated from a wide variety of aquatic animals in both freshwater and seawater including fish, crustaceans and mollusks. However, only the fish reovirus isolates with a genome of 11 segments of dsRNA are included in the genus *Aquareovirus*. Crustacean reovirus isolates belong to the genus *Cardoreovirus* (Attoui et al., 2012) or are not yet assigned to a genus (Chen et al., 2011; Zhang et al., 2015), whereas mollusk (oyster or clam) isolates such as American oyster isolate 13p2 (Meyers and Hirai, 1980; Meyers et al., 2009) are more likely to be fish reoviruses that simply accumulated in mollusks upon filter feeding of virus-contaminated water (Nibert and Duncan, 2013).

Although aquareoviruses are often isolated from apparently healthy individuals, many of them also play a significant role in the morbidity and mortality of fish, depending on the virus strain and host species (Lupiani et al., 1995; Winton et al., 1987; Blindheim et al., 2015). In some cases the aquareovirus strains that cause disease are sometimes associated with secondary bacterial infections (Lupiani et al., 1989; Baya et al., 1990; Cusack et al., 2001). The clinical presentations shown by fish infected with aquareoviruses are nonspecific and include abdominal distension, lethargy, skin discoloration and hemorrhages of the skin, loss of equilibrium, inappetence, anorexia, abnormal swimming behavior and eventual death, hemorrhages of internal organs, pale liver and kidney, and hepatitis and pancreatitis (Lupiani et al., 1995; Zainathan, 2012). The different fish diseases associated with aquareoviruses are summarized in Table 14.2 together with diseases associated with reoviruses in other aquatic animals. The grass carp reovirus (GCRV) isolates, which occur in the United States and China in several different aquareovirus species (Attoui et al., 2002b; McEntire et al., 2003; Wang et al., 2012), are considered the most pathogenic aquareovirus (Samal et al., 1998).

TABLE 14.3 The dsRNA Genome Segments and Proteins of Genus *Aquareovirus* (species *Aquareovirus A* and HGDRV)

Genome Segment	dsRNA (Size, bp)[a]	Protein Nomenclature	Protein Size (kDa)[a]	Protein Function (and Location)
Seg1	3.8–3.94	VP1	130	Inner capsid protein, guanylyltransferase (core)
Seg2	3.6–3.86	VP2	127	Inner capsid protein, RNA-dependent RNA polymerase (core)
Seg3	3.3–3.73	VP3	126	Inner capsid protein, helicase, NTPase (core)
Seg4	2.21–2.5	VP4 [VP5]	97	Nonstructural, of unknown function [inner capsid protein, NTPase (core)]
Seg5	2.0–2.4	VP5 [VP4]	71	Inner capsid protein, (core) [putative outer capsid protein]
Seg6	1.9–2.2	VP4 [NS66]	73	Inner capsid protein (core) [nonstructural protein]
Seg7[b]	1.5–1.58	NS4 [VP55]	28	Nonstructural, of unknown function
Seg8	1.32–1.4	VP6	46	Inner capsid protein (core)
Seg9	1.14–1.2	NS2 [VP39]	39	Nonstructural, of unknown function
Seg10	0.9–1.12	VP7 [VP38]	35	Major outer capsid[c], may be glycosylated [Unknown]
Seg11[b]	0.8–0.87	NS3 [VP8]	29	Nonstructural, of unknown function
		NS5 [VP15]	15	Nonstructural, of unknown function

[a]*Aquareovirus values are from Subramanian, K., McPhillips, T. H., Samal, S. K., 1994. Characterization of the polypeptides and determination of genome coding assignments of an aquareovirus. Virology 205, 75–81; Attoui et al. (2012) and Fan et al. (2013). The 5′ and 3′UTR are from Fan et al. (2013). 3′UTR does not include the stop codon. All items specific to HGDRV (Fan et al., 2013) are indicated in square brackets.*
[b]*In species Aquareovirus C and Aquareovirus G, Seg7 instead of Seg11 is bicistronic, encoding NS5 in addition to NS4, from nonoverlapping and out-of-phase open reading frames.*
[c]*The aquareovirus particles lack the hemagglutinin spike protein σ1 observed in orthoreovirus particles (Shaw, A.L., Samal, S. K., Subramanian, K., Prasad, B. V. V. 1996. The structure of aquareovirus shows how the different geometries of the two layers of the capsid are reconciled to provide symmetrical interactions and stabilization. Structure 4, 957–967). Thus aquareoviruses lack hemagglutinating activity.*

14.2.2 Virion Properties

The viral fact sheet that describes the virion shape, molecular biology and epidemiology for the genus *Aquareovirus* is found at the ViralZone (Masson et al., 2013). The aquareovirus genome contains 11 segments of dsRNA coding for 12 proteins. Segments size range from 0.8 to 4.0 kb. The genome total size ranges from 18.2 to 30.5 kbp. The genome segments and functions of the aquareovirus proteins are summarized in Table 14.3.

Reoviruses belonging to the same genus have conserved sequences at both 5′ and 3′ termini of their genome segments (Chen et al., 2011; Attoui et al., 2012; Flowers et al., 2016). Table 14.4 compares the terminal conserved sequences of members of the *Aquareovirus* genus with those of members of *Orthoreovirus Cardoreovirus* and *Crabreovirus*. The comparison shows that the 3′ terminal sequence, UCAUC-3′, is conserved among the *Aquareovirus* and *Orthoreovirus* but not in the crustacean reoviruses whereas the conserved 5′ terminal sequence is unique to each group including PRV as separate from members of genus *Orthoreovirus*; thus the five groups of viruses represent five different genera (Chen et al., 2011; Kibenge et al., 2013), which is also supported by phylogenetic analysis (Fig. 14.1).

Reciprocal RNA–RNA blot hybridization and sequence analysis were used to differentiate aquareovirus isolates into seven distinct genetic groups (genogroups) (Lupiani et al., 1993, 1994; Subramanian et al., 1997; Samal et al., 1998; Jaafar et al., 2008) that have since been established into seven ICTV-approved species (designated *Aquareovirus A–G*) (Attoui et al., 2012). There are also three newly proposed species (GCRV-GD108, GCRV-HZ08 and HGDRV) (Zhang et al., 2010; Ye et al., 2012; Fan et al., 2013), and several unassigned reovirus isolates (Attoui et al., 2012; Bandini et al., 1995) (Table 14.2).

14.2.3 Pathology

Fish infected with aquareoviruses generally exhibit low or no pathogenicity for fish; however, some virus isolates have the capacity to cause significant mortality under certain circumstances (Plumb and Hanson, 2011). The different fish disease conditions associated with aquareoviruses are summarized in Table 14.2.

TABLE 14.4 Conserved Terminal Nucleotide Sequences (Positive Strand) of Aquatic Reoviruses (*Piscine Orthoreovirus*, *Aquareovirus*, *Cardoreovirus*, *Crabreovirus*) and *Orthoreovirus* Genome Segments

Reovirus Genus	Reovirus Species/Strain	Conserved Terminal Nucleotide Sequences	
		5′ Terminal Nucleotides	3′ Terminal Nucleotides
Orthoreovirus genus	Piscine orthoreovirus (PRV)[a]	5′-GAUAAA/U	UCAUC-3′
	Avian orthoreovirus-138[b]	5′-GCUUUUU	UCAUC-3′
	Nelson Bay orthoreovirus[b]	5′-GCUUUA	UCAUC-3′
	Mammalian orthoreovirus-1La[b]	5′-GCUA	UCAUC-3′
	Baboon orthoreovirus[b]	5′-GUAAAUUU	UCAUC-3′
	Reptilian orthoreovirus[b]	5′-GUUAUUUU	UCAUC-3′
Aquareovirus genus	*Aquareovirus* A[b]	5′-GUUUUA	UCAUC-3′
	Aquareovirus C[b]	5′-GUUAUU	UCAUC-3′
	Aquareovirus G[b]	5′-GUUUUA	UCAUC-3′
Cardoreovirus genus	*E. sinensis* reovirus (*Es*RV905)[c]	5′-GGAUUUA	UAUAACAGAC-3′
Crabreovirus genus	Mud crab reovirus (MCRV)	5′-AUAAAU/C	A/CAACG/UAU-3′
	Scylla serrata reovirus (*Ss*RV)[d]	5′-AUAAAU	ACGAU-3′
	Callinectes sapidus reovirus 1 (*Cs*RV1)[e]	5′-AUAAAYWHH[e]	GAWCAACKAU-3′[e]

[a]*Genome sequence information is from Palacios et al. (2010).*
[b]*Genome sequence information is from Attoui et al. (2012).*
[c]*Genome segment 1 of EsRV, which has only one segment sequence available in GenBank (Deng et al., 2012).*
[d]*Genome sequence information is from Chen et al. (2011).*
[e]*Genome sequence information is from Flowers et al. (2016). Ambiguity code: Y = C/U; W = A/U; H = A/C/U; K = G/U.*

14.2.3.1 Tasmania Salmon Reovirus

Following its first isolation in the early 1990s, a preliminary infectivity trial indicated that Tasmania salmon reovirus (TSRV) was nonpathogenic to approximately 1-year-old Atlantic salmon under experimental conditions (Humphrey et al., unpublished in Zainathan, 2012). This first experimental study showed viral replication and persistence of TSRV in Atlantic salmon in the absence of clinical disease or severe pathological changes. Interestingly, a subsequent experimental infection of TSRV in Atlantic salmon fry (less than 6 months old) resulted in relatively high mortality rates (Crane et al., unpublished), thus demonstrating that TSRV could be highly pathogenic under certain conditions (Zainathan, 2012). Other reports later describe that elevated water temperatures (18°C vs 10°C) were associated with increased mortality in juvenile Atlantic salmon experimentally infected with TSRV (Carlile, 2013 in Carlile et al., 2014). Gross and behavioral signs described are distended abdomen, lethargy, loss of equilibrium and inappetence (Carlile, personal communication in Zainathan, 2012). Zainathan (2012) also describe enlarged spleen and pale liver, liver abscess, granular spleen, dark hindgut, hemorrhages of internal organs, multifocal lesions in pyloric caeca, congestion, adhesions of internal organs and ascites.

The histopathology of TSRV infection was mild-to-severe, multifocal, acute hepatic necrosis, mild-to-severe multifocal necrosis of the renal hematopoietic tissue and moderate-to-severe multifocal acute pancreatic necrosis with foci of liquefactive fat necrosis also observed in the most severe examples (Carlile, personal communication in Zainathan, 2012; Carlile et al., unpublished in Carlile et al., 2014). Zainathan (2012) also reported random foci of edematous separation of cardiomyofibers predominantly in the outer layer of myocardium. There were moderate-to-high numbers of mixed lymphoid and white blood cell aggregates along the pericardium, inner layer and outer compact layer of myocardium and inflammation of pericardium suggestive of pericarditis.

14.2.3.2 Chinook Salmon Reovirus DRC

Parisot and Pelnar (1962) reported a virus-like disease characterized by abrupt and rapid rise in mortalities. The fish had bright, cherry red lesions on the dorsal surface of the body posterior to the head and anterior to the dorsal fin, markedly swollen abdomens and were darker in color. The histopathological findings reported were necrosis of parenquimal and

hematopoietic cell in the kidney, muscle hemorrhaging, and necrosis in the pancreas and inclusion bodies of varying sizes, both intranuclear and intracytoplasmic.

14.2.3.3 Chum Salmon Aquareovirus

Chum salmon aquareovirus (CSV) was isolated from asymptomatic fish in CHSE-214 cell line (Winton et al., 1981). In-vivo studies by injection showed no substantial mortality or gross pathology for chum salmon (*Oncorhynchus keta*), Chinook salmon (*Oncorhynchus tshawytscha*) fry or kokanee salmon (*Oncorhynchus nerka*) fry. Histological examination provided evidence of pathology in chum salmon and Chinook salmon fry. Areas of focal necrosis were observed in the liver of infected fish.

14.2.3.4 Channel Catfish Reovirus and Channel Catfish Reovirus 730

Channel catfish reovirus was first isolated from healthy channel catfish (Amend et al., 1984). The virus was nonpathogenic when injected into channel catfish (Plumb and Hanson, 2011). Xu et al. (2013) reported a reovirus, designated as channel catfish reovirus 730, isolated from channel catfish, *Ictalurus punctatus*, and fingerlings suffering a severe hemorrhage in China's Hubei province. Experimental infection confirmed the pathogenicity of the virus and proved it to be the causative pathogen. The diseased channel catfish fingerlings (6–12 cm) were lethargic, separately floating at the surface with spiral swimming movements and finally died within 24 h, showing severe hemorrhage at operculum, lower jaw and fin bases, body color depigmentation, bilateral exophthalmia and abdominal distention. On postmortem examination, gills of diseased fish presented blood loss anemia and became pale. The liver was pale and contained small hemorrhages, and the spleen was dark and enlarged. The digestive tract contained no food, but had hemorrhages on the intestinal wall. Clear yellow to blood-tinged fluid was presented in the body cavities of abdominal distention individuals.

14.2.3.5 Golden Shiner Reovirus

Golden shiner reovirus (GSRV) was first isolated from moribund cultured golden shiners (*Notemigonus crysoleucas*) in Arkansas (United States) in 1977 (Plumb et al., 1979) and is a significant pathogen of farmed grass carp (*Ctenopharyngodon idellus*) and fathead minnows (FHMs) (*Pimephales promelas*) and has also been isolated from wild "creek chub" (*Semotilus atromaculatus*) in the United States (Goodwin et al., 2006). Shiners infected with GSRV may swim lethargically at the surface, dive when they are disturbed, hold their fins close to the body and lie on the bottom of aquaria or tanks (Plumb et al., 1979). External clinical signs are not obvious, but the back and head of infected fish are reddish (erythema) as a result of hemorrhage in the skin and dorsal musculature. Petechial are present in visceral fat and intestinal mucosa. Open ulcerative lesions will occasionally develop in the skin; however, these lesions may be due to secondary bacterial infections (Plumb and Hanson, 2011). The moribund FHM had abnormally red dorsal musculature visible through an intact dermis (Goodwin et al., 2006).

14.2.3.6 American Grass Carp Reovirus

American grass carp reovirus (AGCRV) has been reported in association with a single fish kill involving grass carp (*Ctenopharyngodon idella*) during the winter months (Goodwin et al., 2010).

14.2.3.7 Grass Carp Reovirus

GCRV was first isolated from dying grass carp (*C. idella*) in China during the 1970s. The clinical signs of this infection were hemorrhages of organs showing spots or plate forms, in combination with some or all of the following signs: exophthalmia, body darkening, hemorrhage of the mouth cavity, hemorrhagic or pale gills and hemorrhage at the base of fins and gill covers. Internal hemorrhage may occur throughout the musculature, liver, spleen, kidney and intestines (Jiang, 2009). Some farmers tried to classify this disease into three types according to clinical signs: "red muscles," "red intestine" or "red fin and gill cover." In fact, these are only some of the signs; sick fingerlings show one or all (Jiang, 2009). The virus causes an acute hemorrhagic disease in yearling and fingerling grass carp during the summer months when water temperatures range between 24°C and 30°C (Jiang and Ahne, 1989; Jun et al., 1997).

Liang et al. (2014) reported grass carps infected by GCRV-CL showed spots or plate hemorrhages in the tissues and this infection was more virulent than the GCRV-HZ08 strain. The grass carps infected by GCRV-CL had higher viral RNA copy numbers than those infected by GCRV-HZ08 in different tissues and organs, particularly in the liver, spleen, kidney,

intestine and muscle, which had a higher number of viral RNA copies during the sixth to ninth days. However, 10 days after infection there were not high viral RNA copy numbers.

14.2.3.8 Turbot Aquareovirus

Turbot aquareovirus (TRV) was isolated in northwest Spain from turbot (*S. maximus*) suffering a mixed bacterial and viral infection (Lupiani et al., 1989), causing low but economically serious mortalities in a few turbot farms. Since then, the virus has been repeatedly isolated from the same and other fish farms. These later isolations were consistently made in fish after stress and/or concomitant with bacterial infections (Rivas et al., 1996).

14.2.3.9 Turbot Reovirus (SMReV)

Scophthalmus maximus reovirus (SMReV) was recently isolated from a diseased turbot. It is the first isolated aquareovirus from a marine fish in China. Amino acids comparison and phylogenetic analysis suggested that SMReV was a new aquareovirus in the species *Aquareovirus A* (Ke et al., 2011), and therefore different from TRV, which belongs to *Aquareovirus E*.

14.2.3.10 Atlantic Halibut Reovirus

Atlantic halibut reovirus caused 58–98% mortality with severe multifocal liver necrosis in captive/farmed juvenile Atlantic halibut (*Hippoglossus hippoglossus*) in Nova Scotia, Canada (Cusack et al., 2001), Scotland (Ferguson et al., 2003) and most recently in Norway (Blindheim et al., 2015). The moribund larvae (start-feeding phase) showed multifocal necrosis in the liver and pancreas and areas of syncytial formation in these tissues. Large subcellular inclusions were present in the syncytial areas. There were no signs of inflammation in these areas. Food material could be observed in the gut of some individuals (Blindheim et al., 2015).

Sequence analysis of the putative FAST protein by Blindheim et al. (2015) revealed the highest amino acid sequence identity (46.8%) to Green River Chinook virus, which is an unassigned member of the genus *Aquareovirus*, while a comparison with isolates belonging to the species *Aquareovirus A* showed 33% sequence identity.

14.2.3.11 Reovirus From White Bream (Blicca bjoerkna)

Schachner et al. (2014) reported a new reovirus isolated exclusively from white bream (*B. bjoerkna* L.) (formerly *Abramis bjoerkna* L.). White bream of different sizes exhibited skin and gill damage to varying degrees including scale loss, fin erosion, increase in mucus production, epidermal swelling as well as hemorrhages mainly along fin and gill rays. Gill histology was characterized by an extremely delicate respiratory epithelium that tended to either detach from capillaries by edematous swelling or be replaced by mucus-secreting goblet cells. The interlamellar epithelium exhibited some infiltration. The most marked and unique finding in individual white bream gill tissue was extravasation of an eosinophilic substance, resembling agglutinated erythrocytes extruding from destroyed lamellar capillaries and respiratory epithelium. There was accumulation of eosinophilic granular cells at the base of the lamellae and of rodlet cells lining the endothelium of afferent arteries next to the gill arch-supportive tissue. While proliferation of goblet and granular cells was found in other cyprinid species, these were different from the extravasation observed in white bream (Schachner et al., 2014).

14.2.4 Immunity

Rivas et al. (1996) demonstrated that TRV survived and replicated within turbot macrophages in vitro without destroying the cell monolayer, suggesting that aquareoviruses elude the recognition system of the macrophages thus preventing their clearance from the host. It was suggested that macrophages may be a site for viral persistence in fish. It appears that surviving fish remain carriers; hence clinical disease is a most likely in fry, fingerlings and yearlings.

Detailed studies on immunity have been limited to the most pathogenic aquareoviruses. The intracellular antiviral immune signaling in fish following infection with GCRV was recently reviewed (Rao and Su, 2015). Overexpression of grass carp high-mobility group box 1 (HMGB1) proteins, a newly discovered family of nucleic acid sensors important in the regulation of innate immunity (Yanai et al., 2009), significantly delays the GCRV-induced cytopathic effect (CPE) in *C. idella* kidney (CIK) cells without affecting the viral titer at 24 h and 48 h post-GCRV infection, indicating that HMGB1 did not possess direct antiviral activity (Yang et al., 2013a). Previously, Joseph et al. (2004) demonstrated that infectious

salmon anemia virus (ISAV)-infected TO cells showed leakage of HMGB1 protein from the nucleus, which is characteristic of cells undergoing necrosis (Scaffidi et al., 2002), suggesting that CPE in these cells is associated with necrosis. It follows therefore that the GCRV-induced CPE in CIK cells is due to necrosis and not apoptosis. Antiviral activation of interferon-I (IFN-I) following GCRV infection occurs via toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiation factor 88 (MyD88)-dependent pathways in toll-like receptor (TLR) signaling and by IFN-β promoter stimulator-1 (IPS-1)-dependent (in retinoic acid-inducible gene (RIG) I-like receptor (RLR) or in melanoma differentiation-associated gene 5 (MDA5)) or independent (in laboratory of genetic and physiology 2 (LGP2)) signaling (Su et al., 2008, 2010, 2011; Yang et al., 2012a, 2013b; Chen et al., 2013b, 2015). These pathways of GCRV-triggered IFN-I response were inhibited by the immune adapter molecule sterile alpha and toll/IL-1R motif containing 1 (SARM1), which also promoted GCRV-induced cell death (Yan et al., 2015). GCRV infection also upregulated expression of nucleotide-binding domain (NOD)-like receptors (NLRs) (Chen et al., 2013a). Overexpression of Mx genes significantly inhibits the replication of GCRV and delays the CPE induced by GCRV infection (Peng et al., 2012). Overexpression of the viperin gene also confers significant protection against GCRV infection (Wang et al., 2014). The specific immune-stimulated genes (ISGs) GCRV-induced genes 1 and 2 (Gig 1 and Gig 2) were first identified as novel fish ISG from ultraviolet (UV)-inactivated GCRV-infected CAB cells (Zhang and Gui, 2004), and because they are not induced by recombinant carp IFN, they may be induced in an IFN-independent pathway (Sun et al., 2013). Adenosine deamiase acting on RNA, an RNA editing enzyme that targets both coding and noncoding dsRNA, is upregulated by GCRV in vivo and in vitro (Yang et al., 2012b). Such innate immune defense mechanisms initiated by aquareovirus infection may be the basis for the increased survival of aquareovirus-preexposed fish when challenged with infectious hematopoietic necrosis virus (LaPatra et al., 1995).

14.2.5 Diagnostic Methods

Classical whole virus detection methods for aquareoviruses consist of virus isolation in fish cell lines and detection via purification and electron microscopy. Aquareoviruses replicate well in several fish cell lines forming syncytia in monolayer cultures (Winton et al., 1987; Samal et al., 1990, 1998; Lupiani et al., 1995; Cusack et al., 2001; Fan et al., 2013; Schachner et al., 2014; Blindheim et al., 2015). The exceptions are: AGCRV, a member of the species *Aquareovirus G*, which does not form syncytia, but produces a pattern of cell rounding that is very similar to that produced by fish rhabdoviruses (Goodwin et al., 2010); and GCRV-GD108 and GCRV-HZ08 which are noncytopathic in cell culture (Ye et al., 2012; Zhang et al., 2010). Thus aquareoviruses normally do not cause great difficulty in the regulatory health inspections on aquatic animals using cell culture (USFWS and AFS-FHS, 2014); they are easy to detect because of the ease of virus isolation in fish cell lines (Brady and Plumb, 1991; Lupiani et al., 1995; Rivas et al., 1996; Neukirch, 2000) and by virtue of the large syncytia that they produce, readily distinguishing them from the nonsyncytial CPEs typical of the highly regulated fish pathogens (Goodwin et al., 2006; Batts and Winton, 2012).

Viral antigen detection methods for aquareovirus include immunoenzyme staining, staphylococcal coagglutination and immunofluorescence and enzyme-linked immunosorbent assays (reviewed by Seng et al., 2004). Antigenic serodiagnosis using the antibody of grass carp IgM is used for GCRV detection (He et al., 2013; Hongli et al., 2014).

More sensitive nucleic acid-based assays have also been developed to specifically detect aquareoviruses. A rapid, sensitive and highly specific reverse-transcription polymerase chain reaction (RT-PCR) detection method for different aquareoviruses was reported by Seng et al. (2004). The assay, which uses specific degenerate primers, was shown to be very useful for rapid and accurate detection of a variety of aquareovirus strains isolated from different host species and origin, and could detect aquareoviruses in low titer virus-infected cell cultured cells and in tissue samples of aquareovirus-carrier fish. A reverse-transcription loop-mediated isothermal amplification requiring no complicated instrumentation has been developed for GCRV diagnosis (Zhang et al., 2013). Goodwin et al. (2010) reported a quantitative real-time RT-PCR assay that can be used to identify AGCRV in cell cultures or directly in fish tissues to differentiate it from rhabdoviruses (viral hemorrhagic septicemia and spring viremia of carp virus) with similar CPE. The assay detected as few as two copies of the plasmid template and did not cross-react with any other aquareoviruses tested (Goodwin et al., 2010). Zainathan et al. (2015) developed an aquareovirus generic conventional RT-PCR and a nested RT-PCR as well as RT-qPCR assay specific for TSRV and then performed intralaboratory and interlaboratory (between two labs) comparison of the PCR assays and virus isolation in cell culture using finfish cell lines, CHSE-214 and *Epithelioma papulosum cyprinid* (EPC), for the detection and identification of TSRV using field samples. All the diagnostic methods (in both laboratories) had high specificity, while the estimated sensitivity varied between tests with RT-qPCR being the most sensitive (95.2%) method followed by virus isolation and then conventional nested RT-PCR; RT-qPCR was 1000-fold more sensitive than virus isolation (Zainathan et al., 2015).

14.2.6 Prevention and Control

14.2.6.1 Control

The presence of aquareoviruses is likely to be under-reported due to the fact that these viruses are typically associated with subclinical infections, and most isolations of aquareoviruses occur incidentally during routine virus screening for other, more significant pathogens (Crane and Carlile, 2008). However, many of these viruses also play a significant role in the morbidity and mortality of fish populations. Therefore, molecular characterization of each new aquareovirus isolate is essential in order to quickly identify the virus so that countermeasures can be executed to prevent further spread of the disease. The control of aquareovirus infections requires the use of chemical disinfectants to destroy the virus in hatcheries, tanks, ponds, effluent water or equipment. Detection of the AGCRV in golden shiners still in the hatchery was considered evidence for vertical transmission (Goodwin et al., 2010). Rivas et al. (1996) examined the susceptibility of TRV to five commercially available disinfectants. Complete inactivation of virus was obtained with 2% formalin within 5 min and with 42.5% isopropanol within 15 min at 15°C. Although chlorine and iodine are recognized as potent virucidal agents, with a wide range of applications in fishery management (Finlay, 1978), they were not effective at inactivating TRV (Rivas et al., 1996).

14.2.6.2 Regulatory Measures

AGCRV was removed from the Significant Diseases/Pathogens List of Title 14, California Code of Regulations because there was no documented impact on any California fish populations due to the presence of this virus; no literature was found that discussed the economic impacts associated with disease outbreaks or management processes (Fischer, 2008). In contrast, GCRV is one of the major fish pathogens for freshwater species (catfish, carp, etc.) in Asia.

14.2.6.3 Vaccines and Vaccination

Vaccination has been attempted mostly in China since 1960s to control GCRV (reviewed by Rao and Su, 2015), the most pathogenic aquareovirus (Samal et al., 1998) and the cause of grass carp hemorrhagic disease. The different vaccine formulations to date include inactivated and attenuated vaccines. Experimental recombinant subunit vaccines containing GCRV outercapsid proteins VP5 and VP7 expressed in *Escherichia coli* (Shao et al., 2011) and recombinant VP4 of GCRV-GD108 (Tian et al., 2013), and DNA vaccines of GCRV VP6 (Zhou et al., 2014) and VP7 (Zhu et al., 2015) are under development. Vaccine development is also being undertaken due to the devastating losses caused by the marine threadfin aquareovirus (TFV) and guppy aquareovirus (GPV) in Singapore's fish farming industry (Sim et al., 2004; Seng et al., 2005a,b).

Two recombinant proteins representing the inner and outer coat proteins of TFV (~75 and ~35 kDa, respectively) were used to study their effects on the immunity of freshwater guppy *Poecilia reticulata*, against GPV. Immunization of guppy with the proteins resulted in the production of neutralizing antibodies. The neutralization titers were significantly higher in guppy immunized with a combination of both proteins than with either protein alone (Sim et al., 2004).

14.2.6.4 Novel Antiviral Strategies

RNA interference, a highly conserved gene-silencing mechanism caused by dsRNA in both plants and animals (Li et al., 2013), can suppress GCRV replication both in vitro and in vivo (Shen et al., 2013).

14.3 PISCINE ORTHOREOVIRUS

14.3.1 Virion Properties

The genome segments and functions of the PRV proteins are summarized in Table 14.5 (Kibenge et al., 2013). Table 14.4 compared the terminal conserved sequences of members of the *Aquareovirus* genus to those of members of the *Orthoreovirus* genus, PRV, *Cardoreovirus* and *Crabreovirus* supporting the designation of PRV to a separate genus (Kibenge et al., 2013). PRV was recently assigned to the genus *Orthoreovirus* (Rimstad and Wessel, 2015).

Kibenge et al. (2013) used phylogenetic analysis of the Seg-S1 genes, for which the largest number of complete sequences from different "isolates" is available, to group Norwegian PRV strains into a single genotype, Genotype I, with subgenotypes, Ia and Ib; the Canadian PRV strains matched subgenotype Ia and Chilean PRV strains from Atlantic salmon matched subgenotype Ib. This classification appears to be very stable because 60 additional Norwegian PRV Seg-S1 sequences (Garseth et al., 2013a) supported this grouping (Godoy et al., 2016). The recent Chilean PRV strains from

TABLE 14.5 The dsRNA Genome Segments and Proteins of Piscine Orthoreovirus (PRV)

Genome Segment[a]	dsRNA (Size, bp)[b]	5′UTR (bp)[b]	3′UTR (bp)[b]	Protein Name[c]	Protein Size (aa) and Mass (kDa)[d]	pI Value	Predicted Protein Function (and Location)
L1	3911	18	44	Core shell (*T*=1) [λ1]	1282, 141.41 kDa	5.47	Major inner capsid protein, Helicase, RNA triphosphatase (core)
L2	3935	18	44	Core turret [λ2]	1290, 143.75 kDa	4.81	Core spike, guanylyl transferase, methyltransferase "turret" protein (core spike)
L3	3916	7	48	Core RdRp [λ3]	1286, 144.24 kDa	8.68	Minor inner capsid protein, RNA polymerase (core)
M1	2383	21	79	Core NTPase [μ2]	760, 86.09 kDa	8.23	Minor inner capsid protein, nucleoside triphosphate phosphohydrolase (core)
M2	2179	26	89	Outer shell (*T*=13) [μ1]	687, 74.26 kDa	6.27	Outer capsid protein, membrane penetration, apoptosis (outer capsid)
M3	2403	83	61	NS factory [μNS]	752, 83.53 kDa	5.00	NS, binds ssRNA and virus cores, genome packaging? (nonstructural)
S1	1081	28	60	Outer clamp [σ3]	330, 37.08 kDa	7.43	Major outer capsid protein, dsRNA binding protein, translation control, modulation of cellular interferon, zinc-binding (outer capsid)
				NS, p13 [σ1s]	124, 12.99 kDa	4.88	NS, block cell-cycle progression, cytolytic in PRV (nonstructural)
S2	1329	21	45	Core clamp [σ2]	420, 45.93 kDa	9.02	Major inner capsid protein, morphogenesis? (core)
S3	1143	28	50	NS RNA [σNS]	354, 39.07 kDa	7.76	NS, ssRNA-binding, genome packaging? (nonstructural)
S4	1040	38	54	Outer fiber [σ1]	315, 34.60 kDa	6.04	Outer capsid protein, virus attachment, cell tropism, pathways of viral spread in vivo, virulence (outer capsid)

[a] *Genome segment nomenclature used by Palacios et al. (2010) for PRV.*
[b] *Values obtained from cDNA sequenced (Kibenge et al., 2013). The 3′UTR does not include the stop codon.*
[c] *Protein nomenclature used by Key et al. (2013) for PRV. Homolog of Mammalian orthoreovirus protein is given in square brackets.*
[d] *PRV gene products are calculated from sequence data (Kibenge et al., 2013).*

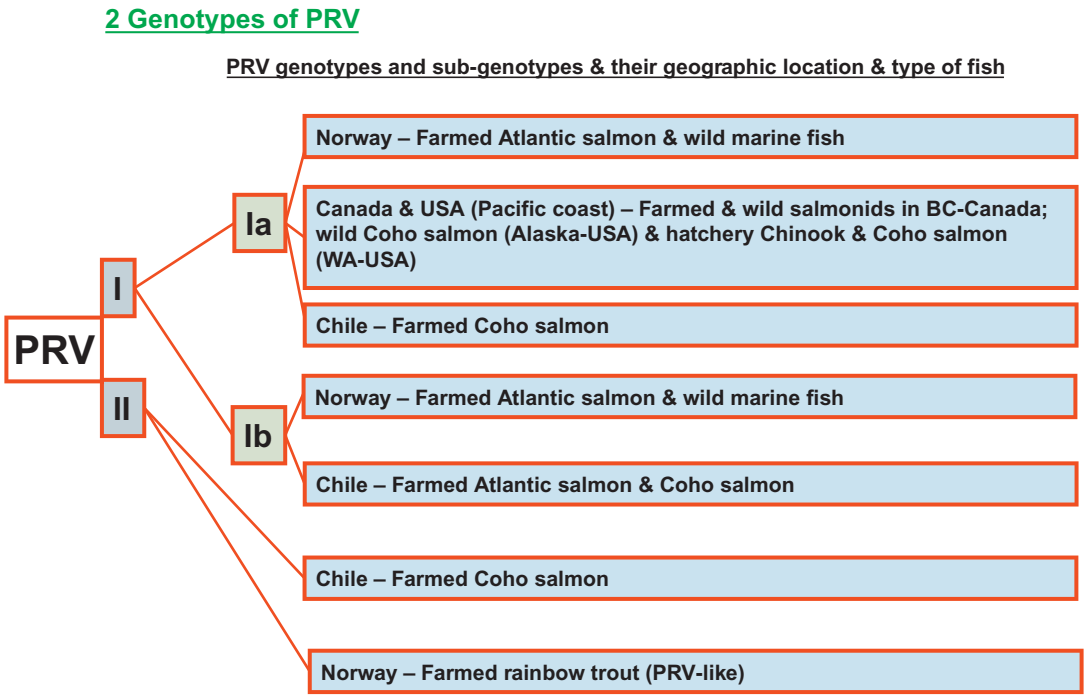

FIGURE 14.4 Genetic diversity of PRV "isolates" based on phylogenetic analysis of the Seg-S1 genes and their geographical distribution worldwide. *PRV*, piscine orthoreovirus. *Godoy et al., 2016.*

coho salmon are more genetically diversified than those from Atlantic salmon, and some form a distinct new phylogenetic cluster, designated Genotype II that includes the PRV-related virus associated with the new HSMI-like disease in rainbow trout (Godoy et al., 2016) (Fig. 14.4).

14.3.2 Clinical Significance

PRV is associated with HSMI (Palacios et al., 2010), an emerging disease of marine-farmed Atlantic salmon in Norway and Scotland. HSMI was first recognized in 1999 in western Norway (Kongtorp et al., 2004a) and subsequently in Scotland (Ferguson et al., 2005), Chile (Godoy et al., 2016) and Ireland (Biering and Garseth, 2012). The virus was first identified through high-throughput pyrosequencing of clinical samples from fish experimentally injected with tissue homogenate from HSMI-diseased fish, and viral RNA was unequivocally detected using in situ hybridization in the experimentally reproduced HSMI lesions (Palacios et al., 2010). The virus can be propagated in the GF-1 cell line and cardiac and skeletal muscle pathology typical of HSMI can be reproduced in naïve Atlantic salmon by experimental inoculation with the supernatant from cell culture passaged PRV (Mikalsen et al., 2012). Finstad et al. (2012) used immunohistochemical staining to demonstrate a correlation between PRV antigens and HSMI lesions in the hearts of Atlantic salmon coinciding with the course of HSMI, thereby strengthening the hypothesis of PRV being the causative agent of HSMI. Recently, Atlantic salmon erythrocytes have been shown to be major target cells for PRV replication, accompanied by cytoplasmic inclusions (Finstad et al., 2014), which resemble those described in erythrocytic inclusion body syndrome (EIBS) (Leek, 1987; Michak et al., 1992). Most recently, integrated technologies were used to diagnose a potential HSMI in farmed Atlantic salmon samples collected from an aquaculture facility in 2013–2014 in British Columbia-Canada (DFO, 2016). Other reports doubt the pathogenicity of PRV, describing PRV as an opportunistic (Løvoll et al., 2010; Wiik-Nielsen et al., 2012a) or nonpathogenic virus (Garseth et al., 2013b; Marty et al., 2015).

The ambiguity in the pathogenicity of PRV may be in part due to the nature of samples studied and the inability to fulfill Koch's (River's) postulates (Rivers, 1937). The clinical relevance of samples used to determine PRV pathogenicity has never been in question, and yet a majority of samples used previously in PRV surveillance studies were collected for different purposes, for example, IPNV surveillance or ISAV surveillance, or pancreas disease (PD) surveillance, or general fish health surveillance. In some cases attempts have been made to reproduce the disease experimentally using fish samples

from non-clinical cases. If the correct HSMI samples are used in the analyses, it may be possible to determine conclusively the etiological role of PRV in HSMI disease.

Most recently, PRV was identified as a probable cause of melanized spots in white skeletal muscle of farmed Atlantic salmon in Norway (Bjørgen et al., 2015), further elevating the economic importance of this virus for the aquaculture industry. Furthermore, a new disease in rainbow trout similar to HSMI and associated with a PRV-related virus was also reported in Norway (Olsen et al., 2015). A specific real-time RT-PCR TaqMan assay developed for laboratory detection of the virus revealed high amounts of the virus (Ct< 25) in diseased fish at all affected farms and low amounts (Ct> 31) in low prevalence at the broodfish farms (Olsen et al., 2015). No controlled laboratory studies have been done to link the PRV-like virus to the pathological findings.

14.3.3 Geographical Distribution and Species Susceptibility

PRV is widespread in Norwegian salmon aquaculture (Løvoll et al., 2012) and is also found in wild fish populations of Atlantic salmon (Palacios et al., 2010). The virus was found in escaped farmed Atlantic salmon in a nearby river shortly after escaping, reinforcing the potential for its spread to wild salmonids (Madhun et al., 2015). PRV was also detected in 3% of anadromous trout (sea-trout) "*Salmo trutta*" tested, but not in anadromous Arctic char "*Salvelinus alpinus*" (Garseth et al., 2013b). It has been detected by RT-qPCR at a low prevalence in certain marine fish species (Atlantic herring, Capelin "*Mallotus villosus*," Atlantic horse mackerel "*Trachurus trachurus*" and Great silver smelt "*Argentina silus*") along the coast of Norway (Wiik-Nielsen et al., 2012b). The authors speculated that there is probably a more complex relationship that involves several carriers and virus reservoirs because none of the positive samples were from fish caught close to fish farms that had registered outbreaks of HSMI (Wiik-Nielsen et al., 2012b). In Norway in 2014, the disease was diagnosed in 181 farms (171 ongrowing sites, seven hatcheries and three broodstock farms), this is an increase from 2013 when 130 ongrowing sites, three hatcheries and one broodstock farm were affected (National Veterinary Institute, 2014). The virus has also been detected by RT-qPCR in farmed Atlantic salmon in Chile (Kibenge et al., 2013), Ireland (Rodger et al., 2014) and Denmark (Mikkelsen et al., 2014), in farmed Atlantic salmon and wild chum salmon "*O. keta*," rainbow trout "*O*ncorhynchus *mykiss*" and cutthrout trout "*Oncorhynchus clarkii*" in British Columbia (Kibenge et al., 2013), in wild coho salmon from Alaska (Marty et al., 2015) and recently, in hatchery Chinook salmon "*O. tshawytscha*" and coho salmon "*Oncorhynchus kisutch*" in Washington (WFRC, 2014) and in farmed coho salmon in Chile (Godoy et al., 2016). A recent report showed that PRV is enzootic in farmed and wild salmonids on the Canada/US Pacific coast (Siah et al., 2015). In Chile, in the first half of 2015, 44 cases of PRV in Atlantic salmon were reported by diagnostic laboratories (Sernapesca, 2015).

14.3.4 PRV Growth in Cell Culture

Several attempts have been made to isolate and propagate PRV in cell culture. To date the virus is reported to grow, albeit poorly, in several fish cell lines including EPC, FHM (Kongtorp and Taksdal, 2009) and GF-1 (Mikalsen et al., 2012) derived from the fin tissue of orange-spotted grouper "*Epinephelus coioides*" (Chi et al., 1999). In all cases, the CPE could not be sustained beyond the fourth passage, and no increase in PRV titer could be demonstrated. Thus, it has not been possible to obtain virus concentrations high enough to purify or quantify the virus or visualize the virus by electron microscopy. Most recently, PRV has been shown to replicate in primary cultures of Atlantic salmon erythrocytes over 21 days for two passages (Wessel et al., 2015). The amount of PRV within infected erythrocytes was substantial and could be visualized by electron microscopy; however, it was not accompanied by cell lysis (Wessel et al., 2015); this is consistent with in vivo observations where neither a PRV infection nor an HSMI outbreak is associated with anemia (Kongtorp et al., 2004b).

14.3.5 PRV Cytoplasmic Inclusions Are Indistinguishable From Those for EIBS Virus

Electron microscopy images of cytoplasmic inclusions of PRV in Atlantic salmon erythrocytes (Finstad et al., 2014) strongly resemble those reported for EIBS in Chinook salmon erythrocytes (Meyers, 2007) and coho salmon erythrocytes (Michak et al., 1992) (Fig. 14.5). Moreover, the EIBS virus has also been associated with HSMI in Atlantic salmon from western Norway, where typical virus particles were present in vacuoles within erythrocytes and unidentified head kidney cells (Watanabe et al., 2006). Isolation of the EIBS virus from the HSMI cases was attempted on Atlantic salmon kidney cells, but the CPE could not be sustained beyond the second passage (Watanabe et al., 2006), thus preventing further characterization. It is not clear whether EIBS represents one virus or several related or unrelated viruses (Finstad et al., 2014).

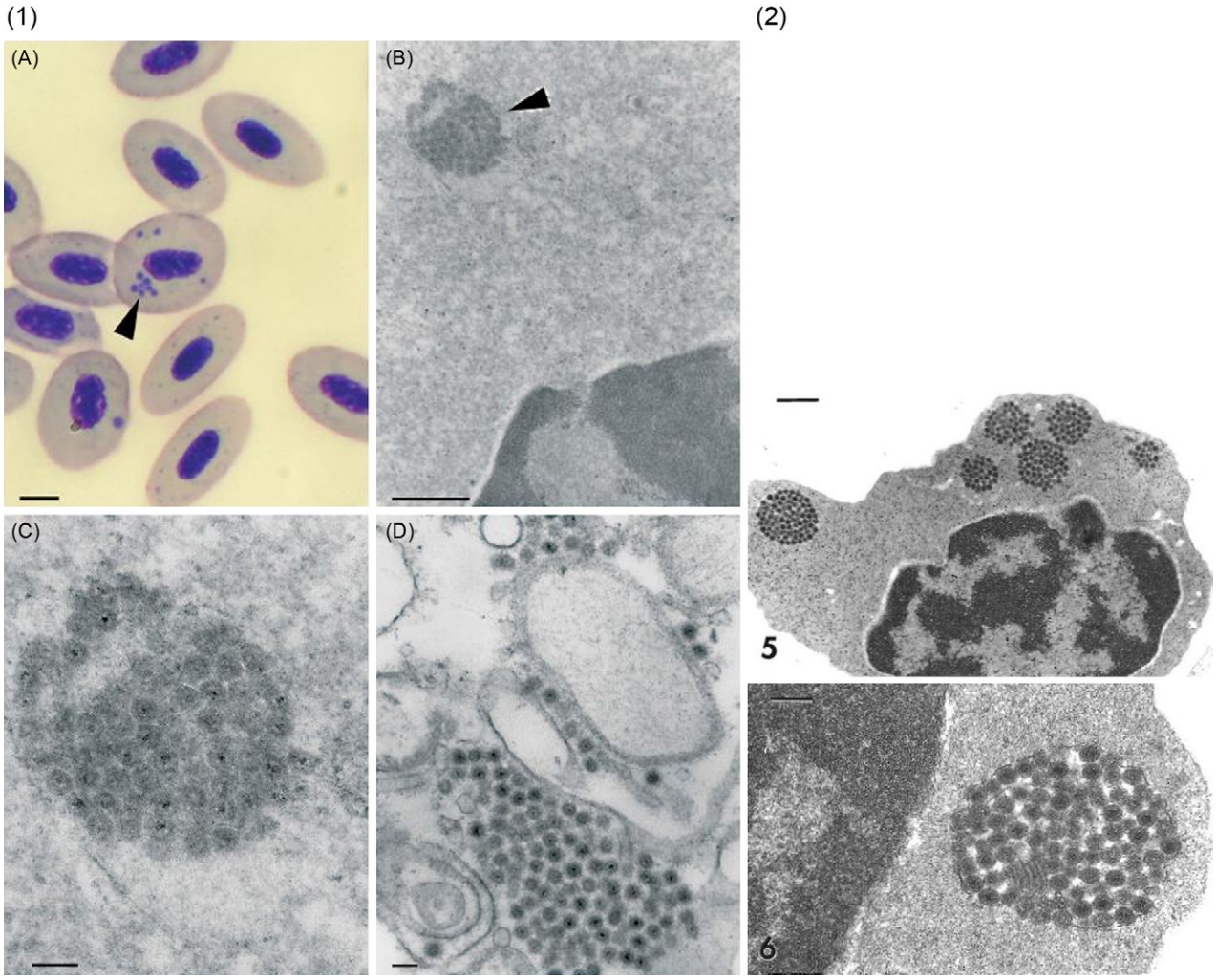

FIGURE 14.5 Cytoplasmic inclusions in fish erythrocytes. **Panel 1: EIBS virus in *Oncorhynchus tshawytscha***. (A) Peripheral blood smear with single and multiple cytoplasmic erythrocytic inclusion bodies (arrowhead). Scale bar = 5 µm. (B) TEM of a single virus inclusion body (arrowhead) in erythrocyte cytoplasm. Scale bar = 0.5 µm. (C) Magnification of virus particles (60–70 nm) in the inclusion body of (B). Scale bar = 100 nm. (D) Extracellular virus particles associated with cell debris in peripheral blood. Scale bar = 100 nm. **Panel 2: *Onchorhynchus kisutch***. Electron micrographs of blood from Lewis River Hatchery yearling coho salmon with a high prevalence of intracytoplasmic inclusions, as associated with erythrocytic inclusion body syndrome. Fig. 14.5. An atypical erythrocyte shows six viral inclusions within the cytoplasm. Bar = 0.5 µm. Fig. 14.6. Membrane-bound intact inclusion. Bar = 0.16 µm. **Panel 3: Transmission electron micrographs of infected RBC**. (A) Erythrocyte with viral inclusions (arrow). (B) Inclusions containing reovirus-like particles. (C) Inclusions with lamellar structure. (D) Inclusions with a mixture of viral particles and lamellar structures (arrowhead). Some of the inclusions are surrounded by an intact, or partially intact membrane-like structures. (E) Inclusions containing numerous reovirus-like particles. **Panel 1:** *From Meyers, T.R., 2007. First report of erythrocytic inclusion body syndrome (EIBS) in chinook salmon Oncorhynchus tshawytscha in Alaska, USA. Dis. Aquat. Organ. 76, 169–172.* Fig. 14.1. *Copyright 1 Inter-Research (2007), with permission;* **Panel 2:** *From Michak, P., Smith, C.E., Hopper, K., 1992. Erythrocytic inclusion body syndrome: a light and electron microscopic study of infected erythrocytes of chinook Oncorhynchus tshawytscha and coho O. kisutch salmon. Dis. Aquat. Organ. 12, 229–233.* Figs. 14.5–14.6. *Copyright 1 Inter-Research (1992), with permission;* **Panel 3:** *From Finstad, Ø.W., Dahle, M.K., Lindholm, T.H., Nyman, I.B., Løvoll, M., Wallace, C., Olsen, C.M., Storset, A.K., Rimstad, E., 2014. Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes. BMC Vet. Res. 45, 35.* Fig. 14.6. *Copyright 1* Finstad et al. (2014), *with permission* http://www.biomedcentral.com/download/license.pdf *no changes were made.*

14.3.6 Pathology and Epidemiology

14.3.6.1 Heart and Skeletal Muscle Inflammation

14.3.6.1.1 Clinical Signs

HSMI appears 5–9 months after Atlantic salmon are transferred from freshwater to marine pens. The affected fish cages present an increase of mortality and the most prominent signs of disease are that the fish are lethargic and usually positioned

(3)

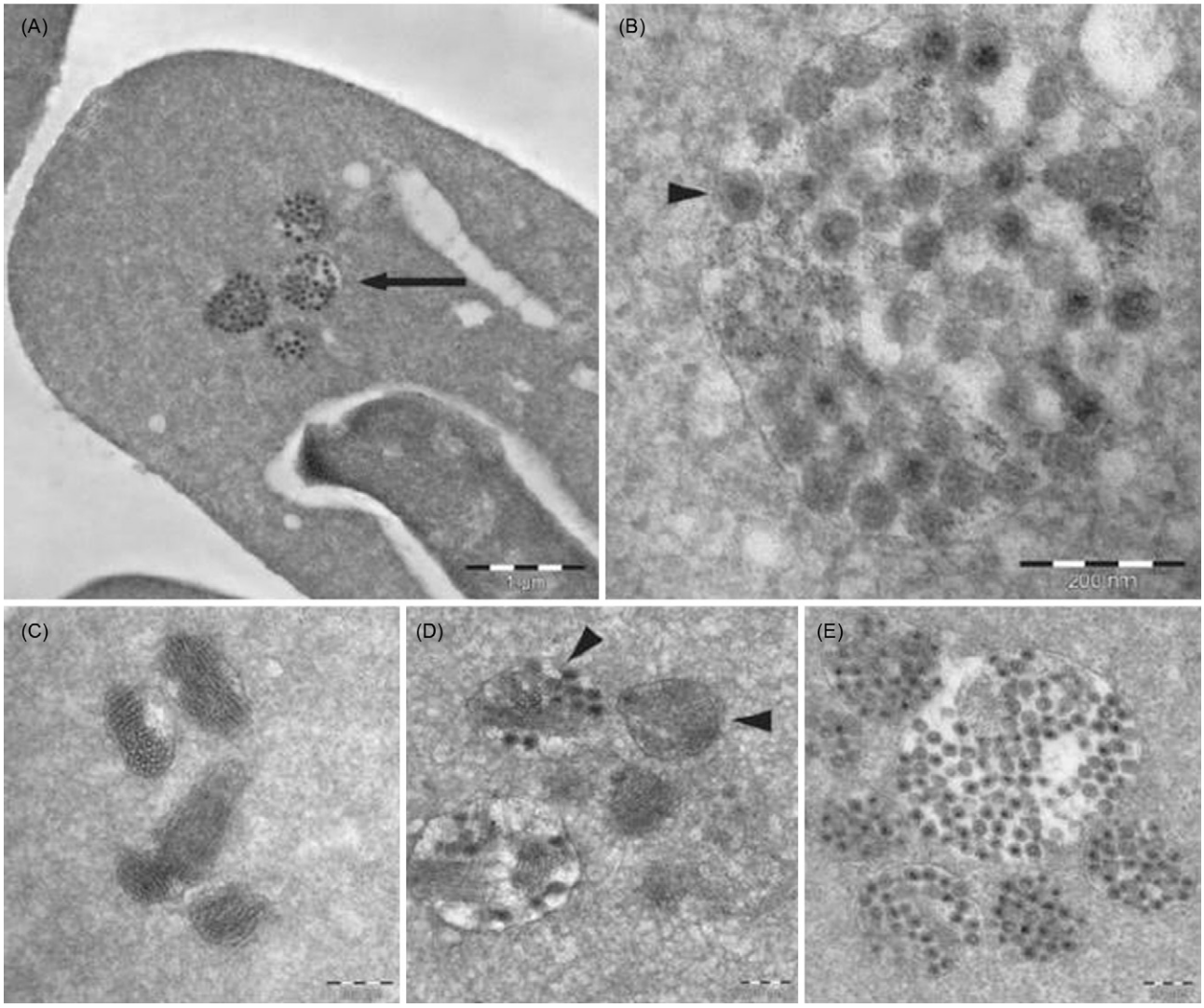

FIGURE 14.5 (Continued)

near the cage wall and facing the sea current, aberrant swimming with up to 20% mortality, but usually no external signs (Kongtorp et al., 2004a, 2006).

14.3.6.1.2 Gross Pathology

The gross findings noted in Atlantic salmon include pale heart, pale heart with loose texture, pericardial hemorrhage, hemopericardium, hepatomegaly, pale to yellow liver, nutmeg liver, liver petechial hemorrhaging, fibrinous coat on the liver surface (Fig. 14.6), splenomegaly, ascitic fluid and petechiae in the perivisceral fat and pale gills, raised scales and exophthalmia (Kongtorp et al., 2004a, 2006; Ferguson et al., 2005, Watanabe et al., 2006) petechial hemorrhaging in gas bladder and jaundice (Godoy et al., 2016). HSMI-like disease was also reported in farmed coho salmon in Chile. Grossly, affected coho salmon had pale gills, hemopericardium, pale hearts and yellow liver (Godoy et al., 2016).

14.3.6.1.3 Histopathology

The most significant histopathological lesions of HSMI are found in heart and red skeletal muscle (ie, myocarditis and necrosis of red skeletal muscle) (Fig. 14.7). In the heart, degeneration and necrosis of muscle fibers and a massive inflammatory response involves both compact and spongy layers of the ventricle. Infiltrations are mainly mononuclear. An extensive epicarditis is usually found in association with myocarditis. Myocardial degeneration and necrosis is observed in

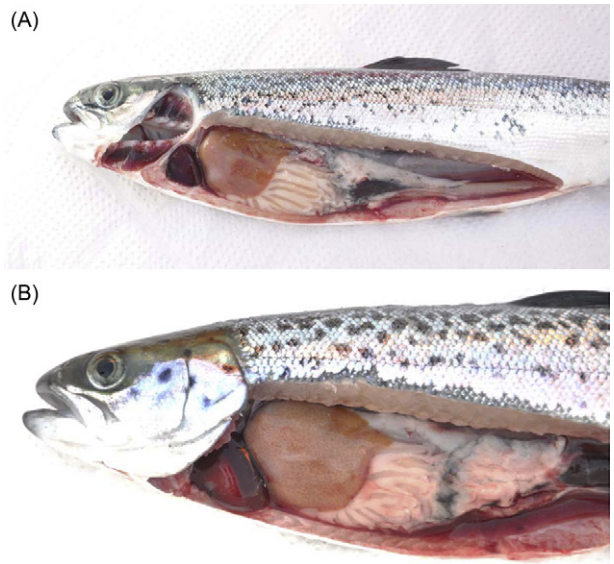

FIGURE 14.6 Marine-farmed Atlantic salmon (*Salmo salar*) in Chile affected with HSMI. (A) Fish presenting with hemopericardium and fibrinous coat on the liver surface. (B) Fish presenting with hemopericardium, fibrinous coat on the liver surface and nutmeg liver. *Courtesy of Dr. Marcos G. Godoy, CIBA, Puerto Montt, Chile.*

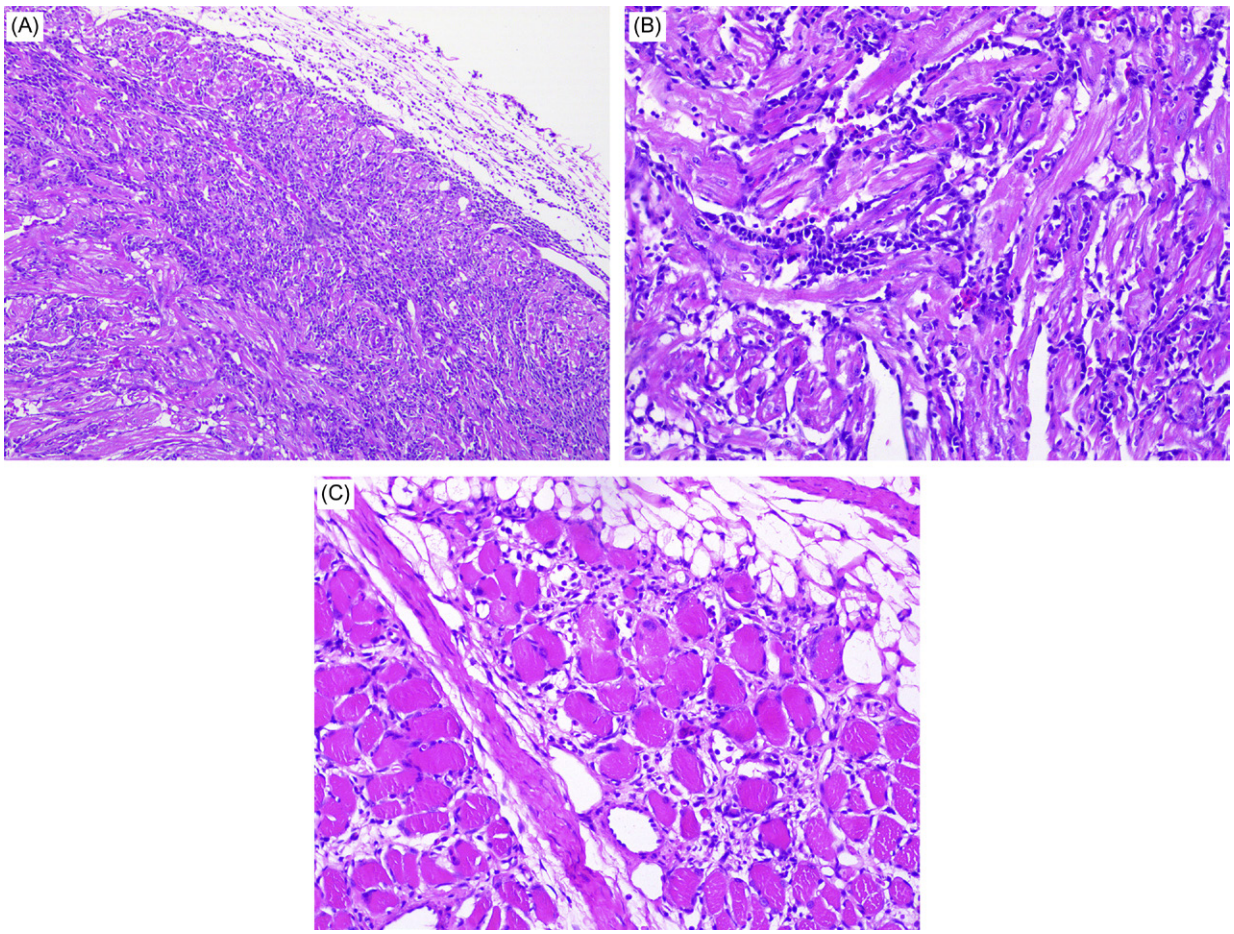

FIGURE 14.7 Marine-farmed Atlantic salmon (*Salmo salar*) in Chile affected with HSMI. (A) Micrograph of cardiac lesion (H&E, 100X). Ventricle showing severe diffuse epicarditis and myocarditis. (B) Micrograph of cardiac lesion (H&E, 200X). Ventricle showing severe diffuse myocarditis. (C) Micrograph of red muscle lesion (H&E, 200X). Muscle showing severe diffuse degeneration and myositis. *Courtesy of Dr. Marcos G. Godoy, CIBA, Puerto Montt, Chile.*

advanced stages of the disease. Red muscle inflammation follows the same pattern as seen in the heart, but is not a consistent finding; affected myocytes show signs of degeneration, loss of striation and eosinophilia, vacuolation, central nuclei localization and karyorhexis. Other lesions include focal liver necrosis and circulatory disturbances, such as edema and erythrocyte accumulation in several organs (Kongtorp et al., 2004a,b, 2006; Ferguson et al., 2005; Kongtorp and Taksdal, 2009; Watanabe et al., 2006; Finstad et al., 2012; Yousaf et al., 2012, 2013; Yousaf and Powell, 2012). The histopathology lesions in coho salmon with HSMI-like disease were different from those reported in Atlantic salmon, and in rainbow trout with the new HSMI-like disease in Norway. In the Chilean coho salmon cases, the heart inflammation was diffuse and presented generally in the spongy layer; the red muscle inflammation was mild and not always present (Godoy et al., 2016).

14.3.6.2 Epidemiology

14.3.6.2.1 Transmission

HSMI appears to be a severe disease with high morbidity and prolonged duration. A gradual increase in lesion severity in the months prior to onset of clinical signs indicates that infection may be present subclinically in the farm for a very long time (Kongtorp and Taksdal, 2009). Horizontal transmission has been confirmed in cohabitant transmission trials (Kongtorp et al., 2004b; Kongtorp and Taksdal, 2009). Vertical transmission is less studied and it thus appears that vertical transfer is not a major route of transmission for PRV (Wiik-Nielsen et al., 2012b).

14.3.6.2.2 Risk Factors

Aldrin et al. (2010) showed the effect of distance to an infectious fish farm was positive and significant for all diseases, implying that the risk involved with proximate infectious fish farms increased with decreasing distance. There was a significant effect of sharing a contact network with an infectious farm and there was a significant effect of previous infected cohorts on the same farm. Kristoffersen et al. (2013) showed that the risk of developing HSMI increased with increasing cohort lifespan, increasing infection pressure and increasing cohort size, and was mostly low for cohorts grown on farms in southern Norway, high for farms in mid-Norway and variable for farms in northern Norway (based on the geo-index). The final model was used to explore three different scenarios with regards to the risk of developing HSMI, and to calculate the probability for each cohort of developing HSMI, independent of their actual disease status.

The possibility of PRV transmission by marine species cannot be excluded; Wiik-Nielsen et al. (2012b) describe the detection of PRV sequences from marine fish.

14.3.7 Immunity

PRV infection was shown to induce expression of the antiviral Mx protein and a cell-mediated immune response in the heart (CD8, IFNγ and granzyme A genes were significantly upregulated, likely accounting for the myocarditis lesion) (Mikalsen et al., 2012). The Mx expression and the T-cell response are probably important in controlling PRV replication in infected fish. Atlantic salmon erythrocytes, target cells for PRV replication (Finstad et al., 2014), may have a direct role in immune response (Morera and Mackenzie, 2011). Previously, Workenhe et al. (2008) were the first to report that Atlantic salmon erythrocytes produced IFN-α in response to ISAV. Wessel et al. (2015) have since shown that Atlantic salmon erythrocytes generate antiviral immune gene activation after PRV infection, with significant upregulation of IFN-α, RIG-I, Mx and PKR genes.

14.3.8 Diagnostic Methods

A diagnosis of HSMI is based on histological findings of myocarditis affecting the epi- and endocardium, myocardial necrosis, myositis and necrosis of red skeletal muscle (Kongtorp et al., 2004b). Most recently, integrated technologies were used to diagnose a potential HSMI in farmed Atlantic salmon samples collected from an aquaculture facility in 2013–2014 in British Columbia-Canada (DFO, 2016). The PRV prevalence surveillance is exclusively conducted by screening for PRV genetic material by RT-PCR. Viability of the virus is not typically assessed. To date, PRV is reported to grow, albeit poorly, in several fish cell lines (refer to Section 14.3.4), but the virus is readily detected by RT-PCR (Palacios et al., 2010; Kibenge et al., 2013) or by immunohistochemistry (Finstad et al., 2012). HSMI is an important differential diagnosis for pancreas disease (McLoughlin and Graham, 2007) and cardiomyopathy syndrome (Ferguson et al., 1990).

14.3.9 Prevention and Control

14.3.9.1 Control and Regulatory Measures

HSMI, in association with PRV infection, has only been reported in Norway (Palacios et al., 2010; Mikalsen et al., 2012) and Chile (Godoy et al., 2016). HSMI was removed from the National list 3 and is therefore now defined as not notifiable

in Norway (National Veterinary Institute, 2014). In Chile, HSMI will be recommended to be included on the List 3 of high-risk diseases (Anonymous, 2012), thus ensuring its surveillance in Chilean aquaculture. The virus is ubiquitous and HSMI in farmed Atlantic salmon appears to depend on the existence of other factors (including environment, stress, PRV/host contact types, PRV infection titer). These factors do not appear to be present on the pacific coast of North America, and the virus is considered to pose a low risk to wild species of Pacific salmonids (The Pacific Northwest Fish Health Protection Committee, and Meyers, 2014). HSMI is not listed by OIE.

14.3.9.2 Vaccines and Vaccination

The large number of HSMI outbreaks in Norway has fueled the development of a PRV vaccine, but none is yet available commercially. A patent was filled by the discoverers of PRV (Lipkin et al., 2011).

14.4 GENUS CARDOREOVIRUS

The genus *Cardoreovirus* consists of *E. sinensis* reovirus 905 (*Es*RV905) isolated from a freshwater crab, the Chinese mitten crab *E. sinensis* in China (Zhang et al., 2004). *Carcinus mediterraneus* W2 virus (*Cc*RV-W2) and *Macropipus depurator* P virus (*Md*RV-P) (Montanié et al., 1993) in marine crabs are also listed as possible members of the genus *Cardoreovirus*, but are not yet approved as species (Attoui et al., 2012), and differ from *Es*RV905 in cytopathology, virus size and electrophoresis pattern (Zhang et al., 2004). The cardoreovirus genome consists of 12 segments of linear dsRNA, and members have only been isolated from diseased crabs. The *Es*RV particles are 55 nm in diameter, and their morphology is similar to members of the subfamily *Sedoreovirinae* with which the genus *Cardoreovirus* clusters in phylogenetic analysis using amino acid sequences of the putative RdRp (Attoui et al., 2012).

*Es*RV905 was associated with "trembling disease (TD)," an economically important disease in farmed Chinese mitten crab. Clinical signs of the disease include trembling of the legs, sluggishness and loss of appetite. The virus infects connective tissue of the gills, midgut, hepatopancreas, and hemocytes, and mortality reached 30% in experimental infection using hemolymph of diseased crabs filtered at 0.22 µm; however, the trembling clinical signs were not reproduced in crabs (Zhang et al., 2004).

14.5 PROPOSED GENUS *CRABREOVIRUS*

Mud crab reovirus (MCRV), the causative agent of "sleeping disease" (SD) in the mud crab *S. serrata* (Weng et al., 2007), has a genome of 12 segments of linear dsRNA, but is distinct from members of genus *Cardoreovirus* (Zhang et al., 2004), and is proposed to belong to a new genus in family *Reoviridae*, tentatively named *Crabreovirus* (Deng et al., 2012). A second virus, *Ss*RV has been isolated from the same disease outbreaks, also referred to as "waterclear disease" (CD) (Chen et al., 2011). A third member of virus of the proposed genus *Crabreovirus* (Fig. 14.1), is *Callinectes sapidus* reovirus 1 (CsRV1, also called RLV for reo-like virus) from blue crab *C. sapidus* (Bowers et al., 2010); its 12 genome segments were recently sequenced using next-generation sequencing (Flowers et al., 2016). The *Cs*RV1 RNA-dependent RNA polymerase sequence is similar to those of *Ss*RV, *Macropipus depurator* P virus (*Md*RV-P; P from Paralysis virus) from Mediterranean swimming crab *M. depurator* (Vago, 1966), and *E. sinensis* reovirus WX-2012 (*Es*RV) from the Chinese mitten crab *E. sinensis* in China with "TD" (Shen et al., 2015). Other reoviruses of crabs with properties similar to MCRV include *C. mediterraneus* W2 virus (*Cc*RV-W2; W from W virus found in Weymouth) from shore crab *C. mediterraneus* (Mari and Bonami, 1988), although the genomes of both *Md*RV-P and *Cc*RV-W2 have not yet been sequenced (Montanié et al., 1993) and were proposed members of genus *Cardoreovirus* (Mayo and Haenni, 2006).

SD/CD is a severe disease of marine-cultured mud crab in southern China caused by MCRV and *Ss*RV (Weng et al., 2007; Chen et al., 2011). The virus, which is considered highly pathogenic, infects connective tissue cells of hepatopancreas, gills and intestines, with mortalities of approximately 70% on affected farms (Weng et al., 2007). Affected mud crabs were lethargic, anorexic, with a gray discoloration, and with an atrophied hepatopancreas, empty intestines and loose yellow gills. The disease, including a mortality of 100% within 10 days of oral or intramuscular inoculation, has been reproduced experimentally (Weng et al., 2007). Virus exposure by cohabitation resulted in 80% mortality within 20 days (Weng et al., 2007).

TD of *E. sinensis* is an economically important disease of freshwater-cultured Chinese mitten crab in Jiangsu Province, caused by *Es*RV WX-2012 (Shen et al., 2015). This is the third reovirus (after *Es*RV905 and *Es*RV816) isolated from farmed Chinese mitten crab with "TD" (Shen et al., 2015). Experimental infections with either *Es*RV905 or *Es*RV816 resulted in approximately 30% mortality, but no trembling clinical signs (Zhang et al., 2004; Zhang and Bonami, 2012).

It is not known if TD can reproduce with *Es*RV WX-2012 because no experimental infections have been reported. Near-full-length genome sequences of 12 segments of *Es*RV WX-2012 were reported (Shen et al., 2015). The total genome sequence of this virus has a 69% identity with MCRV, and both have conserved the 5′- and 3′-terminal sequences (Shen et al., 2015), supporting its placement in the proposed genus *Crabreovirus*.

In the blue crab fishery, which occurs on the Atlantic coast of the United States and in South America (Williams, 1974), *Cs*RV1 causes disease leading to high mortalities in both wild and farmed (soft shell production facilities and crab hatcheries) populations of blue crab (Bowers et al., 2010; Messick and Kennedy, 1990). Experimental infection of disease-free blue crabs resulted in 100% mortality within 15 days of injection, with hemocyte infiltration of tissues (Bowers et al., 2010), which is characteristic of reo-like virus infections in blue crabs (Johnson and Bodammer, 1975; Johnson, 1977; Tang et al., 2011). An in situ hybridization procedure with a digoxigenin-labeled probe (Tang et al., 2011) and a highly sensitive real-time quantitative RT-PCR (RT-qPCR) assay (Flowers et al., 2015) have been developed for diagnosis of *Cs*RV infection in crabs. In wild blue crab populations, *Cs*RV1 occurs at an average prevalence of 20% (Flowers et al., 2015).

The *Cc*RV-W2 was isolated from diseased shore crabs *C. mediterraneus* from the Mediterranean coasts (Mari and Bonami, 1988). Disease was experimentally reproduced by injection of partially purified W2 virus suspension previously prepared from naturally infected animals. Clinical signs included absence of aggressiveness, increasing weakness and lack of appetite; the connective tissue of many organs (hepatopancreas, digestive tract, gills and hematopoietic organs) showed severe damage, particularly the connective tissue surrounding the tubules of the hepatopancreas, but these are not pathognomonic of reovirus infection (Mari and Bonami, 1988).

14.6 UNASSIGNED CRUSTACEAN REOVIRUSES

Since the first report of a crustacean virus by Vago (1966), many disease outbreaks associated with reovirus infections have been reported (Bonami and Zhang, 2011), but the genomes of these viruses either have not been completely sequenced to allow assigning to a genus (Zhang et al., 2015) or, as in the case of *Ss*RV (Chen et al., 2011), might have been misidentified. According to Deng et al. (2012), *Ss*RV should be considered a member of the proposed new genus *Crabreovirus*, with 12 segments of linear dsRNA, not 13 as reported by Weng et al. (2007) and Chen et al. (2011). The reclassification of *Ss*RV to *Crabreovirus* is supported by the fact that the conserved 5′ and 3′-terminal nucleotide sequences of *Ss*RV are similar to those of members of *Crabreovirus* (see Table 14.4).

Eriocheir sinensis reovirus 816 (*Es*RV816) is the second reovirus (after *Es*RV905) isolated from the Chinese mitten crab *E. sinensis* in China with "TD" (Zhang and Bonami, 2012). This virus differs from *Es*RV905 and members of the genus *Cardoreovirus* in virion size (it is ~60 nm in diameter), in having only 10 segments of linear dsRNA, and in causing cytoplasmic eosinophilic inclusion bodies in connective tissues (Zhang and Bonami, 2012). However, the virus had similar pathogenicity to *Es*RV905 in causing approximately 30% mortality, but no trembling clinical signs in experimental infection.

With regard to shrimp, reoviruses appear to have limited pathogenicity, occurring either in healthy shrimp or in mixed infections with other *bona fide* crustacean viral pathogens (reviewed by Krol et al., 1990). Unlike crab reoviruses, which target connective tissues, all the shrimp reoviruses target epithelial hepatopancreatic cells (Zhang et al., 2015). The reo-like virus found in white shrimp *Penaeus vannamei* was said to resemble *Cc*RV-W2 from shore crab *C. mediterraneus* (Krol et al., 1990).

*Mn*RV, which has been associated with severe mortality in farmed freshwater prawn (oriental river prawn) *M. nipponense*, has a genome of 10 segments of dsRNA, but is not yet assigned to a genus (Zhang et al., 2015). It is not included in the phylogenetic tree that shows the relationships within family *Reoviridae* (Fig. 14.1) because the available sequence of 250 nt (GenBank Accession no. KM405244) is too short. In a preliminary analysis, *Mn*RV clustered with *Mimoreovirus*/NC_008172 (personal observations). A specific conventional RT-PCR has been developed for detection of *Mn*RV (Zhang et al., 2015).

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Chapter 15

Birnaviruses of Aquatic Organisms

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15.1 INTRODUCTION

The *Birnaviridae* family derives its name from the bisegmented, double-stranded RNA (dsRNA) viruses, which are composed of segments A and B. The *Birnaviridae* family has four genera: (1) *Avibirnavirus*, of which the type species is infectious bursal disease virus (IBDV), which infects domestic and wild birds (Fauquet et al., 2005; Kasanga et al., 2008); (2) *Aquabirnaviruses*, isolated from different fish and shellfish species, with infectious pancreatic necrosis virus (IPNV) being the type species (Bovo et al., 1984); (3) *Entomobirnavirus*, in which drosophila X virus of the fruit fly (*Drosophila melanogaster*) is the type species (Chung et al., 1996; Dobos and Roberts, 1983); and (4) *Blosnarvirus*, with the type-specific strain being blotched snakehead virus (Fauquet et al., 2005; King et al., 2011). Apart from being bisegmented, another feature that separates the birnaviruses from other segmented viruses (such as the reoviruses) is that the 5′ terminus for segments A and B of the former's genome is linked to the viral protein (VPg) (Calvert et al., 1991; Muller and Nitschke, 1987; Revet and Delain, 1982). Given that this chapter deals with only the birnaviruses of aquatic organisms, the focus will be on *Aquabirnaviruses*, the only genus whose members infect aquatic organisms.

From about 2002 to 2003, there have been attempts to redefine the nomenclature of aquabirnaviruses infecting fish, especially focusing on which isolates should be called IPNV (Rimstad, 2003). This was against the background that isolates infecting nonsalmonid species did not yield classical pancreatic necrosis. It was suggested, therefore that only species that infect or cause disease in salmonid should be referred to as IPNV, while all the others were to be called marine aquabirnaviruses (MABV) (Rimstad, 2003). The International Committee on the Taxonomy of Viruses (ICTV) (King et al., 2011), however, subdivided the aquabirnaviruses into three viral species based on susceptible host species infected by the viruses, namely (1) IPNV, as the type species for *Aquabirnaviruses*; (2) yellowtail ascites virus (YTAV), which causes diseases in yellowtail fish (*Seriola quinqueradiata*) (Hirayama et al., 2007; Kobayashi, 2010; Sorimachi and Hara, 1985); and (3) Tellina virus 1 (TV-1), which infects *Tellinus telius* mollusks (King et al., 2011; Brown, 1986; Dobos et al., 1979; Gudding, 2014; Nobiron et al., 2008). In addition, MABV have been identified that infect a wide range of fish, crustaceans and mollusks (Bovo et al., 1984; Dobos and Roberts, 1983; Rodriguez Saint-Jean et al., 2003).

15.2 VIRAL PROPERTIES OF AQUABIRNAVIRUSES

The viral properties of aquabirnaviruses can be divided into (1) biophysical properties, (2) genome organization and (3) structural chemistry, as discussed next.

15.2.1 Biophysical Properties of Aquabirnaviruses

The biophysical properties of IPNV, which is the type species of aquabirnaviruses, has been investigated in detail by Dobos and Roberts (1983). The virus has a hydrodynamic diameter of 64.0 nm and an electron microscopy diameter of 60 nm composed of an icosahedron capsid (Dobos et al., 1979). The inner core is approximately 45 nm in diameter (Dobos and Roberts, 1983). It has an average molecular weight (MW) of 55×10^6 and a buoyant density of CsCl of 1.33 g/cm^3, and it is stable at pH values 3–9 (Dobos, 1976). It is resistant to 1%SDS at 20°C and pH 7.5 for 30 min (Dobos, 1976). IPNV has two segments consisting of molecular weights 2.5×10^6 and 2.3×10^6, respectively, which sediment as a 14S RNase-resistant component in sucrose gradients (Dobos, 1976). Similarly, TV has hydrodynamic and electron microscope diameters of 64.0 nm and 58.8 nm, respectively. It sediments at a buoyance density of 1.32 g/mL in CsCl (Underwood et al., 1977), and its two segments have MW of 2.5×10^6 and 2.3×10^6 in sucrose gradients, respectively, similar to IPNV (Dobos et al.,

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00015-2
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1979). In general, these properties show that aquabirnaviruses have very close physical and biochemical properties that make them belong to the same family and genus.

15.2.2 Genome Organization of Aquabirnaviruses

In terms of genome organization, IPNV has double-stranded linear segments A and B. Segment A consists of 3092 bp, containing two open reading frames (ORFs). The short ORF encodes the VP5 (17-kDa) nonstructural protein. The long ORF encodes a 104-kDa fragment, polyprotein NH_2-pVP2-VP4-VP3-COOH, which is cotranslationally cleaved by the VP4 protease to produce the precursor pVP2 (62 kDa), VP4 (29 kDa) and VP3 (31 kDa), and an X protein for TV-2 (Duncan and Dobos, 1986; Duncan et al., 1987). The pVP2 is further processed at its C-terminus by VP4 to produce VP2 (54 kDa). VP2 forms the outer capsid and small fragments of viral peptides (P1, P2 and P3). Fig. 15.1 shows a comparison of genome organizations for IPNV, YTAV and TV-1. Note that TV-1 has an *X* gene insertion between pVP2 and VP4, while the genome organizations of IPNV and YTAV are similar (Fig. 15.1A). The VP3 surrounds the inner surface of the VP2 capsid (Fig. 15.1B). The VP4 is a protease whose cleavage sites are derived from the conserved Ser-Lys catalytic dyad found in all birnaviruses. The YATV and IPNV cleavage sites are located at $Ala^{508} \downarrow Ser^{509}$ for the pVP2-VP4 junction and $Ala^{734} \downarrow Ser^{735}$ for the VP4-VP3 junction, while the cleavage sites for the TV are shown in Fig. 15.1C.

Segment B of IPNV consists of a 2784-bp single large ORF that is approximately 2900 bp long with a molecular mass 94 kDa (Fig. 15.1B). It codes an internal polyprotein that forms the putative RNA-dependent RNA polymerase (RdRp) (Dobos, 1995). It exists in two forms: as a free polypeptide and as a genome-linked protein (VPg) (Dobos, 1995). Calvert et al. (1991) have shown that the IPNV VPg is linked to the 5′ terminus of each genome segment by a ser-5-GMP phosphodiester bond. For aquabirnaviruses, in vitro RdRp activity for VP1 has been demonstrated for IPNV and TV-2 (Dobos, 1976; Shwed et al., 2002).

15.2.3 Structural Chemistry of Birnaviruses

The crystal structure for birnaviruses has been resolved using IBDV as the prototype model (Coulibaly et al., 2010; Letzel et al., 2007). These studies show that the outer surface of birnaviruses is made of VP2, which spontaneously self-assembles into an outer capsid upon cleavage of the pVP2-VP4-VP3 polyprotein. The VP2 capsid is made of a T = 13*laveo* icosahedron structure comprising 260 trimmer subviral particles (SVPs). Each SVP folds into three distinct domains comprising (1) the base made of NH_2 and C-terminus, (2) a shell made of conserved β-sheets and (3) outer spikes made of surface loops that form the hypervariable regions (HVRs) anchored on β-sheets (Fig. 15.2A). Feeding the atomic model of IPNV into the electron density map of the IBDV crystal structure shows that the outer surface of IPNV is exclusively made of VP2 (Coulibaly et al., 2010). Coulibaly et al. (2010) have shown that IPNV-SVPs, which form the outer capsid, have a similar structural layout as IBDV-SVPs. However, they have pointed out that the layout of the surface loops on the IPNV-HVRs is different from the layout for IBDV-HVR surface loops. For IPNV, surface loops that form the HVRs project outward, unlike IBDV, whose surface loops project inward (Coulibaly et al., 2010). As a result, amino acids responsible for virulence (Santi et al., 2004), immunogenicity (Munang´andu et al., 2013c) and cell culture adaptation (Song et al., 2005) for IPNV are strategically located on the outer surface loops, which project outward, making them easily accessible for binding to antibodies and cell surface receptors.

Combining sequence alignments with structural analysis of the VP2 capsid for different aquabirnavirus reference strains show that the majority of amino acids that code for antigenic diversity are located on the VP2-HVRs (Coulibaly et al., 2010). Detailed analysis of individual loops that form the VP2-HVR shows that Loop P_{DE} spans a fragment of eight amino acids with seven positions having different amino acids and one conserved position, while Loop P_{FG}, which is equally short, spans a fragment of seven amino acids with six positions having different amino acids and one conserved position. On the contrary, loops P_{BC} and P_{HI} span longer fragments with more conserved positions (Fig. 15.2B). In terms of structural layout, it is interesting to note that the more divergent loops P_{DE} and P_{DG} are located on the outer surface, while the less divergent loops P_{BC} and P_{HI} are located inward, suggesting that the antigenic diversity of aquabirnaviruses is highly influenced by amino acids found on the outer surface of HVRs (Fig. 15.2B). While crystallography has paved the way to a better understanding of the protein chemistry of aquabirnaviruses, its combination with the reverse genetics system developed for IPNV (Santi et al., 2004), which is the type species for aquabirnaviruses, is bound to enhance our understanding of the chemical properties of virulence motifs responsible for inducing clinical disease in susceptible hosts. In addition, these advances are bound to enhance our ability to identify immunogenic residues that can produce the most highly protective vaccines for use against aquabirnavirus infections in aquaculture.

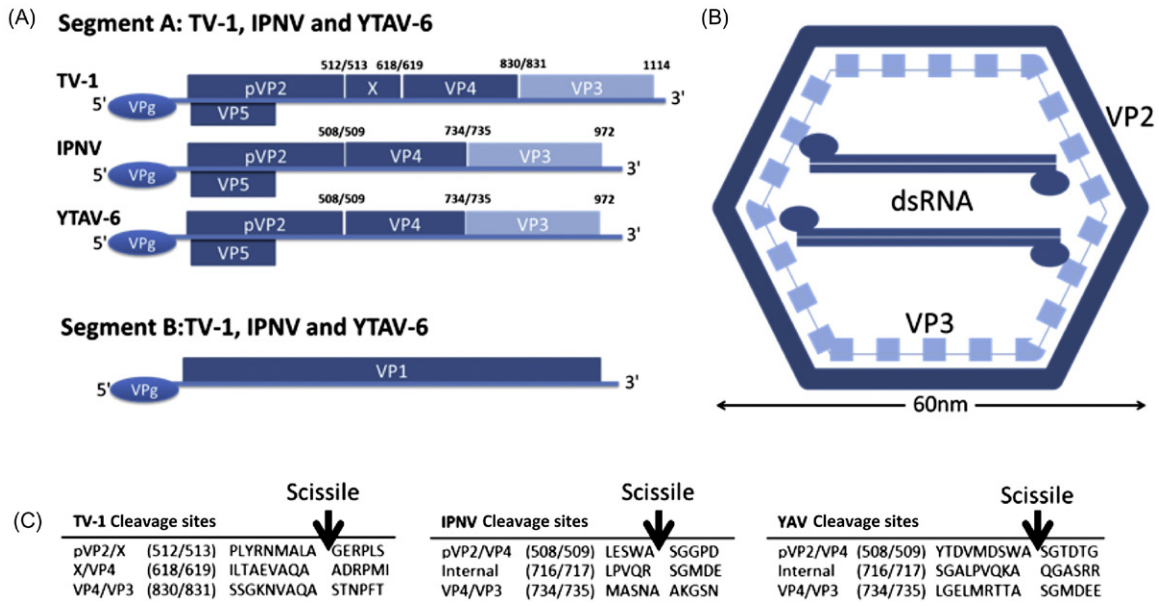

FIGURE 15.1 Shows the genome organizations of three *aquabirnaviruses* (TV [tellina virus], IPNV [infectious pancreatic necrosis virus] and YTAV [yellowtail ascites virus]) and their VP4 cleavage sites. (A) shows a comparison of Segment A organizations for TV, IPNV and YTAV. Note that segment A for the three viruses is made of two open reading frames (ORFs). The large ORF made of the pVP2-VP4-VP3 polyprotein for the YTAV and IPNV, while the large ORF for TV is made of the pVP2-X-VP4-VP3. The smaller ORF is made of VP5 for all three viruses. Segment B is made of a single ORF that forms the VP1. The 5′ termini of both segment A and B is linked to viral protein (VPg). (B) Shows that the outer surface of TV, IPNV and YTAV is made of the VP2 with a 60-nm diameter and is surrounded by VP3 inside. (C) shows the cleavage sites for the VP4 on the segment A polyprotein. The arrows show the scissile cleavage sites. Note that the cleavage sites for the IPNV and YTAV are similar both on the pVP2-VP4 junction, internal VP4 and VP3-VP4 junctions.

15.3 CLINICAL SIGNS, PATHOLOGY AND HISTOPATHOLOGY IN SUSCEPTIBLE HOST SPECIES

Clinical signs, pathology and histopathology induced by aquabirnavirus infections vary with different viral species, strains and host species susceptibility, as discussed next.

15.3.1 Infectious Pancreatic Necrosis Virus

Infectious pancreatic necrosis (IPN) is a contagious disease that predominantly infects members of the *Salmonidae* family, which include the genus *Salmo*, *Oncorhynchus* and *Salvelinus*. A reverse genetics system designed to identify the virulence determinant of IPNV (Santi et al., 2004) has led to a better understanding of the pathogenic mechanisms of the virus in Atlantic salmon. Using the highly virulent *Norwegian* Sp strain NVI015, encoding the virulence motifs identified by Santi et al. (2004), the sequential stages of infection progression from virus entry via mucosal surfaces, dissemination to primary replication sites, deposition in target organs and the ultimate establishment of disease have been studied in detail by Munang´andu et al. (2013a, 2014a). The virus gains entry through the gills, gut and skin, followed by dissemination to the internal organs via the hematogenous route (Novoa et al., 1995; Munang´andu et al., 2012, 2013a). Predilection sites for the primary replication of IPNV in internal organs include the head kidney, spleen and pancreas (Munang´andu et al., 2013a). Baron (1996) pointed out that deposition of virus in target organs is often correlated with an increase in viral loads that correspond with disease establishment. This phenomenon has led to the target organs being called "sites of disease establishment." In the case of IPNV, it has been shown that concurrent infection of the pancreas and liver results in a significant increase in viral loads, which correlates with the establishment of pathology (Munang´andu et al., 2013a). Munang´andu et al. (2013a) showed that an increase in viral loads to greater than $1 \times 10^7/TCID_{50}/mL$ corresponds with detection of tissue damage in target organs. Viral loads below this threshold do not result in any pathology, allowing this cutoff limit to serve as a correlate of pathology for IPNV infection in target organs.

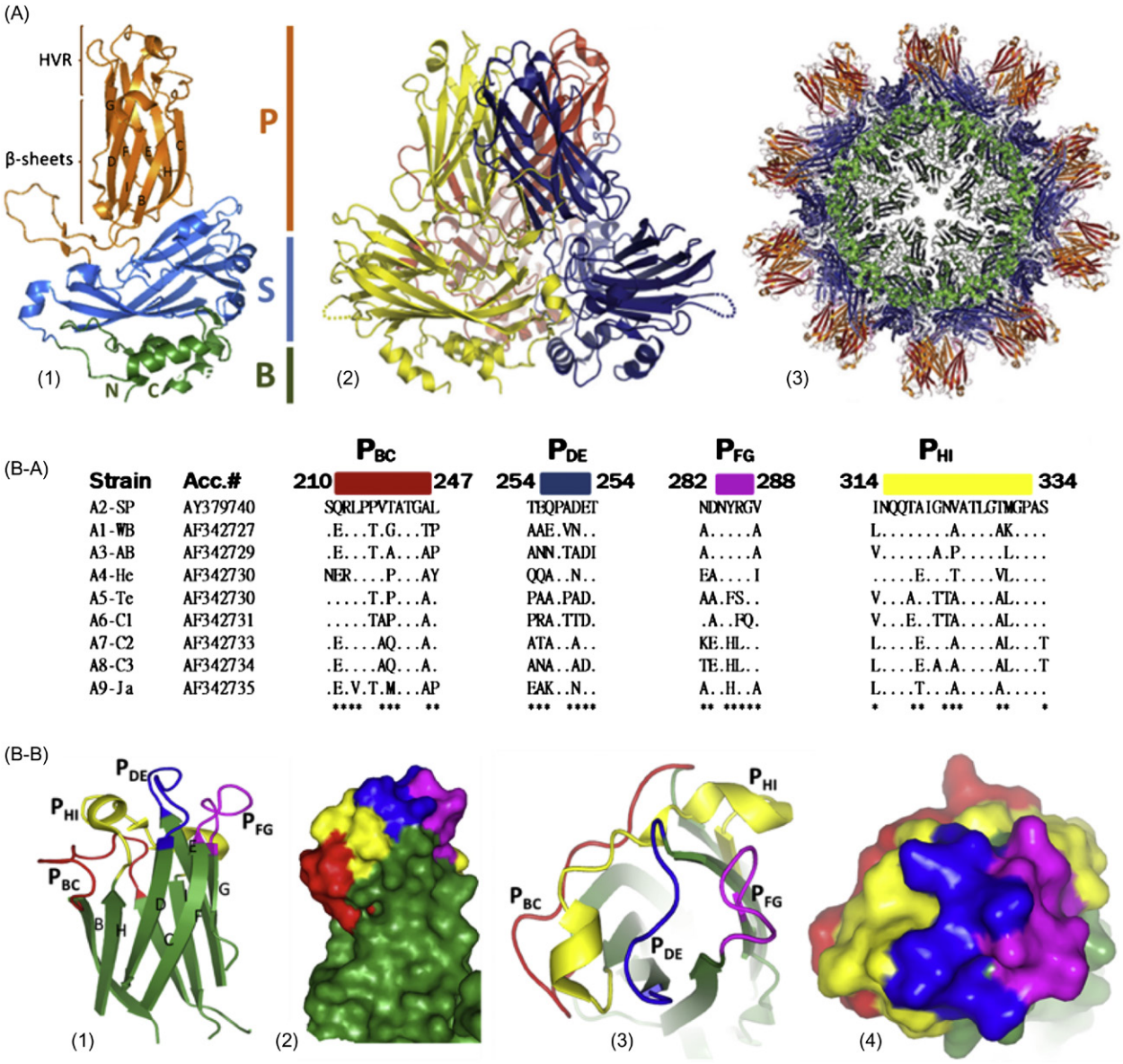

FIGURE 15.2 (A) Structural layout of the VP2 capsid for birnaviruses. (1) shows the subviral particle (SVP) made of three domains. B-domain (base) shows the base made of the N- and C-terminal ends while the S-domain (mid-part) forms the shell made of ß-sheets. The P-domain (upper part) is made of eight ß-sheets that support the four surface loops that form the hypervariable regions (HVRs). (2) shows a trimmer made of three SVPs (light, medium, and dark grey). (3) shows the VP2 capsid made as an interlink of several trimmers in which the base (B-domain, inner part) is located inside having the N- and C-terminals pointing inward. The shell is made of interlinked S-domains (mid part) that surrounds the base and provides anchorage to the P-domains (outward pointing). The P-domains on the VP2 capsid are made of interspaced ß-sheets radiating outward whose outermost surface form the HVRs (outmost loops). (1) was generated using Pymol v99 (Delano and Lam, 2005; Delano, 2009) using the 3IDE template while (2) and (3) were adapted from Coulibaly et al. (2010). (B) A comparison of sequence alignments and the structural layout of surface loops that form the VP2-HVRs for different aquabirnaviruses. (B-A) shows sequence alignments for loops P_{BC}, P_{DE}, P_{FG}, and P_{HI}. Note that positions that have amino acid variations are designated with a star (*) on each loop. (B-B) shows two- and three-dimensional structures of the P-domain. (1) shows that the loops that form the HVRs are located on the surface of the P-domain that correspond with the sequence alignments shown in (B-A). (2) shows that the apex of the HVRs is made of loops P_{DE} (mid loop) and P_{FG} (right loop), while P_{BC} and P_{HI} are located on the periphery. (3) and (4) show the surface of the P-domain at a 90° turn of (1) and (2), respectively. Note that in (3) and (4), loops P_{DE} and P_{FG} occupy the surface of the HVR, while P_{BC} and P_{HI} are on the periphery. All figures were generated using Pymol v99 (Delano and Lam, 2005; Delano, 2009) using the 3IDE template for infectious pancreatic necrosis virus (IPNV).

Clinical signs include darkening of the skin, distended abdomen and abnormal swimming. Gross pathology is characterized by hemorrhages in the pancreas, as well as congestion in the liver, spleen and head kidney. Histopathological changes include vacuolation, necrosis and pyknotic nuclei in the pancreas and liver (Munang´andu et al., 2013a, 2014a).

15.3.2 Yellowtail Ascites Virus

Different scientists have described the clinical signs of YTAV in yellowtail fish (*Seriola quinqueradiata*) (Hirayama et al., 2007; Kobayashi, 2010; Sorimachi and Hara, 1985). Virulence is associated with strain variability, of which the recent isolation and characterization of the highly virulent YTAV-06 strain has led to a better understanding of the pathogenic mechanisms of YTAV in susceptible fish (Hirayama et al., 2007). Infected fish show signs of circling swimming, darkened pigmentation, loss of balance and distended abdomen (Hirayama et al., 2007; Kobayashi, 2010; Sorimachi and Hara, 1985). In infected fish, the virus has been detected in the gills, brain, heart, spleen, kidney, pancreas and lateral muscles (Kobayashi, 2010). Postmortem findings include ascites in the abdominal cavity, excessive mucus in the gills, congestion in the liver, the heart being covered by a white membrane layer and hemorrhagic spots on body surfaces, brain and abdominal adipose tissues. (Hirayama et al., 2007; Kobayashi, 2010; Sorimachi and Hara, 1985). Histopathological changes include congestion in the liver, while severe conditions are characterized by sinusoid dilatation, edema and necrotic hepatocytes showing karyopyknosis (Kobayashi, 2010). In addition, kidneys show severe tubular necrosis, while the heart shows fibrinous pericarditis (Kobayashi, 2010). Sorimachi and Hara (1985) showed that histopathological changes linked to pathognomonic features of the disease were characterized by severe necrotic foci in the pancreas and liver, which is similar to observation seen for IPNV in Atlantic salmon (Table 15.1). Mortality in less virulent strains is often <10%, while mortality induced by the YTAV-06 strain is >70% under experimental conditions (Hirayama et al., 2007). In addition, as shown in Table 15.2, several asymptomatic carriers of YTAV exist that could serve as a source of infection to susceptible species.

15.3.3 TV Subtype 1

Buchanan (1973) described the clinical signs of TV-1 in the marine bivalve mollusk *Tellina tenuis*, in which shells of infected mollusks were markedly thinner and chalkier than in uninfected mollusks. The digestive glands of infected mollusks were pale yellow, unlike the usual dark brown color of healthy mollusks. Histopathology showed necrotic cells in the digestive glands, which had inclusion bodies in the cytoplasm that were filled with icosahedron particles of 70-nm diameter characteristic of birnaviruses.

15.3.4 Other Aquabirnaviruses

Aquabirnaviruses from marine species distinct from IPNV, YTAV and TV by genotyping based on amino acid differences on the VP2/NS junction have been classified as MABV (Hosono et al., 1996), while those infecting eels in Europe are called eel virus European (EVE) (Sano, 1976). In eels, EVE shows different clinical signs that include twinkling movement, darkened skin, swimming at the surface, exophthalmos, edema of the head and operculum and abnormal trunks (van Beurden et al., 2012). Gross pathology includes ascites, bleeding in intestinal walls, enlarged internal organs, congestion of the gills and nephritis (van Beurden et al., 2012). As for MABVs, they infect a wide range of host species, causing different clinical conditions linked to mortality in susceptible species, although there are several marine species that exhibit asymptomatic infections of MABVs which could serve as sources of infection in susceptible farmed species (Hosono et al., 1994; Jung et al., 1999; Kobayashi et al., 2005; Kusuda et al., 1989, 1993, 1994; Suzuki et al., 1995).

15.4 EPIZOOTIOLOGY

The geographical distribution of aquatic birnaviruses is worldwide, with a wide host range including fish, mollusks and crustaceans, as already stated previously. These viruses have been isolated from at least 55 fish species (Reno, 1999), while Munro and Midtlyng (2011) suggested that the host range could be more than 100 species. The viruses are capable of survival outside the host in the environment for extended periods of time, as demonstrated by Barja et al. (1983), who showed virus survival with infecting ability in fresh water for up to 20 days at 15°C. This, however, is inversely related to temperature, as the duration of the ability to remain infective reduces when the virus is kept at 20°C. The virus is, however, more stable in marine water, whereby temperature has little or no effect on its survival (Barja et al., 1983; Toranzo et al., 1983). This explains, at least in part the virus's ability to infect fish over generations at seawater sites.

TABLE 15.1 Comparison of Symptoms, Pathology and Histopathology of Aquabirnaviruses

Parameters	Aquabirnavirus					
	Infectious Pancreatic Necrosis Virus	**References**	**Yellowtail Ascites Virus**	**References**	**Tellina Virus 1**	**References**
1. Susceptible spp.	Salmonids		Yellowtail fish	Sorimachi and Hara (1985)	Mollusks	Buchanan (1973)
2. Symptoms	Aberrant swimming		Circling		Thin and chalky shells	Buchanan (1973)
	Distended abdomen		Distended abdomen			
	Loss of skin surface color		Loss of skin surface color			
3. Pathology	Hemorrhages in pancreas Congestion in liver, spleen and head kidney	Munang'andu et al. (2013a,c)	Ascites in abdomen	Kobayashi (2010)	Pale yellow digestive glands instead of the usual dark brown color	Buchanan (1973)
			Congestion in liver			
			Hemorrhages in brain, abdominal cavity, gills, body surface and opercula; white membrane covering the heart			
4. Infected organs	Pancreas, liver, head kidney, spleen, intestines, gills and muscle	Munang'andu et al. (2012, 2013a)	Gills, heart, kidney, liver, spleen, stomach, skin, muscle, pancreas and intestine	Kobayashi (2010)	Digestive glands	Buchanan (1973)
5. Target organs	Pancreas and liver		Kidney and liver		Digestive glands	Buchanan (1973)
6. Histopathology in target organs	Pancreas: necrosis, pyknotic nuclei, vacuolation, in the pyloric ceca	Munang'andu et al. (2013a,c)	Kidney: shows tubular necrosis, vacuolation, hyaline droplets and cloudy swelling of tubular epithelial cells	Kobayashi (2010)	Necrosis of cell of digestive glands and inclusion bodies in the cytoplasm showing viral particles	Buchanan (1973)
	Liver: necrosis, vacuolation, pyknotic nuclei in the hepatocytes.	Munang'andu et al. (2013a,c)	Liver: shows dilation, sinusoids and hepatocyte edema and fibrin deposition	Kobayashi (2010)		Buchanan (1973)

TABLE 15.2 Fish and Shellfish Species Infected by Infectious Pancreatic Necrosis Virus (IPNV), Yellowtail Ascites Virus (YTAV) and Tellina Virus (TV)

Disease	Fish Species (Scientific Name)	Clinical Condition	References
IPNV	Atlantic salmon (*Salmo salar* L.)	Susceptible	Munang'andu et al. (2012)
	Brown trout (*Salmo trutta* L.)	Susceptible	Rexhepi et al. (2011)
	Rainbow trout (*Oncorhynchus mykiss*)	Susceptible	Bebak and McAllister (2009)
	Amago salmon (*Oncorhynchus rhodurus*)	Susceptible	Jung et al. (1999)
	Chum salmon (*Oncorhynchus keta*)	Susceptible	Jeon et al. (2011); Suebsing et al. (2011)
	Coho salmon (*Oncorhynchus kisutch*)	Susceptible	Lopezlastra et al. (1994)
	Brook trout (*Salvelinus fontinalis*)	Susceptible	Dorson et al. (1991)
	Arctic char (*Salvelinus alpinus*)	Susceptible	Dorson et al. (1991)
	Lake trout (*Salvelinus namaycush*)	Susceptible	Dorson et al. (1991)
	Turbot (*Scophthalmus maximus*)	Susceptible	Mortensen et al. (1993)
	Atlantic halibut (*Hippoglossus hippoglossus*)	Susceptible	Biering et al. (1994); Biering and Bergh (1996)
	Atlantic cod (*Gadus morhua*)	Asymptomatic	Garcia et al. (2006)
	Pike (*Esox lucius*)	Asymptomatic	Liu et al. (2008)
	Spotted wolfish (*Anarhichas minor*)	Asymptomatic	Sommer et al. (2004)
	Gilthead seabream (*Sparus aurata*)	Asymptomatic	Fernandez-Trujillo et al. (2011)
	Red banded seabream, (*Pagrus auriga*)	Asymptomatic	Lopez-Jimena et al. (2010)
	Common seabream, (*Pagrus pagrus* (L.))	Asymptomatic	Lopez-Jimena et al. (2010)
	Saithe (*Pollachius virens*)	Asymptomatic	Wallace et al. (2008)
	Blue mussel (*Mytilus edulis*)	Asymptomatic	Molloy et al. (2013)
YTAV	Yellowtail fish (*Seriola quinqueradiata*)	Susceptible	Sorimachi and Hara (1985); Sorimachi and Egusa (1986)
	Japanese flounder (*Paralichthys olivaceus*)	Susceptible	Kusuda et al. (1989)
	Amberjack (*Seriola dumerili*)	Susceptible	Kusuda et al. (1993); Isshiki et al. (2001)
	Ayu (*Plecoglossus altivelis*)	Susceptible	Jung et al. (1999)
	Japanese parrot-fish (*Oplegnathus fasciatus*)	Susceptible	Isshiki and Kusuda (1987)
	Spotted parrot-fish (*Oplegnathus panctatus*)	Susceptible	Nakajima et al. (1998)
	Red sea bream (*Pagrus major*)	Asymptomatic	Kusuda et al. (1994)
	Tiger puffer (*Takifugu rubripes*)	Asymptomatic	Kamakura et al. (1995); Isshiki et al. (2001)
	Saffron cod (*Eleginus gracilis*)	Asymptomatic	Kobayashi et al. (2005)
	Rainbow smelt (*Osmerus eperlanus* m.)	Asymptomatic	Kobayashi et al. (2005)
	Snowy scuplin (*Myxocephalus blandti*)	Asymptomatic	Kobayashi et al. (2005)
	Japanese dace (*Tribolodon hakonesis*)	Asymptomatic	Kobayashi et al. (2005)
TV	Bivalve mollusk (*Tellina tenuis*)	Susceptible	Buchanan (1973)

15.4.1 Infectious Pancreatic Necrosis Virus

The host range of IPNV includes salmonids and marine pleuronectiformes, including turbot, halibut, cod, Pacific flounder, Senegalese sole, striped bass, common carp and loach (reviewed in Smail, 2012). Among the salmonids, susceptibility varies, but the most affected species are rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), brown trout

(*Salmo trutta*), Atlantic salmon (*Salmo salar*) and several Pacific salmon species (*Oncorhynchus* spp.) (Smail, 2012). Other fish species that are infected by IPNV are listed in Table 15.2.

Within species, age has been shown to play a role in susceptibility or resistance. The best-studied species here are farmed salmonids, where it has been shown that fry at start feeding are the most susceptible. As the fish grows, they become more resistant. The smolts stage, 3–6 weeks following transfer to sea (Roberts and Pearson, 2005), is another window where susceptibility has been observed, probably because of the physiological stress that the fish undergo at this stage. Thereafter, the fish become more resistant until they reach market size.

15.4.2 Transmission

Evidence for vertical transmission of IPNV is documented for brook trout (Wolf et al., 1963), zebrafish (Seeley et al., 1977), rainbow trout (Dorson and Torchy, 1985) and arctic char (Ahne and Negele, 1985). For Atlantic salmon, there is no conclusive evidence of vertical transmission of IPNV yet. As many farmers test brood fish, eggs and milt, discarding those that test positive, the risk of vertical transmission is minimized. Moreover, several studies in Atlantic salmon have shown that brood stations are the least likely sources of the introduction of IPNV into the production line (Mutoloki and Evensen, 2005). Horizontal transmission of IPNV is well known. The mode of virus release from infected fish to the external environment is via the gills, gut, skin and excreta (Hill, 1982; Yamamoto, 1975). One study suggested a minimum infectious dose of less than 10^{-1} $TCID_{50}$/mL to induce infection by bath challenge.

Mutoloki and Evensen (2005) showed that the freshwater stage was the most important for the introduction of IPNV into an Atlantic salmon production line. These authors also showed that seawater infections were largely a recurrence of freshwater infections, especially in that infected fish remained carriers for the rest of their lives.

In general, the prevalence of the virus under intensive rearing conditions is significantly higher than in wild populations (Kim et al., 2013; Wallace et al., 2008). This host enrichment not only leads to changes in virus virulence, but also increased prevalence. Indeed, the high virus prevalence in farmed fish has been blamed for the rising prevalence of infections in wild populations (Gregory et al., 2007d).

15.4.3 Yellowtail Ascites Virus

There are few studies that comprehensively define the geographical distribution of YTAV. Phylogenetic studies place these isolates in genogroup 7 (Nishizawa et al., 2005). By extrapolation from reports of phylogenetic studies, and also by examining the published literature (for example, Table 15.2), YTAV is largely found in the Far East (mainly Japan and South Korea), and probably southeast Asia as well, given the volume of trade between these areas (Shin et al., 2011; Zhang and Suzuki, 2004). The host range and susceptible/resistance of species are given in Table 15.2.

YTAV is believed to be transmitted both vertically and horizontally (Isshiki et al., 1993). As with IPNV, infected fish can display clinical symptoms or remain asymptomatic carriers (Table 15.2), and the virulence of different isolates has been shown to vary. Mortality in less virulent strains is often <10%, while the YTAV-06 strain induced >70% mortality under experimental conditions (Hirayama et al., 2007).

15.4.4 Tellina Virus

TV has been isolated from different species of mollusks in Europe, Asia and Alaska, but classical outbreaks are only reported in *Tellina tenuis* and Japanese oyster (*Crassostrea gigas*) (Hill, 1976; Jung et al., 1999). Experimental infections have also been reported in various bivalves species, while molecular characterization of the virus has been done using different fish cell lines, such as blue gill fry cells (BF-2) and chinook salmon embryo (CHSE-214) cells (Jung et al., 1999). Infection of flat oysters with virus isolated from bivalves on fish cells resulted in histological changes consistent with infection of TV (Chou et al., 1998; Hill, 1976).

TV is regarded as an opportunistic virus because isolates from mollusks have shown weak pathogenicity, while the presence of environmental stressors such as heavy metal contamination and changes in water temperature have been shown to contribute to the increase in host susceptibility and increased mortality (Chou et al., 1994, 1998; Suzuki et al., 1998a,b).

15.5 PREVENTION AND CONTROL

The estimated cost of IPNV on the salmon aquaculture industry annually in Norway alone was approximately US$100 million during the last decade of the 19th century (Olsen, 2002). In the United Kingdom, the disease mostly affected rainbow trout in the early1980s, but it has since spread to other fish species (Ruane et al., 2007). In 2002 losses due to IPN in the

United Kingdom were estimated to be at least £2 million (Anonymous, 2003). In order to mitigate these losses, disease control measures were introduced, including the use of biosecurity control measures, vaccination and selective breeding of genetically resistant fish to disease infection, as discussed next.

15.5.1 Vaccination

Vaccination is one of the most effective disease prevention strategies used in aquaculture (Gudding, 2014). However, not all vaccines developed for aquabirnaviruses are highly protective (Munang´andu et al., 2012). As a result, recent studies have focused on optimizing vaccine performance with the view to improve the level and duration of protective immunity induced by vaccination. In the case of IPNV, recent studies have focused on (1) identifying the correlates of protective immunity for optimizing vaccine performance (Munang´andu et al., 2013a), (2) identifying immunogenic proteins on the VP2 capsid for use in producing highly protective vaccines (Munang´andu et al., 2013c), (3) identifying the most effective antigen delivery systems (Munang´andu et al., 2012), (4) elucidating the immunological mechanisms of vaccine protection (Munang´andu et al., 2013a) and (5) identifying elements of adaptive immune responses responsible for protective immunity (Munang´andu et al., 2013a,b, 2014a). Although different vaccine delivery systems have been explored, inactivated whole viral (IWV) vaccines remain the most widely used because of their safety (Munang´andu et al., 2014b). There are currently no licensed live IPNV vaccines in use, and DNA vaccines have not shown superior protection over IWV vaccines (Mikalsen et al., 2004; Munang´andu et al., 2012). Other vaccine delivery systems explored include subunit, viruslike particles, PLGA nanoparticles and fusion protein vaccines (Munang´andu et al., 2012; Munang´andu and Evensen, 2015).

The measures of protective immunity for IPNV vaccines were recently elucidated by Munang´andu et al. (2014a), who showed that a threshold antibody titer that would serve as a correlate of vaccine protection can be established for IPNV vaccines. Munang´andu et al. (2014a) have shown that an antibody titer $>1.20_{OD490}$ (1:50 dilution, ELISA) corresponded with postchallenge survival proportions (PCSP) >90%, while antibody titer $>1.40_{OD490}$ corresponded with PCSP>94%. In terms of underlying mechanisms of vaccine protection, they showed that a titer $>1.20_{OD490}$ was linked to the prevention of virus spreading to primary replication sites and target organs. In infected fish, vaccine protection was based on suppressing virus replication to levels below the correlate of pathology in target organs in order to prevent disease establishment (Munang´andu et al., 2013a, 2014a). It is likely that similar correlates of protective immunity can be established for YTAV, EVE and MABV, and that the underlying mechanisms of vaccine protection for these viruses could be similar to mechanisms of vaccine protection for IPNV in Atlantic salmon.

15.5.2 Biosecurity Control Measures

Biosecurity control measures play an important role in preventing the transmission of infectious diseases on hatcheries and fish farms, as well as preventing the international transmission of infectious diseases through export or import of eggs, live fish and fish products. In the case of IPNV, some of the important factors that exacerbate the need for designing effective biosecurity control measures are that (1) the virus is highly contagious in susceptible species (Urquhart et al., 2008), (2) it induces persistent infections in survivors of clinical infections (Gadan et al., 2013), (3) the virus exists as a mixed population inclusive of avirulent and virulent strains in different ecosystems (Bain et al., 2008; Mutoloki and Evensen, 2011; Mutoloki et al., 2013), (4) avirulent strains can revert to virulence under stress conditions (Mutoloki et al., 2013), (5) there are concerns of vertical transmission from parent stocks to eggs (Chaves-Pozo et al., 2010; Mulcahy and Pascho, 1984; Munro et al., 2010; Smail and Munro, 2008) and (6) asymptomatic wild reservoirs exist that could serve as a source of infection in farmed fish (Wallace et al., 2008). Although it is possible to design biosecurity control measures customized for the control of IPNV to address these outlined problems, implementation of biosecurity control measures designed for the prevention of other viral diseases described elsewhere in this book (Munang´andu et al., 2016) include the capacity to prevent the transmission of aquabirnaviruses in farmed fish.

15.5.3 Selective Breeding of Genetically Resistant Fish to Aquabirnavirus Infections

The use of high-throughput genome mining techniques such as RNA-seq has accelerated the ability to identify genetic markers for disease resistance in fish species (Gheyas et al., 2010; Houston et al., 2010; Moen et al., 2015; Ozaki et al., 2001). In Norway, advances in selective breeding have led to generating of a strain of Atlantic salmon that has high genetic resistance to IPNV infection (Moen et al., 2015). Although selective breeding as a disease control strategy is still in its early developmental stages, reports from the Norwegian Veterinary Institute (NVI) show that the incidence of IPNV in farmed Atlantic salmon has significantly reduced in Norway for the period between 2009 and 2014 (Bornø and Lie Linaker, 2015; Fig. 15.3). This reduction is attributed to introduction of a genetically resistant strain of Atlantic salmon to IPNV infection

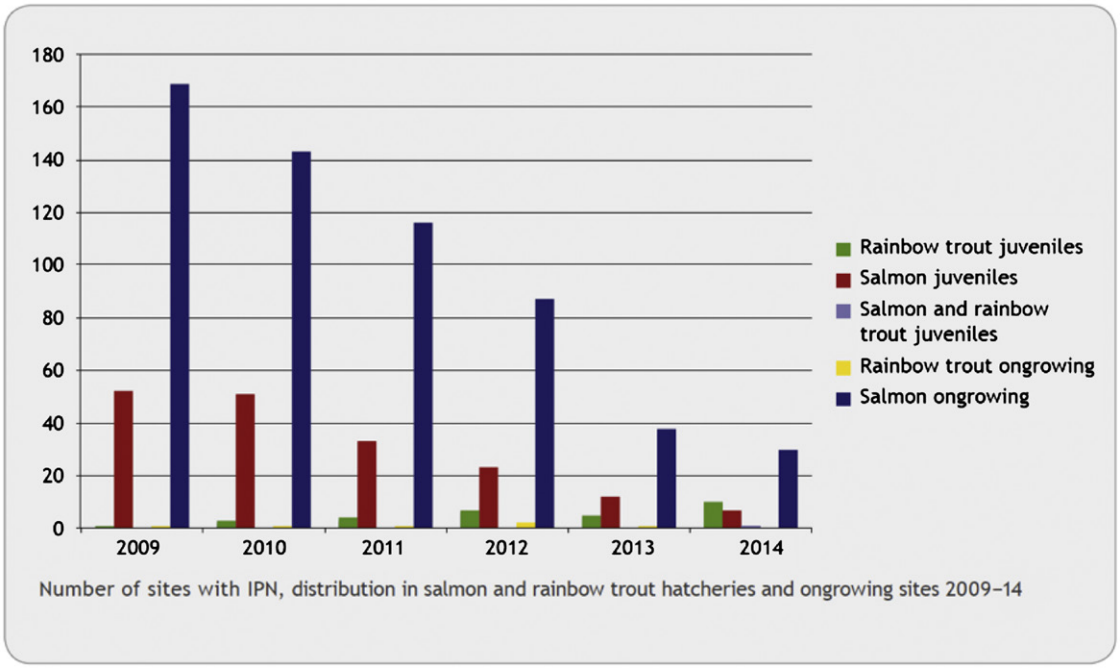

FIGURE 15.3 Shows the number of infectious pancreatic necrosis virus (IPNV) cases for the period 2009–14 in Norway. Note that the numbers of IPNV cases were highest in the ongrowing salmon, which is the age that includes post smolts. Juvenile salmon account for the second group with high mortality, which covers the age between fingerling and parr. Both in the juveniles and ongrowing salmon the number of cases show a tremendous decline for the period 2009–14. Note that the number IPNV cases in juvenile rainbow trout is slowly increasing, although the overall incidence is lower than cases reported in Atlantic salmon.

in the Norwegian aquaculture industry (Bornø and Lie Linaker, 2015; Moen et al., 2015). Although IPNV levels in rainbow trout have generally been lower than in Atlantic salmon, the disease in rainbow trout is slowly increasing (Bornø and Lie Linaker, 2015; Fig. 15.3). This can be attributed to the lack of a strain of rainbow trout that is genetically resistant to IPNV infection for use in fish farming in Norway. The decreasing trend of IPNV cases in farmed Atlantic salmon in Norway suggests that selective breeding of disease resistant fish is an effective disease control strategy that can be used to reduce the impact of infectious diseases.

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Chapter 16

Totiviruses of Fish

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16.1 INTRODUCTION

The family *Totiviridae* is composed of small (approximately 40 nm), nonenveloped icosahedral viruses with nonsegmented, linear double-stranded RNA (dsRNA) genome of 4.6–6.7 kb. There are currently five genera—the *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus* and *Victorivirus*—all infecting unicellular organisms like protozoa and fungi. During the past decade, new and more complex totivirus-like viruses have been described; three of which infect arthropods, the *infectious myonecrosis virus* (IMNV) infecting shrimp (Poulos and Lightner, 2006), *Armigeres subalbatus totivirus* (AsTV) infecting mosquito (Zhai et al., 2010) and *Drosophila melanogaster totivirus* (DTV) infecting common fruit fly (Wu et al., 2010). The last characterized, *Piscine myocarditis virus* (PMCV), is the first potential member of *Totiviridae* infecting a vertebrate host, the Atlantic salmon (*Salmo salar* L.) (Haugland et al., 2011). None of these are yet officially assigned to the virus family. A totivirus-like virus was also recently described from arboreal ants, *Camponotus yamaokai* (Koyama et al., 2015).

16.2 SPECIFIC FISH DISEASES

PMCV is currently only shown to cause disease in Atlantic salmon. The disease has been described as a disease entity in Norway since 1985 (Amin and Trasti, 1988; Ferguson et al., 1990) and was originally in popular terms called "heart rupture," due to observed rupture of the thin atrial wall of affected fish. The name of the disease is cardiomyopathy syndrome (CMS), which is more of a layman's term and does reflect the true heart pathology seen from PMCV infections. These are characterized by a necrotizing myocarditis, involving the atrium and the spongious part of the heart ventricle. At later stages, the sarcoplasm of cardiomyocytes can be completely lost and replaced with fibrous tissue, likely representing a reparative process where muscle fibers are replaced with fibrous tissue.

16.3 STRUCTURE AND COMPOSITION OF VIRUS

PMCV virions are spherical with a diameter of approximately 50 nm (Fig. 16.1). As for other members of the family *Totiviridae*, the particles seem to be simple, consisting of a nonenveloped protein shell surrounding the RNA genome. No details of the virion have been revealed, but there are indications of a structural symmetry on the viral surface (Fig. 16.1). The buoyant density of viral particle is 1.3842 g/mL (Haugland et al., 2011). The protein shell is tentatively composed of multiple copies of a coat protein, similar to the icosahedral structures of *Totiviridae* members and other dsRNA viruses (Naitow et al., 2002).

The genome encapsidated by the viral particle is a nonsegmented dsRNA of 6688 base pairs. The positive-sense (+) strand has three open reading frames (ORF1, 2 and 3) (Fig. 16.2; Haugland et al., 2011). ORF1 is encoding a protein of 861 amino acids (aa) with a predicted molecular mass of 91.8 kilodalton (kDa) and a pI of 5.4. The protein is believed to represent the coat protein, based on comparison with the organization of the genomes of *Totiviridae* (Haugland et al., 2011). It has also been shown to result in virus-like particles when recombinant-expressed using a baculovirus vector in insect cells (Marit Rode, personal communication, PHARMAQ AS, Oslo, Norway). ORF2 is encoding a protein of 726 aa, which has a predicted molecular mass of 83.1 kDa and a pI of 9.42. The amino acid sequence shows similarity to the RNA-dependent RNA polymerases (RdRp) of the *Totiviridae* family and for this reason and the position in the genome compared with *Totiviridae*, it is believed to represent a RdRp (Haugland et al., 2011). Similar to the other family members, the organization

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00016-4
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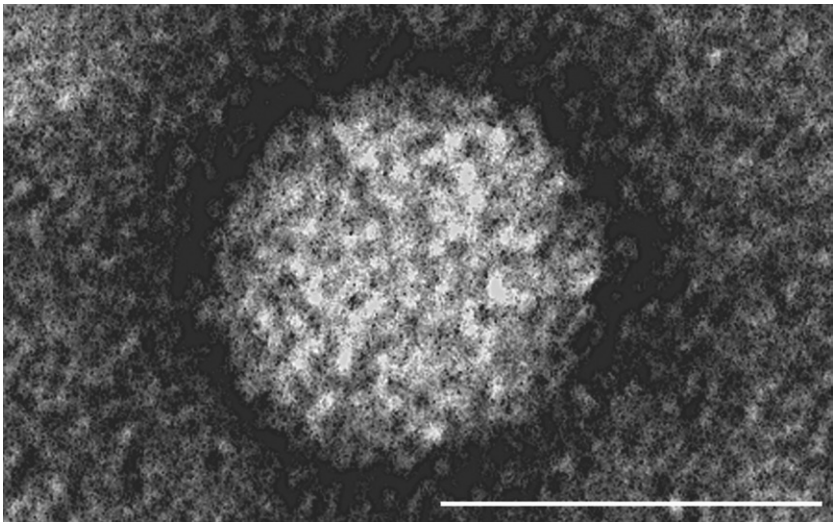

FIGURE 16.1 Negative staining of virus isolated from heart tissue homogenate. A virus-like particle of 50 nm with regular surface structures and potential surface projections is seen. Bar = 50 nm.

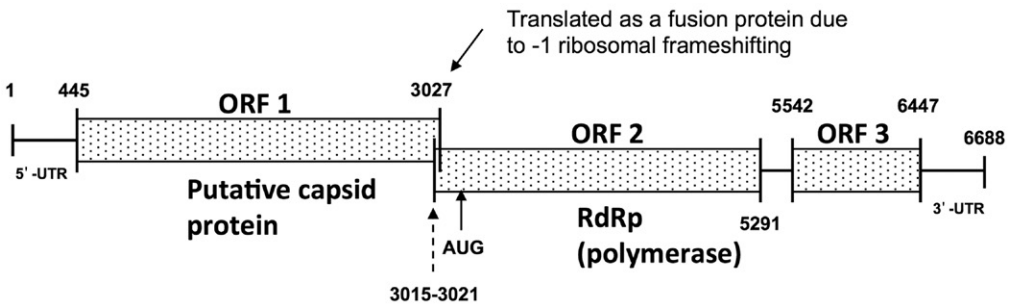

FIGURE 16.2 Suggested genome organization of *piscine myocarditis virus* (PMCV). The genome consists of three large open reading frames (ORFs). ORF1 and ORF2 resemble two potential overlapping ORFs. ORF2 is likely translated as a fusion protein with ORF1 because of a ribosomal -1 frameshifting. It can also be translated as a separate protein (start codon indicated). ORF3 is a nonoverlapping frame.

of ORF1 and 2 resemble two overlapping frames with a site for -1 ribosomal frameshift found in the overlapping region (Fig. 16.2). As for the *Totiviridae* members, this might direct the translation of a major coat protein from ORF1 and a minor fusion protein of coat and the following RdRP from ORF2, but this has not yet been confirmed. The presence of the third ORF in the PMCV genome is a feature exclusively seen for PMCV compared with other family members or potential members. The encoded protein is 302-aa long, with a predicted molecular mass of 33.4 kDa and a pI of 6.99, and shares no sequence homology with proteins of known totiviruses or other viruses/organisms (Haugland et al., 2011). The role of the ORF3-encoded protein in virus particle and/or infected cells remains elusive. A role in formation of surface structures of the viral particle has been proposed because the predicted molecular mass is similar to the predicted molecular mass of one unit of a trimer forming fiber-like protrusions on the IMNV virion. Both viruses are shown to infect using of extracellular transmission, which deviates from the classical virus species of the family *Totiviridae* (Haugland et al., 2011; Tang et al., 2008). Such fiber-like protrusions are suggested to participate in the extracellular transmission of the virus and thus play a role in viral pathogenesis (Tang et al., 2008). Yet another possibility is that the encoded protein is nonstructural and only present in the infected cell. Several roles are possible (eg, enhancing or modulating the inflammation observed in CMS and the ORF3-encoded protein showed no significant similarity with any other known proteins). There is a putative chemokine superfamily motif in the N terminus of the ORF3 gene product (Haugland et al., 2011), or it could be that the protein promotes release of viral particles from infected cells. Additional studies are needed to define the importance of the ORF3-encoded protein in viral pathogenesis.

16.4 CLASSIFICATION AND VIRUS REPLICATION

PMCV, together with IMNV (Poulos et al., 2006), AsTV (Zhai et al., 2010) and DTV (Wu et al., 2010), is not yet officially assigned to a virus family. They have all been proposed as members of the family *Totiviridae*, based on size and characteristics of virion and similar genetic characteristics of the two first ORFs encoding the capsid protein (ORF1) and RdRp (ORF2). PMCV shares the feature of extracellular transmission with these unassigned viruses. This is a feature that is not shared with the official *Totiviridae* members in general. The only difference is the *Giardia lamblia virus* (GLV), which is transmitted extracellularly (Sepp et al., 1994) and is also the totivirus with the closest relationship to PMCV and the other unassigned viruses (Haugland et al., 2011). It is suggested that PMCV, the arthropod infecting viruses and GLV represent a discrete clade of "toti-like" viruses that carry components for cell entry and might deserve a definition as a separate virus family or subfamily (Dunn et al., 2013).

PMCV is shown to replicate in cultured fish cells at low levels with release of infectious virus to the culture supernatant (Haugland et al., 2011). Extracellular release is also shown in its natural host, Atlantic salmon, where in vivo experiments show that salmon injected intramuscularly with PMCV transfer the virus through water to cohabitating fish, where the virus shows increased replication over time, and with time, the cohabiting fish develop heart changes typical of CMS (Haugland et al., 2011). The histopathological changes were similar to those seen in the injection challenged fish and from clinical cases. The virus is shown to cause a systemic infection with presence in heart, kidney, liver, spleen, peripheral blood leukocytes and red blood cells (Timmerhaus et al., 2011), and heart is considered the target organ for virus replication (Haugland et al., 2011). It is not known which cells (of the heart) are the prime replication sites, but degenerate/necrotic cardiomyocytes are found with high amounts of viral genome in the sarcoplasm (Haugland et al., 2011). Details of the replication mechanisms of PMCV are not known. It is anticipated that similarities in the genome characteristics (ORF1 and 2) with other totiviruses would indicate similar steps in the replication of the virus. For these, transcription of the dsRNA genome to a single strand (+) RNA copy by the viral polymerase (from the negative strand) occurs inside the virion, which means that the dsRNA genome is not exposed in the cytoplasm (or only at late stages of replication). This plus-strand transcript is transported out of the virion and used as template for translation of the major coat protein and some copies with RNA polymerase fuse to the coat protein. To what extent the mRNA is polyadenylated and noncapped, as for GLV (Janssen et al., 2015), is not known. Mechanisms of initiation of translation are also not known. + RNAs are encapsidated in a sub-viral particle, and these particles synthesize the (−) strand on the (+) strand template to yield dsRNA, and the virus cycle is completed.

Mature virions are transmitted to new cells during cell division, sporogenesis, cell fusion (totivirus) or are released from the host cell (Giardiavirus) (Janssen et al., 2015). PMCV carry characteristics in the genome that support that the RNA polymerase can be produced fused to the coat protein. However, the fact that PMCV uses extracellular release and the presence of third ORF questioned to code for a protein involved in transmission of the virus points to certain differences in replication mechanisms—at least when it comes to spread between cells.

16.5 EPIDEMIOLOGY

CMS primarily affects Atlantic salmon in the second year in seawater, close to harvest and therefore the economic impact of the disease is significant. It has been estimated that CMS outbreaks cause financial losses of 4–8 million € (or 33–66 million NOK) each year in Norway, but this number is likely an underestimate of the problem (Brun et al., 2003). CMS was described as a disease entity in 1985 from Norway (Amin and Trasti, 1988; Ferguson et al., 1990), and has subsequently been diagnosed in the Faroe Islands and Scotland (Poppe and Sande, 1994; Rodger and Turnbull, 2000) and recently in Ireland (Rodger et al., 2014). The first field survey of CMS outbreaks in Norway was published by Brun et al. (Brun et al., 2003). In this survey, 25 cases were diagnosed during the first year of the study. The number of reported cases increased and by 2001, 103 cases were diagnosed and in 2002, 101 cases. A summary of the number of reported cases per year from 2007 to 2013 is shown in Table 16.1 (with some years missing information). There was also a high number of reported cases in 2013 (100 cases).

The average cumulative mortality from CMS under clinical conditions is around 6.1% compared with 2.5% in farms without CMS, but other factors clearly contribute to these differences so exact figures are difficult to obtain. The highest cumulative mortality rate was reported to be 19.9%. Most of the cases occurred approximately 400 days post-sea transfer, with a range from 253 to 595 days (Brun et al., 2003). CMS occurs along the entire Norwegian coastline.

A recent study addressed the genetic variation of the PMCV genome in different geographic regions in Norway (Wiik-Nielsen et al., 2013). The study showed that PMCV infecting salmon in Norway is genetically homogenous and belongs to a single genogroup. However, it could also indicate that the virus has been introduced to farmed salmon fairly recently and thus diversification into subclades has not yet occurred.

TABLE 16.1 Number of Reported Cases of Important Viral Infections of Atlantic Salmon in Norway, 1998–2013

Disease/ Year	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
ISA	13	14	23	21	12	8	16	11	4	7	17	10	7	1	2	10
PD	7	10	11	15	14	22	43	45	58	98	108	75	88	89	137	99
HSMI							54	83	94	162	144	139	131	162	142	134
CMS				103	101	n.a.	n.a.	n.a.	n.a.	85	75	76	53	74	89	100
IPN					174	178	172	208	207	165	158	223	198	154	119	56

Source: Norwegian Veterinary Institute, Norway (Fiskehelserapporten, 2006–13).
ISA, infectious salmon anemia; PD, pancreas disease; HSMI, heart and skeletal muscle inflammation; CMS, cardiomyopathy syndrome; IPN, infectious pancreatic necrosis virus; n.a., not available.

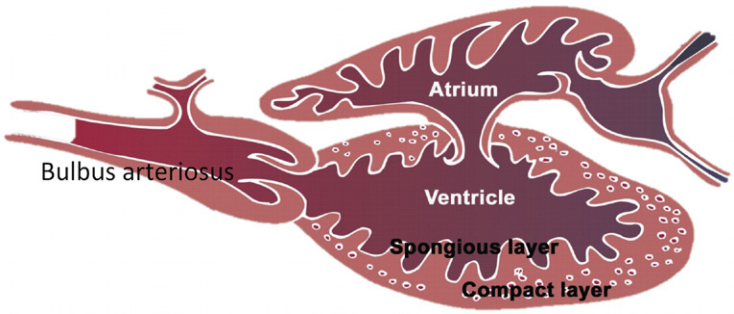

FIGURE 16.3 Schematic outline of the heart anatomy of Atlantic salmon. Atrium receives blood from hepatic veins and blood to the gills flow through the *bulbus arteriosus.*

16.6 PATHOLOGY AND IMMUNITY

Heart changes are inflammatory in nature and are first seen in the atrium post-experimental challenge, extending to the spongious part of the heart ventricle (Fig. 16.3) at later stages (Fritsvold et al., 2009; Haugland et al., 2011; Martinez-Rubio et al., 2014). Early histopathological changes are characterized by a multifocal pattern with infiltration of inflammatory cells, mainly lymphocytic cells of the atrium (Fig. 16.4A) accompanied by degeneration and/or necrosis of individual cardiomyocytes. Multifocal lesions gradually develop into diffuse and extensive lesions (Fig. 16.4A). At later stages, affected fish display extensive inflammation and severe myocardial damage, leaving almost no intact cells in the atrium and the spongy layer of the ventricle (Fig. 16.4B). Inflammatory infiltrates consist of mononuclear cells, lymphocytes and macrophages. The compact layer of the ventricle remains unaffected (Haugland et al., 2011). Myocardial cells show loss of striation, eosinophilia and lysis of contractile fibers. The endothelium covering the trabecules becomes hypertrophic and is also hyperplastic (Fig. 16.4B). Fibrosis is also observed, and is thought to represent a reparative process. The epicardium is usually unaffected.

Immune responses to PMCV infection have be described at transcript level (Timmerhaus et al., 2011), but there are no functional studies (antibodies/T cell responses) yet. The transcriptome showed a systemic induction of antiviral and IFN-related responses as early as 2 wpi, declining at later stages (Timmerhaus et al., 2011). Later, there was a biphasic B-cell pathway activation and upregulation of MHC mRNA. Peak pathology coincided with upregulation of T-cell response, but what role these responses play in clearing the infection remains to be documented. Further, it is not known if infected fish are able to mount an immune response that will protect against development of pathology or if the fish would be able to clear the virus, and regenerate the damaged cardiomyocytes.

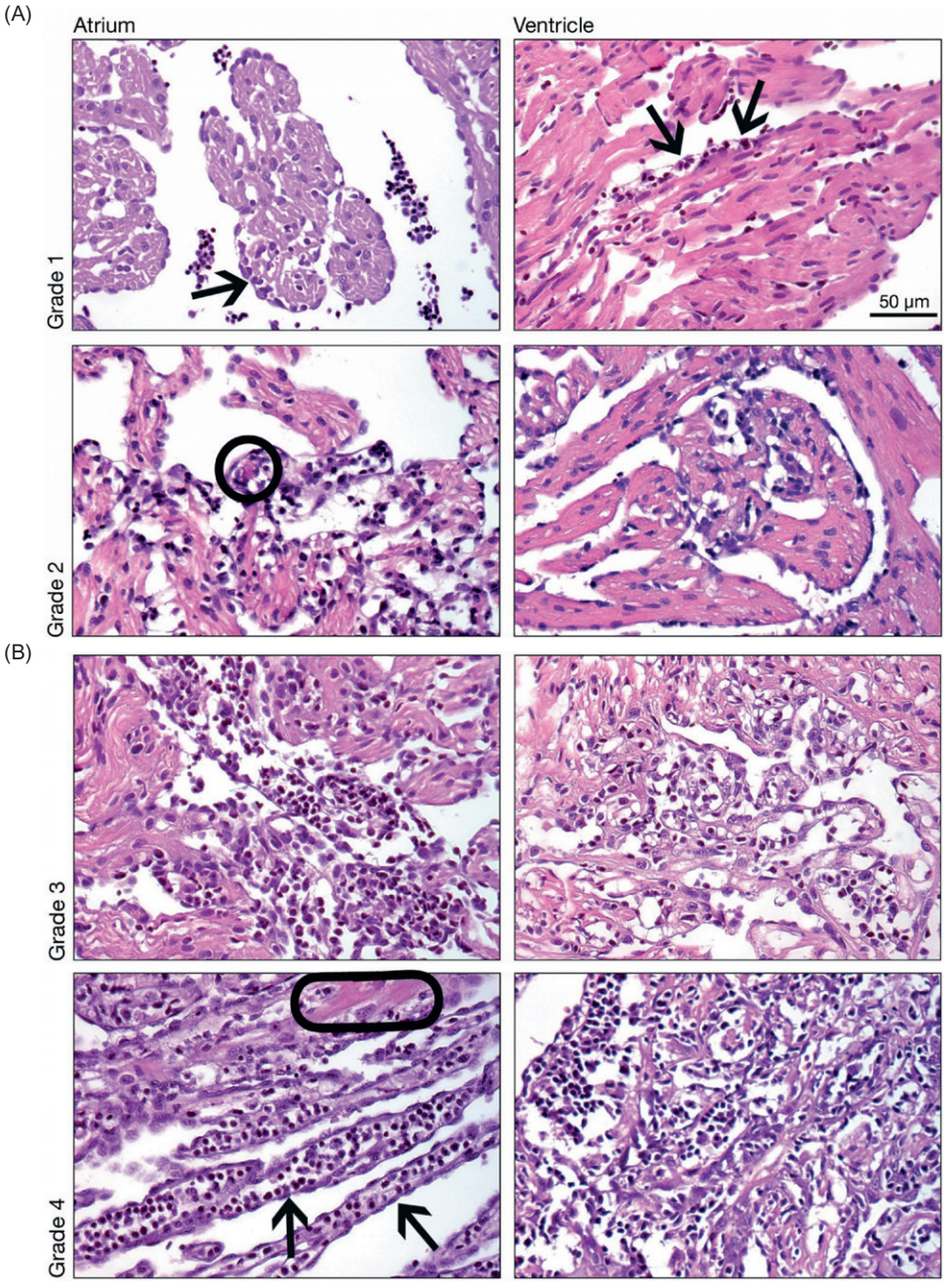

FIGURE 16.4 Histological changes and scoring of lesions in atrium and ventricle. (A) Grade 1: arrows indicate minor inflammatory lesions consisting of sparse, focal subendocardial infiltration by mononuclear leukocytes in atrium and ventricle. Grade 2: several lesions with small to moderate infiltration of leukocytes. Myocyte degeneration and necrosis are encircled in the atrium. (B) Grade 3: Multifocal to confluent lesions with moderate to severe leukocyte infiltration. Grade 4: Arrows indicate hyper-trophic endocardial cells in the atrium, forming empty tubes where almost all muscle fibers have been replaced by inflammatory cells, dominated by small mononuclear lymphocyte-like cells. Myocyte degeneration and necrosis are encircled in the atrium. Ventricular example is from a focal Grade 4 lesion. Hematoxylin and eosin staining. Scale bar (applies to all panels) = 50 µm. *From: Fritsvold, C., Kongtorp, R.T., Taksdal, T., Orpetveit, I., Heum, M., Poppe, T.T., 2009. Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon Salmo salar. Dis. Aquat. Organ. 87, 225–234.*

16.7 DIAGNOSTIC METHODS

The diagnosis of CMS is based on histomorphological assessment of heart specimens by light microscopy. Histopathological changes of the heart can be combined with real-time PCR analysis, preferentially of heart samples, for an etiological diagnosis (Haugland et al., 2011). There is good correlation between virus genome levels in heart samples detected by real-time PCR methods and histopathology scores of heart ventricle (Fig. 16.5; Haugland et al., 2011). The viral genome has also been detected in fish with histopathological changes typical of PMCV infection (Haugland et al., 2011; Lovoll et al., 2010), but in situ hybridization is not used for routine diagnostics. Clinical signs are seldom observed and sick fish die suddenly from a tamponade (bleeding into the heart sac) caused by a rupture of the atrium wall or *sinus venosus* (Fig. 16.6). Other signs, such as skin hemorrhage, raised scales and skin edema, have also been reported in some cases of CMS (Rodger and Turnbull, 2000). Farmed fish are usually affected the second season in seawater (ie, when they get close to slaughter size). Fish may also die from CMS at smaller sizes. Thus CMS mostly affects large fish a few months before reaching slaughter weight. In most cases, apparently healthy fish die without any clinical signs.

Ultrasound examination and echocardiography of anesthetized fish show dilated atrium and compressed cardiac ventricle in terminal stages of CMS. At necropsy, atrial thrombosis, ascites, fibrin exudates and blood clots in the peritoneum and heart are typical findings (Ferguson et al., 1990; Rodger and Turnbull, 2000; Rodger et al., 2014). Congestion of the

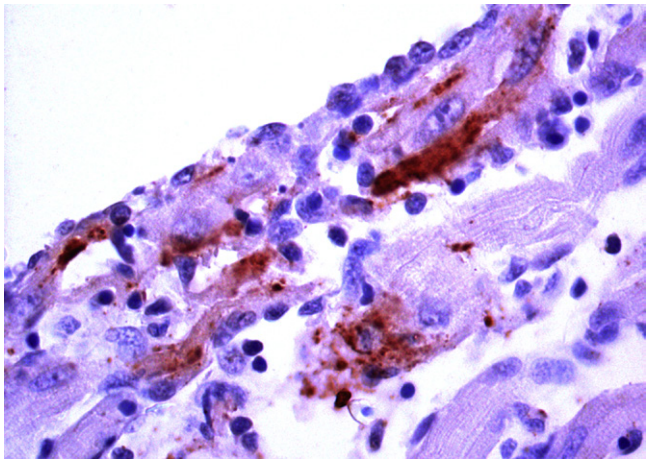

FIGURE 16.5 In situ hybridization that shows presence of viral genome in cardiomyocytes (reddish (dark gray in print versions) coloration). Note: Endothelial cells are negative. Counterstaining with hematoxylin.

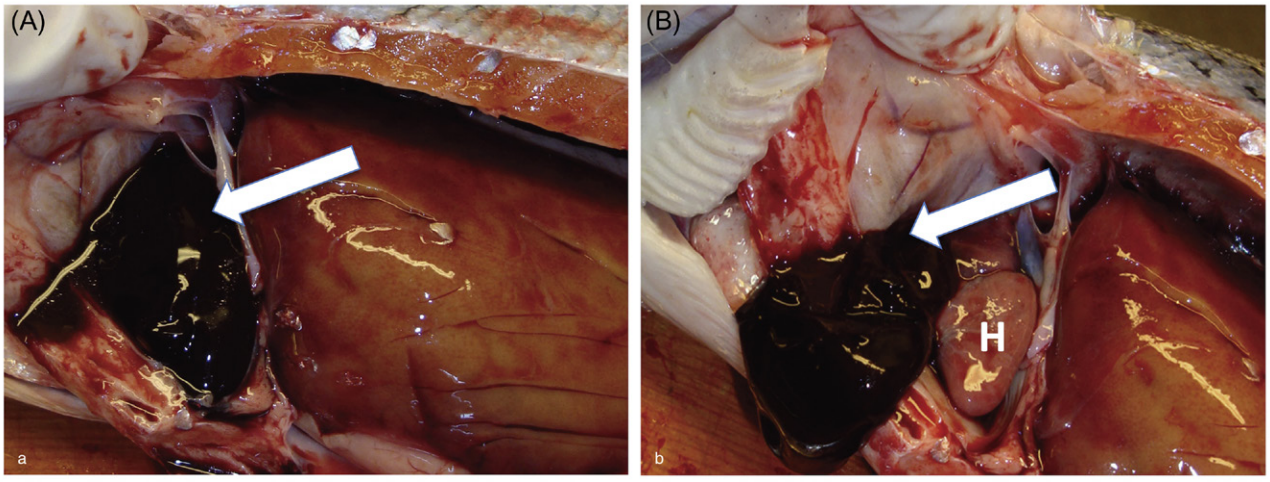

FIGURE 16.6 Atlantic salmon with heart tamponade (*arrow*). (A) The entire heart sac is filled with blood (*arrow*), (B) The blood coagulum has been removed outside the heart sac with the heart (H) displayed.

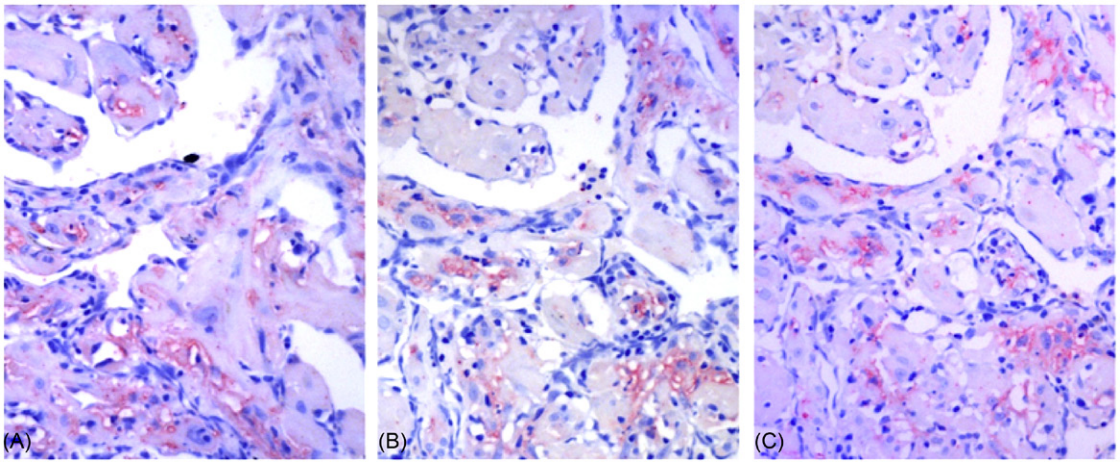

FIGURE 16.7 Immunohistochemistry of heart samples from Atlantic salmon with a histopathological diagnosis of CMS. (A) Red (dark gray in print versions) coloration in cardiomyocytes is indicative of the presence of PMCV using polyclonal antibodies based on recombinant proteins from ORF1 and ORF3 (kindly provided by PHARMAQ AS). The signal strength obtained using ΔORF-1 (A), ORF-1 (B) and (C) ORF-3 expressed proteins for generation of antibodies resulted in staining of variable distribution and intensity. All antibodies were raised in rabbits and diluted 1:500. Developed with a Streptavidin alkaline phosphatase method.

spleen and in the gills, likely caused by circulation collapse, are also typical signs (Rodger and Turnbull, 2000; Rodger et al., 2014). Differential diagnoses are pancreas disease and heart and skeletal muscle inflammation, and both can be differentiated from CMS by heart histopathology and real-time PCR analysis.

Immunohistochemistry (IHC) has not been established as a routine diagnostic tool. It has proven difficult to obtain good antibodies to the capsid protein and the ORF3-encoded protein (own observation). Preliminary studies show that IHC could serve as an adjunct to the diagnostic methods currently available (Fig. 16.7).

16.8 PREVENTION AND CONTROL

The source of infection is not known. It is possible that wild fish species are carriers of the virus (Bockerman et al., 2011) and transmit the infection to farmed salmon. CMS has also been described in wild-caught Atlantic salmon (Poppe and Seierstad, 2003), but little is known and those totivirus-like viruses that have been detected in wild fish species are different from what is found in Atlantic salmon (Tengs and Bockerman, 2012), wild or farmed. In addition, the fact that farmed salmon develop the disease toward the late stage of the grow-out cycle points toward the fish not being infected in freshwater. There are also no documented cases of CMS or PMCV infection at the freshwater stage (in hatcheries).

For these reasons, it is difficult to implement control measures against PMCV infection. However, there are studies showing that inflammatory responses and induced pathological changes after experimental challenge can be modulated by functional feeds (Martinez-Rubio et al., 2014). The study showed that there were significant differences in immune and inflammatory responses in groups fed reduced lipid content and increased eicosapentaenoic, and induced pathology in heart tissue was alleviated in these fish over the course of the infection. Fish fed the functional feeds had milder and delayed inflammatory responses and thus showed less severity of heart lesions at early and late stages post-experimental infection (Martinez-Rubio et al., 2014). These findings would indicate that clinical course of the infection can be modulated by feed intervention.

No vaccines are currently available although studies are ongoing (research and development stage) to develop vaccines against PMCV infection, but there are no publications on vaccine efficacy against the disease.

ACKNOWLEDGMENT

Financial support has been received from the Research Council of Norway project no. 216177, *Studies of virulence mechanisms and host responses to infection with piscine myocarditis virus (PMCV)* to ØE. The assistance from Dr. Ida Skaar, Norwegian Veterinary Institute, Oslo, Norway, in preparing Fig. 16.3 is appreciated. The efforts of Snorre Gulla and Simen Nørstebø DVMs, in testing the different antibodies against PMCV for immunohistochemical detection in situ, are appreciated.

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Chapter 17

Paramyxoviruses of Fish

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17.1 INTRODUCTION

The family *Paramyxoviridae* (*para*=beyond; *myxo*=mucus or slime) within the order *Mononegavirales* contains two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, comprising 9 recognized genera and 36 species. These viruses have an affinity for mucoproteins and enzyme activity against mucoprotein substrates. Many of the viruses within this group are important pathogens infecting humans and other vertebrate mammals, birds and reptiles, causing a range of diseases (most notably human measles and mumps, Newcastle disease in birds, distemper in canids and seals and respiratory diseases in primates, cetaceans, rodents and snakes). However, only two aquatic paramyxoviruses have been genetically characterized from fish: Pacific salmon paramyxovirus (PSPV) and Atlantic salmon paramyxovirus (ASPV). A new genus, *Aquaparamyxovirus*, is represented by ASPV as the type species (ICTV, 2014).

PSPV was initially isolated from the pooled kidney and spleen (but not ovarian fluids) of healthy adult Chinook salmon *Oncorhynchus tshawytscha* returning to Oregon coastal hatcheries in 1982 and 1983 (Winton et al., 1985). In Chinook salmon embryo cells (CHSE-214), the virus was slow to replicate, taking up to 4 weeks to show cytopathic effects (CPEs). CPEs consisted of clustered rounding of cells with increased refractility becoming generalized throughout the cell monolayer (Fig. 17.1) with detachment of cells in the medium. Cell cultures can also become persistently infected. The CPEs appeared similar to cell senescence and are easily overlooked in older cell cultures. The virus proved to be avirulent for at least four species of salmonids with no evidence of replication following injection of high titers of cultured virus (Lannan et al., 1989). Subsequently, pathologists at several fish health laboratories located in coastal areas from California to Alaska isolated paramyxoviruses in cell cultures inoculated with internal tissues or reproductive fluids of normal fish during routine surveillance. These isolates were almost exclusively from adult Chinook salmon returning to spawn, except for one isolate from coho salmon *Oncorhynchus kisutch* (Batts et al., 2008). However, based on the prolonged incubation period and the rounding/refractile CPE, presumed isolates that have not been confirmed genetically have been made from several other salmonid species, including pink *Oncorhynchus gorbuscha*, sockeye *Oncorhynchus nerka* and coho salmon from Washington State and coho salmon and steelhead *Oncorhynchus mykiss* from California (Batts et al., 2008).

ASPV was first isolated in Norway in 1995 during an epizootic of gill disease in seapen-farmed, postsmolt Atlantic salmon *Salmo salar* from gill tissues inoculated onto a rainbow trout gill cell line (RTgill-W1). The virus required 9 weeks for CPEs to develop that involved syncytial cell formation with retraction and floating vesicles in the medium (Kvellestad et al., 2003; Fridell, 2003; Fridell et al., 2004). Although the virus was associated with a condition described as *proliferative gill inflammation* (*PGI*), several other potential etiological agents (including bacteria, protozoa and a poxvirus) have also been present and may contribute to the gill pathology syndrome (Kvellestad et al., 2003; Nylund et al., 2008b). Virus inoculation of susceptible salmon did not produce fish mortality (Fridell, 2003) or any clinical pathology, but the fish remained persistently infected and virus could be reisolated. Further immunostaining studies demonstrated that ASPV was present in proliferating epithelial and endothelial cells of Atlantic salmon gills from three farms that suffered mortality from PGI. It was concluded that the cause of PGI was multifactorial, involving ASPV and a chlamydia-like bacteria (Kvellestad et al., 2005). Later studies involving six farms in southwestern Norway with outbreaks of PGI indicated that the disease was indeed multifactorial, but it likely was caused by severe infections of chlamydia, another unknown epitheliocyst-forming organism and a microsporidian protozoan, while ASPV was not detected (Steinum, 2010; Steinum et al., 2010).

Paramyxo-like viruses have also been reported from other salmonid and nonsalmonid species. These have included rainbow trout *O. mykiss* in Germany (Neumann et al., 1986); Japanese black seabream *Acanthopagrus schlegelii*, with ultrastructural observations of a virus associated with epidermal necrosis (Miyazaki et al., 1989); isolation of a virus from

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00017-6
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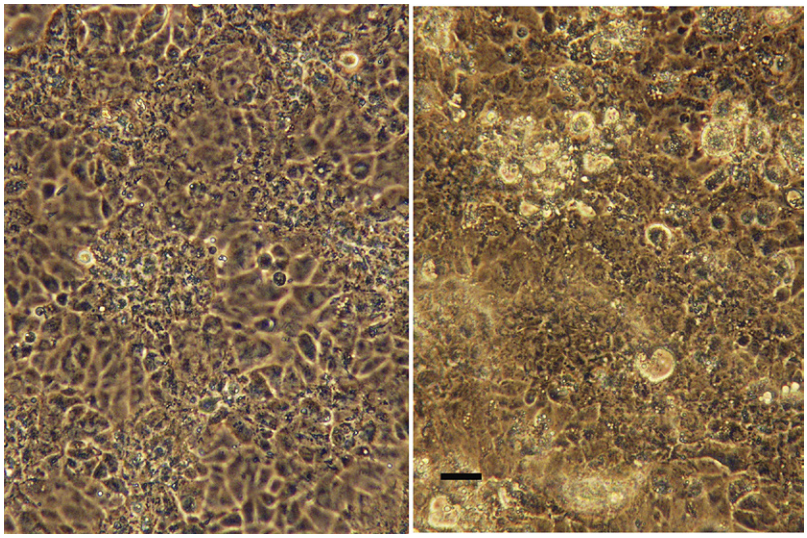

FIGURE 17.1 Cytopathic effects of PSPV from Ninilchik River, Alaska, in CHSE-214 cells at 14°C, demonstrating clustered rounding and refractility of cells (right) at 9 days postinoculation of previously passaged material. Normal cell culture (left). Phase contrast; scale bar = 20 µm.

TABLE 17.1 Fish Paramyxovirus Hosts, Diseases and References

Natural Fish Host	Genus Species	Disease	Reference
Chinook salmon	*Oncorhynchus tshawytscha*	None—avirulent	Winton et al. (1985)
			Batts et al. (2008)
Coho salmon	*Oncorhynchus kisutch*	None—avirulent	Batts et al. (2008)
Atlantic salmon	*Salmo salar*	Proliferative gill inflammation	Fridell et al. (2004)
			Kvellestad et al. (2005)
Rainbow trout	*Oncorhynchus mykiss*	Not known	Neumann et al. (1986)
Black seabream	*Acanthopagrus schlegeli*	Epidermal necrosis	Miyazaki et al. (1989)
Turbot[a]	*Scophthalmus maximus*	Buccal/opercular hemorrhage	Cepeda et al. (1993)
		Erythrocytic inclusions	Lamas et al. (1995)
Koi and common carp[a,b]	*Cyprinus carpio*	Gill necrosis	Body et al. (2000)
Other salmonid species	Virus Not confirmed	None—avirulent	Batts et al. (2008)

[a]*Not likely paramyxovirus based on further characterization (Lamas et al., 1996; Granzow et al., 2014).*
[b]*Recent characterization suggests one carp virus is a new species and possibly new genus of Paramyxoviridae (Haenen et al., 2016).*

cultured turbot *Scophthalmus maximus* in Spain, associated with elevated mortality, buccal/opercular hemorrhaging and erythrocytic inclusion bodies (Cepeda et al., 1993; Lamas et al., 1995); and isolation of a virus from koi carp *Cyprinus carpio*, associated with high mortality and gill necrosis in Belgium, England, Germany and South Africa (Body et al., 2000). Later studies with the turbot virus showed that some characteristics were similar to other paramyxoviruses, while others were not (ie, no hemagglutination activity, smaller particle sizes, different method of host cell penetration), which suggested further investigation is necessary to classify the virus (Lamas et al., 1996). Subsequent investigations with the koi carp virus produced two similar syncytial-forming enveloped RNA virus isolates associated with gill necrosis from common carp *C. carpio* and also in koi from Germany and Belgium. The viruses did not hemagglutinate mammalian erythrocytes and, although having some features of a paramyxovirus, other ultrastructural morphologies were not consistent with members of the family *Paramyxoviridae*, nor with those of other known virus families (Granzow et al., 2014) (Table 17.1). More recent investigation using molecular based results has led to the classification of at least one of these carp viruses as a new species and possibly a new genus within the family *Paramyxoviridae* (Haenen et al., 2016).

17.2 GENETICALLY CHARACTERIZED PARAMYXOVIRUSES IN FISH

The genetically characterized paramyxoviruses in fish are as follows:

- Pacific salmon paramyxovirus—No associated disease
- Atlantic salmon paramyxovirus—Associated with PGI, together with other potential etiological agents in pen-farmed Norwegian Atlantic salmon

The definitive etiology of PGI remains undetermined (Mitchell and Rodger, 2011).

17.2.1 Structure and Composition of the Virus

PSPV is an irregular-shaped, negative-sense, single-stranded RNA virus approximately 125–250 nm in diameter. The virus has an envelope and is inactivated by heat (37°C after 24 h), chloroform and a pH of 2. The virus has a buoyant density of 1.20 g/mL in CsCl and hemagglutinates erythrocytes from several species of mammals, birds and fish (Winton et al., 1985). The coiled nucleocapsid within the virus particle is a left-handed helix of 18 nm in diameter and up to 1000 nm in length, while unassembled nucleocapsids can be observed near the surface of infected cultured cells (Winton et al., 1985; Meyers et al., 2008) (Figs. 17.2 and 17.3).

ASPV is a spherical to irregular-shaped, negative-sense single-stranded RNA virus approximately 150–300 nm in diameter that is enveloped, sensitive to high and low pH, has hemagglutination activity and a neuraminidase receptor–destroying enzyme with a buoyant density of 1.18–1.19 g/mL in CsCl. The nucleocapsid filaments within the virions are 17 nm thick, arranged in a herringbone pattern, and there are five major structural polypeptides with estimated molecular masses of 70, 62, 60, 48 and 37 kDa (Kvellestad et al., 2003). The complete genome is 16,968 nucleotides in length, consisting of six monopartite genes in the order of 3′-N-P-M-F-HN-L-5′ coding for the nucleocapsid, phospho-, matrix, fusion, hemagglutinin-neuraminidase and large polymerase proteins (Nylund et al., 2008a; Falk et al., 2008).

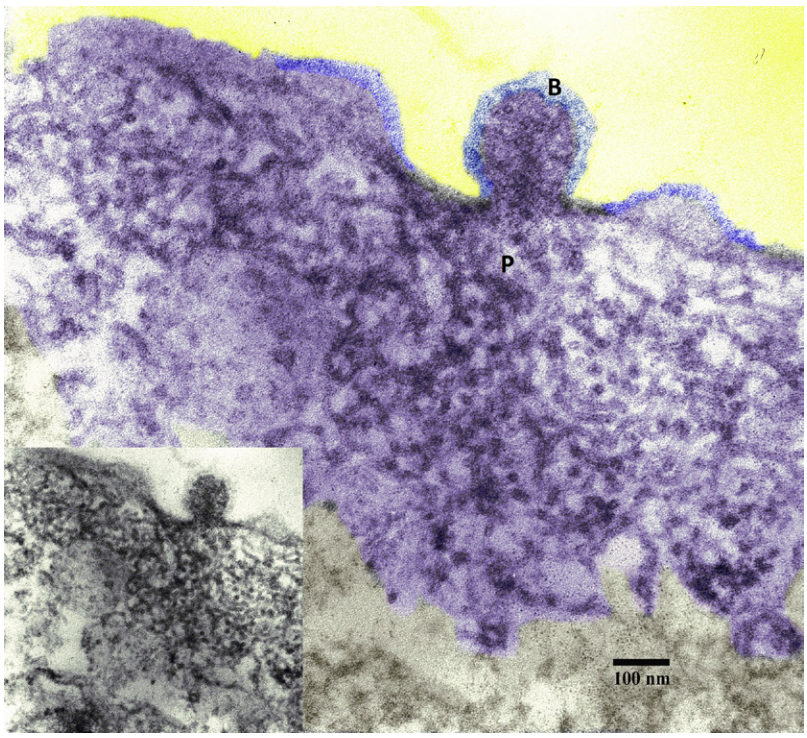

FIGURE 17.2 Chinook salmon, Ninilchik River, Alaska. Falsely colorized ultrastructure of PSPV in infected CHSE-214 cell with three virus particles in different stages of budding from cell membrane; blue (B) = spiked envelope; purple (P) = nucleocapsid within particles from pool of subsurface nucleocapsid material in the cytoplasm. Inset: same image not colorized.

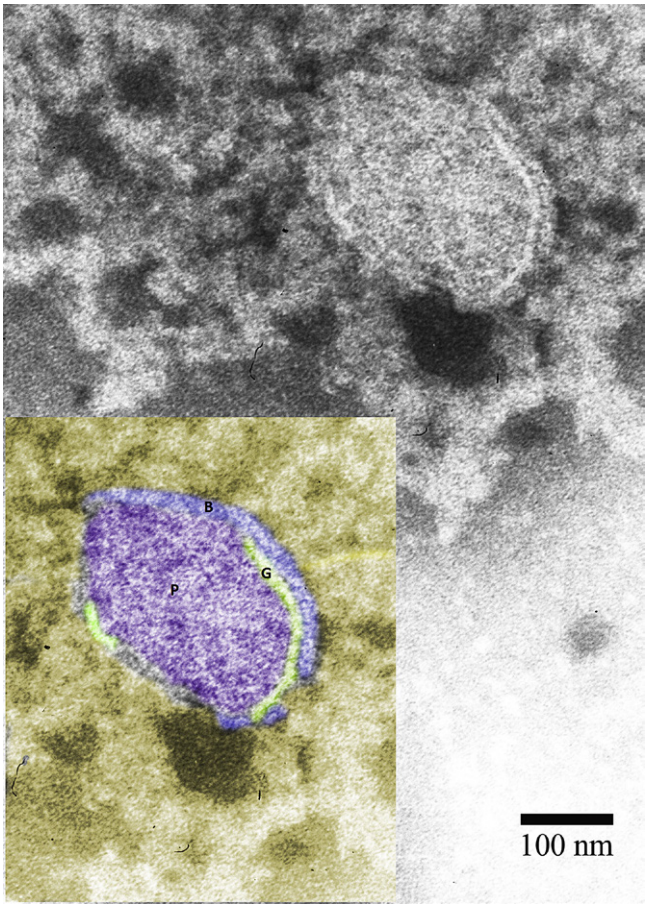

FIGURE 17.3 Negative stain of partially disintegrated particle of PSPV surrounded by nucleocapsid debris cultured from Chinook salmon, Ninilchik River, Alaska. Inset: same negative stain falsely colorized to show the spiked glycoprotein envelope (blue = B), underlying matrix protein (green = G) and nucleocapsid material (purple = P) within the virus particle.

17.2.2 Classification and Virus Replication

When first described, the morphology and biochemical characteristics of PSPV fit most closely within the *Paramyxoviridae* (Winton et al., 1985). Later studies of PSPV and ASPV indicated that both viruses were genetically related but distinct (maximum nucleotide diversity of 26.1% and amino acid diversity of 19.0%) based on the analysis of a 505-base pair region representing 168 amino acids of the polymerase L gene (Batts et al., 2008). When both viruses were compared to homologous sequences of other known paramyxoviruses, they were significantly different from members of the currently established genera within the family *Paramyxoviridae*; that is, each was contained in its own major phylogenetic branch (Batts et al., 2008). This result supported three other genetic studies comparing ASPV with known paramyxoviruses that indicated it was likely a novel virus in the subfamily *Paramyxovirinae* most closely related to members of the genus *Respirovirus* (Fridell et al., 2004; Falk et al., 2008; Nylund et al., 2008a). In 2014, the International Committee on the Taxonomy of Viruses (ICTV) released the creation of a new genus *Aquaparamyxovirus* with ASPV as the type strain (ICTV, 2014). Another aspect of genetic analysis that focused specifically on PSPV phylogenetic relationships included comparisons of 47 isolates from Alaska to California from 1983 to 2007. The results established 17 nucleotide sequence types that could be grouped into two major sublineages of A (13 types) and B (4 types) that differed by 12.1–13.9% at the nucleotide level and only 1.2% at the amino acid level (Batts et al., 2008) (Fig. 17.4).

Typical paramyxovirus replication is in the cytoplasm following fusion to the host cell membrane and entry into the cell. Replication follows the negative-sense RNA model, where the viral nucleotide sequence is complementary to the positive-sense mRNA that it encodes using RNA polymerase "stop-start" transcription for the translation and production of viral proteins. These proteins, assembled within the outer cytoplasm, include late assembly domains that recruit the host cell

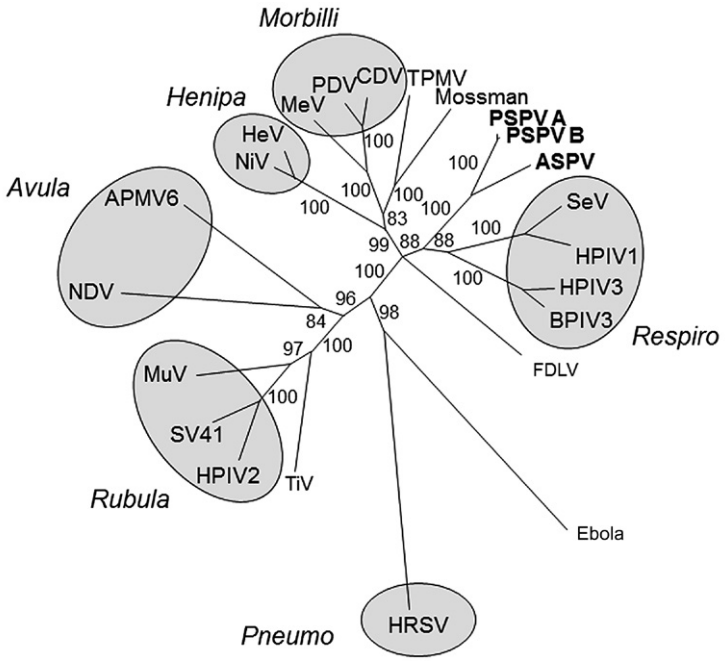

FIGURE 17.4 A representative phylogenetic tree illustrating the relationship of the partial amino acid sequences (168 amino acids) of the polymerase gene of three paramyxoviruses of salmonids (bold print) and other members of the family *Paramyxoviridae*. The tree was generated by neighbor-joining distance methods in which the horizontal branch lengths accurately reflect genetic distance from the *Ebola virus*, a member of the family *Filoviridae*, designated as the outgroup. Abbreviations (with GenBank accession numbers) are: SeV, *Sendai virus* (M30302); HPIV1, *Human parainfluenza virus 1* (AF457102); HPIV3, *Human parainfluenza virus 3* (NC_001796); BPIV3, *Bovine parainfluenza virus 3* (AF178654); FDLV, *Fer-de-Lance virus* (AY141760); TPMV, *Tupaia virus* (AF079780); Mossman, *Mossman virus* (AY286409); PDV, *Phocine distemper virus* (D10371); CDV, *Canine distemper virus* (AF014953); MeV, *Measles virus* (AB012948); NiV, *Nipah virus* (AF212302); HeV, *Hendra virus* (AF017149); TiV, *Tioman virus* (AF298895); SV41, *Simian virus 41* (X64275); HPIV2, *Human parainfluenza virus 2* (X57559); MuV, *Mumps virus* (NC_002200); NDV, *Newcastle disease virus* (AF077761); APMV6, *Avian paramyxovirus 6* (AY029299); HRSV, *Human respiratory syncytia virus* (M11486); Ebola, *Ebola virus* (AF086833). Viruses considered to be members of established genera of the *Paramyxoviridae* are indicated by their inclusion within shaded ovals labeled *Respiro*, *Morbilli*, *Henipa*, *Avula*, *Rubula* and *Pneumo*.

system of endosomal sorting complex required for transport to assist in the budding of a progeny virus through the host cell plasma membrane from which the envelope is formed (Harrison et al., 2010).

PSPV replicates in several fish cell lines cultured in standard Eagle's minimal essential medium supplemented with 10% fetal bovine serum. These cell lines include Chinook salmon embryo (CHSE-214), chum salmon heart (CHH-1), kokanee salmon ovary (KO-6) and coho salmon embryo (CSE-119). Replication of virus at low titer occurs in the fathead minnow (FHM) cell line producing no CPEs. Cell rounding type CPEs take approximately 28 days (14 days initial passage, with a 14-day blind passage) to develop on primary isolation with optimal growth at 18°C. Thereafter, a passaged virus will produce earlier CPEs in approximately 10 days (Winton et al., 1985). Viral CPEs will also occur at 15°C–21°C, but not at 24°C or more, nor will the virus replicate in certain other fish cell lines, including rainbow trout gonad (RTG-2), Atlantic salmon, brown bullhead or *Epithelioma papulosum cyprini* (EPC) (Winton et al., 1985).

ASPV has been isolated in the RTgill-W1 cell line propagated in Leibovitz L-15 medium supplemented with 5% fetal bovine serum but the susceptibilities of other fish cell lines have not been reported. The temperature range for virus replication is 6°C–21°C, and it takes up to 9 weeks postinoculation for syncytial CPEs to develop (Kvellestad et al., 2003). The putative paramyxo-like virus reported in koi also produced syncytial CPEs in EPC and FHM cells after 2–3 weeks postinoculation at 14°C, 21°C and 28°C, but not in RTG-2 cells (Body et al., 2000).

17.2.3 Epidemiology

The established membership within the *Paramyxoviridae* of the A and B sublineages of PSPV and their relatedness to ASPV with equal divergence suggested a common origin from one or more marine fish hosts (Batts et al., 2008). However, both PSPV and ASPV were not sufficiently similar to known genera of paramyxoviruses to determine their evolutionary history. Of further interest, the PSPV sublineages showed no evidence of separation (geographically or temporally), and no

intermediate sequence types were identified. It is also noteworthy that PSPV has not been found in fish from eastern Oregon, Idaho or British Columbia, Canada, where many stocks of Chinook salmon return and spawn, while only three isolates of the same sublineage (A) were cultured in Alaska for three consecutive years from one watershed. Collectively, these data suggest that both sublineages are independently cocirculating in certain stocks of Chinook salmon in specific regions of the Pacific Northwest where fish become infected during their marine life, possibly as adults returning to spawn (Batts et al., 2008). The one isolation of PSPV from coho salmon may have represented a "spillover event" since it belonged to the large sequence type A4*n*14, in which 13 of the 14 identical sequences were obtained from Chinook salmon (Batts et al., 2008).

Prior to the isolation of ASPV, the disease PGI was first reported in the 1980s from all coastal areas of Norway involved with farming of seawater-reared Atlantic salmon. Typically, PGI is most severe during autumn, affecting fish that were smolts transferred to seawater the previous spring and outbreaks may continue for several months (Kvellestad et al., 2005). Although ASPV has been associated with PGI, the disease has not been reproduced experimentally by exposure to the virus and is not always present during disease outbreaks.

17.2.4 Pathology and Immunity

PSPV is not associated with fish mortality or pathological tissue changes (Winton et al., 1985), and there was no reported evidence of replication in four species of salmonid fish experimentally injected with high titers of cultured virus (Lannan et al., 1989). No studies have been published regarding fish immunity to the virus.

ASPV has been associated with PGI in Norwegian pen-farmed Atlantic salmon, but the presence of several other infectious agents (including chlamydia, external protozoa and at least one other virus) has confounded the true etiology of the disease. Because PGI is not a notifiable disease to the Office International des Epizooties (OIE), records have been incomplete regarding the negative effect of fish losses in the Norwegian salmon industry. At best, the cause of PGI has been considered multifactorial, in which ASPV has been hypothesized as a contributing factor (Kvellestad et al., 2005), while other studies have shown no association of the virus with clinical disease (Steinum et al., 2010). Clinical signs for PGI are pale gills with reduced growth rate and varying fish mortality, accompanied by histopathological changes of gill epithelial necrosis and proliferation, and inflammatory exudate within the gill filaments, accompanied by hemorrhage, hemosiderosis and thrombosis with various other bacterial and protozoal agents present (Kvellestad et al., 2005). Although ASPV causes no mortality or disease when experimentally injected into juvenile Atlantic salmon, the virus can persist and be reisolated (Fridell, 2003). However, no studies have been published regarding fish immunity to the virus.

17.2.5 Diagnostic Methods

PSPV can be isolated in fish cell cultures inoculated with kidney/spleen or ovarian fluids from sexually mature salmonids. Several cell lines will support viral replication, including CHSE-214 cells commonly used in many fish health laboratories for routine pathogen surveillance of wild and hatchery salmonids. Slow to develop cell rounding and refractility typically does not occur until 10–28 days, often requiring a blind passage. Electron microscopy of cell culture material can be used to demonstrate paramyxovirus particle morphology with confirmation by PCR using primers developed from a 912-nucleotide partial L gene sequence of the Trask River index PSPV isolate (Batts et al., 2008).

ASPV can be isolated in the RTgill-W1 cell line, resulting in similar delayed CPEs of 9 weeks with syncytial cell formation (Kvellestad et al., 2003). The virus can be confirmed by PCR using primers developed from a 816-nuceotide partial L gene sequence of ASPV (Fridell et al., 2004). Using polyclonal rabbit anti-ASPV serum, additional diagnostic tests performed have included viral neutralization (48 days), hemagglutination inhibition, immunofluorescent staining of infected cell cultures and cryosections of gills and immunohistochemistry of paraffin-embedded sections of gill tissues (Kvellestad et al., 2005).

17.2.6 Prevention and Control

The established avirulence of PSPV for juvenile salmonids and its typical occurrence in clinically normal adult Chinook salmon (and possibly other salmonids) in the US Pacific Northwest has mitigated concerns by fish health regulators regarding its prevention and control. This outcome is fortunate because the relatively irregular occurrence of the virus in certain stocks of fish and the delayed cell senescent-like CPEs would make detection less likely during general surveillance if not routinely practicing blind passage of normal-appearing cell cultures. Furthermore, very little is known about the overall epidemiology and host relationship of the virus, which complicates managing the virus in fish populations. If management was necessary, additional studies would be clearly warranted.

Conversely, ASPV is suspected of being a multifactorial contributor to PGI in the Norwegian Atlantic salmon industry, but the true etiology of the disease requires further study. The virus appears to be widespread among farmed salmon populations with or without PGI, together with other potential causative agents of the disease, making prevention and control problematic and not likely in the near future. Again, much work still needs to be done to determine whether any preventative management of the virus is necessary or practical.

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Chapter 18

Rhabdoviruses of Fish

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18.1 INTRODUCTION

Members of the family *Rhabdoviridae* have an enveloped helical nucleocapsid that is bullet-shaped or cone-shaped, 170 nm long and 70 nm in diameter. The family belongs to the order *Mononegavirales*; it has a single-stranded RNA genome of negative polarity, 11–15 kb in size. Virus multiplication takes place in the cytoplasm and virions bud on plasma membrane or internal membranes; intracytoplasmic inclusion bodies are often formed. The family has eight genera, three (*Vesiculovirus*, *Ephemerovirus*, *Lyssavirus*) contain mammalian, two (*Cytorhabdovirus*, *Nucleorhabdovirus*) plant and three (*Novirhabdovirus*, *Perhabdovirus*, *Sprivivirus*) fish rhabdoviruses. Although all the three genera of fish rhabdoviruses contain viruses that cause disease in both farmed and wild fish populations (Table 18.1), the three most important fish rhabdoviruses are infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in genus *Novirhabdovirus*, and spring viremia of carp virus (SVCV) in genus *Sprivivirus*; these three viruses are covered in this chapter. All three are World Organization for Animal Health (OIE) reportable diseases (OIE, 2015a,b,c) and they can have a considerable economic impact due to clinical disease, production loss and trade or regulatory costs.

18.1.1 Structure and Composition of the Virus

The virions of all three genera are bullet-shaped, typical of the *Rhabdoviridae* (King et al., 2011); IHNV is approximately 110 nm in length and 70 nm in diameter, VHSV is approximately 200 × 75 nm (Coll, 1995a) and SVCV is 60–90-nm wide and 80–180-nm long. All three viruses contain a single-stranded RNA of negative polarity and a genome size varying between 11.0 and 11.2 kb, typically with SVCV < IHNV < VHSV (Bjorklund et al., 1996; Hoffmann et al., 2002; Morzunov et al., 1995; Schutze et al., 1995; Teng et al., 2007; Zhang et al., 2009). There are six genes in the genome of IHNV and VHSV that include in the 3′ to 5′ order the nucleocapsid (N), phosphoprotein (P), matrix protein (M), surface glycoprotein (G), nonvirion protein (NV) and virus polymerase (L) (Bearzotti et al., 1999; Einer-Jensen et al., 2004; Gaudin et al., 1999; Nishizawa et al., 2002, 2006b; Schutze et al., 1995) (Fig. 18.1). SVCV lacks the NV gene, but otherwise has the same gene order (Hoffmann et al., 2002; Teng et al., 2007).

The virus particle or virion is composed of five proteins (N, P, M, G, L) and two major parts. The inner helical ribonucleocapsid core consists of the ribonuclease genome and N, M and L proteins. The matrix protein (M) attaches to both the G protein on the internal side of the membrane and to the ribonucleocapsid. The outer membrane consists of a lipid bilayer membrane and the glycoprotein (G) that projects externally and forms noncovalently bound homotrimer spikes.

The N protein of IHNV contains 413 amino acids and has a molecular mass of 40.5–44 kDa. This is the earliest expressed and most abundant protein produced by the virus during an IHNV infection. The P protein, previously called the M1 protein, contains 231 amino acids and has a molecular mass of 25.6 kDa. The function of the P protein is not known. The M protein, previously called the M2 protein, is a highly basic protein. The M protein contains a number of basic amino acids at the N-terminal end that are conserved among the homologous matrix proteins of other fish rhabdoviruses, including VHSV and hirame rhabdovirus (HIRRV). The G protein forms the spike-like projections on the surface of the mature virion. The predicted amino acid contains 508 residues with a molecular mass of 67–70 kDa. The L encodes a protein of 1986 amino acids with a predicted molecular weight of approximately 225 kDa and shows similarity to the RNA-dependent RNA polymerase genes of other rhabdoviruses. The *NV* gene was discovered first in IHNV between the G and L genes and subsequently in other aquatic rhabdoviruses such as VHSV, HIRRV, snakehead rhabdovirus, eel virus B12 and eel virus C26 (Hoffmann et al., 2005). The *NV* gene encodes a nonstructural protein (Kurath et al., 1985), which can be identified within infected cells but not in purified virions (Hoffmann et al., 2005).

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00018-8
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TABLE 18.1 Rhabdoviruses of Teleosts Recognized by the International Committee on the Taxonomy of Viruses (ICTV)

Genus	Virus	Disease	Major Species Affected
Novirhabdovirus	Hirame rhabdovirus		Flounder[1], grayling[2], various[3]
	IHNV	Infectious hematopoietic necrosis virus[a]	Salmonids
	Snakehead virus		Snakehead[4], warmwater fish southeast Asia[5]
	VHSV	Viral hemorrhagic septicemia virus[a]	Broadly infectious
Perhabdovirus	Anguillid rhabovirus		Eels[6]
	Perch rhabdovirus		Perch[7], various[8]
	Sea trout rhabovirus		*Salmo trutta*[9]
Sprivivirus	Pike fry rhabdovirus	"Red disease"[10]	Pike[10], various[11]
	SVCV	Spring viremia of carp virus[a]	Cyprinids

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[a]*Covered in detail in the present chapter.*

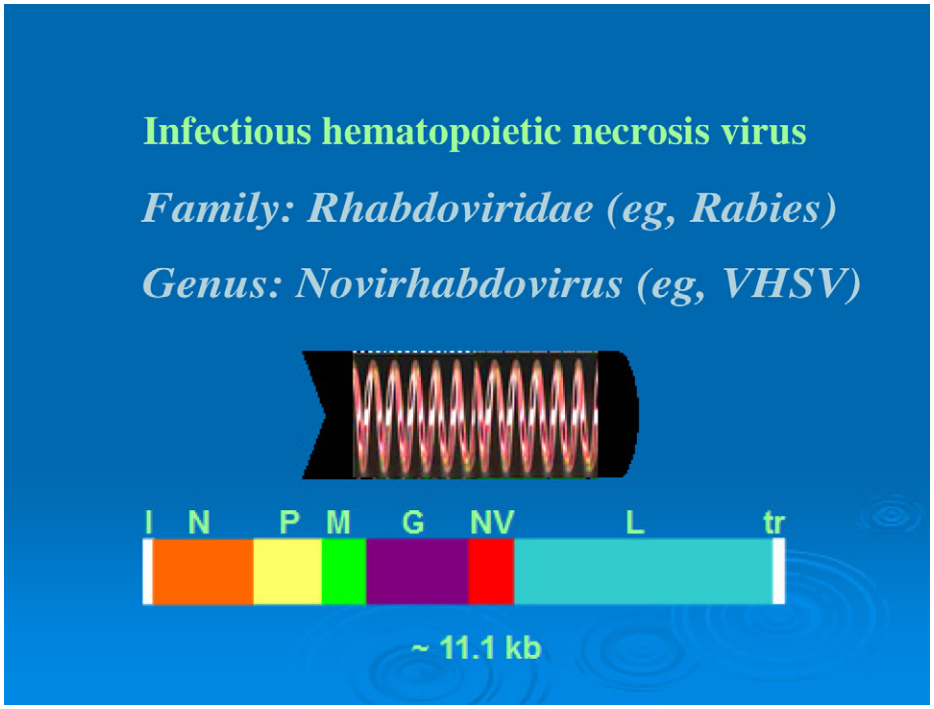

FIGURE 18.1 There are six genes in the genome of infectious hematopoietic necrosis virus (IHNV) that include in the 3′ to 5′ order the nucleocapsid (N), phosphoprotein (*P*), matrix protein (M), surface glycoprotein (G), nonvirion protein (NV) and virus polymerase (*L*).

The encoded proteins of VHSV are the nucleocapsid (35 kDa), polymerase-associated protein (24 kDa), matrix, glycoprotein (65 kDa) and polymerase (220 kDa) (Basurco and Benmansour, 1995; Estepa et al., 1994). The molecular weight of sequence predicted SVCV proteins was 238, 57, 35.6 and 25.6 kDa for the L, G, N and P proteins, respectively, which was slightly different from sodium dodecyl sulfate polyacrylamide gel electrophosphoresis (SDS-PAGE) due to posttranslational modifications (Ahne et al., 2002; Deuter and Enzmann, 1986; Lenoir, 1973; Lenoir and de Kinkelin, 1975). A VHSV virion is composed of approximately 74% protein, 20% lipid, 3% carbohydrate and 3% nucleic acid (Coll, 1995a).

18.1.2 Classification and Virus Replication

The family *Rhabdoviridae* belongs to the order *Mononegavirales* and until the late 1980s only included the genus *Vesiculovirus* and the genus *Lyssavirus* based on electrophoretic protein profiles (Hoffmann et al., 2005). The use of polymerase chain reaction (PCR)-based approaches and sequence analysis, resulted in the addition of 10 genera to the family Rhabdoviridae; *Ephemerovirus, Cytorhabdovirus, Nucleorhabdovirus, Sigmavirus*, Unassigned, Tibovirus, Tupavirus, *Perhabdovirus, Novirhabdovirus* and *Sprivivirus*. The latter three genera contain the fish rhabdoviruses. IHNV is the type species in the genus *Novirhabdovirus* within the family Rhabdoviridae.

IHNV and VHSV are included in the genus *Novirhabdovirus* (International Committee on Taxonomy of Viruses, 2014). The main difference between *Novirhabdovirus* and *Vesiculovirus* and is the presence of the NV gene in *Novirhabdovirus* (ICTV, 2014; Kurath et al., 1997). The NV protein, which is nonstructural, is required for efficient replication of novirhabdoviruses in vivo and North American isolates of VHSV that lacked the NV gene had reduced pathogenicity in olive flounder (*Paralichthys olivaceus*) (Kim et al., 2011) and yellow perch (*Perca flavescens*) (Ammayappan et al., 2011).

SVCV was tentatively placed within the genus *Vesiculovirus* (King et al., 2011); however, it was recently proposed to be included in a new genus, *Sprivivirus* (Adams et al., 2013). There are several additional features that distinguish the SVCV genome, including a smaller noncoding region between the *G* and *L* genes (open reading frames) that is characteristic for fish rhabdoviruses of the genus *Lyssavirus* (King et al., 2011). SVCV, similar to other vesiculoviruses, has highly conserved transcription stop/polyadenylation signals, (TATG(A)7), and an identical transcription initiation sequence (AACAG) that differs from other genera in *Rhabdoviridae*. On the other hand, it is unique in that SVCV has 10 nucleotides of untranslated sequence upstream from the ATG-codon start signal of each gene; this occurs in all SVCV genes except the *P* gene (G instead of C in position-4) (Hoffmann et al., 2002).

The replication cycle of VHSV and other fish rhabdoviruses is largely based on inference from mammalian viruses like VSV and has been summarized in detail elsewhere (Smail and Snow, 2011). Generally, the replication cycle of a rhabdovirus consists of attachment, entry, un-coating, transcription, translation, genome replication, assembly and then release (or lysis). The critical step in virus replication is the viral-host cell membrane attachment through G protein-receptor binding, since the G protein is the target of neutralizing antibodies, as discussed next (Lorenzen and LaPatra, 1999).

The binding of the G protein of VHSV to host receptors, under acidic conditions, initiates the fusion between the viral envelope and cell membrane preceding insertion of the viral ribonucleoprotein into the cytoplasm (Gaudin et al., 1999). The fusion process results in conformational changes to the viral G protein resulting in penetration of the virion inside the host cell. This fusion process can be enhanced by an acidic medium, approximately pH 5.0, in most rhabdoviruses including VHSV (Carneiro et al., 2001; Da Poian et al., 2005; Estepa and Coll, 1996a; Gaudin et al., 1993; Mas et al., 2004).

SVCV infects cells via the glycoprotein spikes that bind to cell receptors and induce endocytosis (Bishop and Smith, 1977). The virus was detected by electron microscopy inside the endosome; however, its uncoating and passage to the cytoplasm was not seen. There was no subsequent formation of inclusion bodies in the cytoplasm after infection as occurred with IHNV and VHSV (Granzow et al., 1997).

Several studies have attempted to identify receptors for rhabdoviruses to account for their very broad host range. Current evidence for VHSV-cell binding mechanisms involve either phospholipids or fibronectin. VHSV binds to phospholipids on host membranes composed of phosphatidylserine (PS), phosphatidylethanolamine and phosphatidylcholine (Coll, 1995c). Binding of the G protein of VHSV to cell membranes does occur at neutral conditions (pH 7); however, better interaction was established under acidic conditions (ie, <pH 6) (Coll, 1995b). Binding of VHSV G protein to PS was mediated by a segment of 28 amino acids (Estepa and Coll, 1996a; Estepa et al., 1999) and antibodies to this G protein peptide inhibited VHSV-mediated cell-to-cell fusion (syncytia) (Estepa et al., 1999). Similar peptides have been observed to be involved in G protein-mediated cell binding in other rhabdoviruses, suggesting that PS-binding is a common feature of the rhabdovirus envelope protein (Estepa and Coll, 1996b).

Fibronectin is a component of the connective tissues of most vertebrates, including fish (Liu and Collodi, 2002; Zhao et al., 2001). Fibronectin can mediate entry into the cell cytoplasm for IHNV (Bearzotti et al., 1999; Liu and Collodi, 2002) and VHSV (Bearzotti et al., 1999). Monoclonal antibodies (MAb) precipitated a complex containing fibronectin in rainbow

trout gonad (RTG-2) cells, but these MAbs protected only salmonid-derived cell lines (Bearzotti et al., 1999). The presence or absence of fibronectin may therefore partly determine the susceptibility of certain fish species or tissues to VHSV.

18.2 INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

18.2.1 Introduction

Infectious hematopoietic necrosis (IHN) is one of the three aquatic rhabdovirus diseases listed as reportable by the OIE and is considered to have a significant economic impact on salmonid aquaculture, both public and private, in North America (Bootland and Leong, 2011). In the United States, IHN impacts natural resource agency efforts to restore and maintain salmon and steelhead (*Oncorhynchus mykiss*) populations and historically has caused millions of dollars in losses to the Idaho rainbow trout industry (Groff and LaPatra, 2000). In British Columbia, Canada, between 2001 and 2003, IHN epizootics caused CAN $200 million loss to the Atlantic salmon (*Salmo salar*) industry (Salonius et al., 2007). These impacts are due to direct mortality, decreased fish performance and production and deformities that occur in the survivors that downgrades the product. The virus now has a worldwide distribution that includes Japan, South Korea, China, Russia and many countries in the European Union (OIE, 2015a).

18.2.2 Disease

The disease caused by IHNV has historically been known by a variety of names, including sockeye salmon virus disease, Columbia River sockeye disease, Oregon sockeye disease and Sacramento River Chinook disease. However, it is now universally accepted that the disease caused by this virus is IHN. The virus infects a broad range of salmonids (Table 18.1) including rainbow trout/steelhead (*O. mykiss*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), cutthroat trout (*Oncorhynchus clarkii*), brown trout (*Salmo trutta*), Atlantic salmon, Chinook salmon (*Oncorhynchus tshawytscha*), sockeye/kokanee salmon (*Oncorhynchus nerka*), chum salmon (*Oncorhynchus keta*), cherry salmon/masou salmon/Yamame trout (*Oncorhynchus masou*), amago salmon (*Oncorhynchus rhondurus*), coho salmon (*Oncorhynchus kisutch*), Japanese charr (*Salvelinus leucomaenis*), Arctic charr (*Salvelinus alpinus*), Arctic grayling (*Thymallus arcticus*) and mountain whitefish (*Prosopium williamsoni*). The susceptibility to IHNV can vary greatly from species to species and between strains within a species. IHNV has also been isolated from European eel (*Anguilla anguilla*) (Bergmann et al., 2003).

18.2.3 Epidemiology

Epidemiological studies of North American IHNV isolates using mid-*G* gene sequences revealed three major genetic subgroups or genogroups, designated as U, M and L, based on the prevalence of the isolates in the upper (U), middle (M) and lower (L) parts of the IHNV geographic range (Garver et al., 2003; Kurath et al., 2003; Troyer et al., 2000). Interestingly, this genetic typing scheme correlated very well with a biochemical typing scheme that was developed more than 15 years earlier. The biochemical typing scheme compared differences in primarily the molecular weight of the N proteins of different IHNV isolates after SDS-PAGE. The electropherotype I isolates had the lowest molecular weight N protein, the type II a slightly higher-molecular-weight N protein and the type IIIs exhibited the highest molecular weight N (Hsu et al., 1986). The U genogroup or type I is the most prevalent in Alaska and British Columbia. However, it is also found in fish in coastal Washington, and the Columbia River basin, including Washington, Oregon and Idaho. The M genogroup or type II occurs in the Columbia and Snake River basins, and recently in Washington coastal watersheds. In contrast, the L genogroup or type III occurs in California and on the southern coast of Oregon. With the current worldwide distribution of IHNV that includes Europe and Asia, additional genetic studies have been conducted on these isolates and while there is some genetic evidence that these isolates may have originated from North America, they appear to have diverged independently (Enzmann et al., 2005; Nishizawa et al., 2006a; Yu et al., 2014).

Although IHNV isolates belonging to each genogroup or electropherotype are capable of infecting multiple host species, there is a general trend in host specificity among IHNV isolates in North America. The IHNV isolates from the U genogroup or type I are highly virulent in sockeye/kokanee salmon and M genogroups or type II cause low mortality in this species. However, in rainbow trout the M genogroup or type IIs are highly virulent and U genogroups cause low mortality (LaPatra et al., 1990). The isolates in the L genogroup or type IIIs appear most virulent for Chinook salmon (LaPatra et al., 1993a).

18.2.4 Pathology and Immunity

After waterborne exposure, transient replication of IHNV occurs in the epithelial cells of the gills, skin and fin bases before spreading to internal organs. In an eloquent piece of work, Harmache et al. (2006) used a recombinant infectious IHNV expressing the Renilla luciferase gene and bioluminescent imaging to show that the fin bases of rainbow trout serve as a major portal entry for IHNV. The manifestation of IHN disease depends on the strain of IHNV, the size and age of the fish, along with the species and strain and the environmental conditions, including water temperature (LaPatra et al., 1994b; LaPatra, 1998). Horizontal or fish-to-fish transmissions through the water and via aerosols are the primary routes through which IHNV spreads in the wild and in aquaculture operations. Although IHNV occurs primarily in fry and fingerlings in aquaculture facilities, young and older salmonids are also susceptible to the virus. There is some evidence that surviving fish can serve as lifelong carriers of the virus, but there is additional evidence that suggests that IHN survivors mount an IHNV-neutralizing antibody response that efficiently clears the virus (LaPatra et al., 2001b).

The classic signs of an acute IHN outbreak are a sudden increase in fish mortality at early life stages. The most common clinical signs include exophthalmia (pop-eye), pale gills due to anemia, skin darkening and lethargy while riding high in the water column. This can be accompanied with internal signs that include a pale liver and kidney (Fig. 18.2). Ascites can accumulate and the stomach can be filled with watery fluid (red to yellow) rather than food. This can be accompanied by petechial hemorrhages in the visceral mesenteries, swim bladder, adipose tissue and peritoneum (Wolf, 1988). This form of the disease progresses to a lethal necrosis of the hematopoietic tissues of the kidney and spleen with a generalized viremia and associated necrosis in all tissues. Death has been attributed to renal failure caused by an electrolyte imbalance (Rodriguez Saint-Jean et al., 2003).

The classical manifestation of IHN that occurs in very small fish (about 0.2–8 g) described previously consists of destruction of the blood-forming tissues and the establishment of a frank viremia with the highest concentrations of virus consistently detected in kidney and spleen tissues, has recently been referred to as "hematopoietic" or classical IHN. Another form of the disease, which generally manifests in larger fish (>8 g), where the virus can only be isolated from brain tissue and is associated with abnormal fish behavior and tetany in a proportion of the affected animals, was also recently described. This form of IHN was referred to as "neurotropic" or brain IHN and is thought to be involved in the manifestation of spinal curvature, primarily scoliosis types of deformities (Fig. 18.3). The current working hypothesis is that fish

FIGURE 18.2 One of the most common clinical signs of classical infectious hematopoietic necrosis (IHN) includes a very pale kidney because of the anemia that is induced and the massive necrosis from the virus replicating to high concentrations in this tissue.

FIGURE 18.3 One of the economic impacts of infectious hematopoietic necrosis (IHN) are spinal curvature types of deformities in 2–4% of the surviving fish of an acute IHN epizootic.

that manifest this form of IHN had gone through a "light" (<10%) hematopoietic IHN epizootic at earlier life stages that elicits low levels of virus-neutralizing antibodies systemically. However, when these fish are re-exposed to virus, the level of protection afforded by these antibodies is enough to prevent systemic or hematopoietic IHN. However, the virus appears to "escape" into the central nervous system (CNS) where there is less immunological surveillance and it replicates to approximately 10^6 plaque-forming units (pfu)/g and destroys brain tissue and causes this neurotropic form of the disease. Experimentally, we have been able to mimic this by passively immunizing with IHNV-neutralizing antibodies at very low concentrations and then waterborne exposing the fish to IHNV. A third form of IHN, more recently observed, occurs in much larger fish (about 50–100 g) and the virus can generally only be isolated from the fin and skin, but primarily the gills. This form of IHN is referred to as "epitheliotropic" or gill IHN. Empirical observations suggest that these larger fish can become infected with IHNV, but the infection does not become systemic because of the life stage of the fish or some other mechanism(s). However, the virus replicates very efficiently in the epithelial cells of the fins, skin and gills and can cause significant gill respiratory problems and predispose fish to secondary bacterial gill disease infections. Mortality is chronic, but because larger fish are affected the kilograms of fish lost can be high. In addition, the fish growth and food conversion impacts can be long term. The different manifestations of IHN (hematopoietic, neurotropic and epitheliotropic) appear to be dependent on the life stage and the immunological status of the host and not due to mutations in the surface glycoprotein (G) of the virus (LaPatra et al., 2008).

The interactions between the salmonid IgM-like humoral immune system and IHNV have been well studied. It had previously been known through empirical observations in the field that were supported by more controlled work in the laboratory, that survivors of IHN were significantly more resistant to reinfection then naïve animals (LaPatra et al., 1991; Wolf, 1988). In addition, it had been shown previously that passive immunization of small, susceptible fish with polyclonal antibodies against IHNV produced in rabbits provided very significant protection against a lethal virus challenge. However, it was very difficult to detect significant IHNV-neutralization activity using serum from fish that had survived IHN. However, when the virus was detected in Europe in the late 1980s, a complement-dependent neutralization test that was originally developed by Paul Vestergård Jørgensen to detect antibodies against VHSV was modified for IHNV. Pierre de Kinkelin's group was the first to consistently detect neutralizing antibodies in the serum from rainbow trout that survived IHN (Hattenberger-Baudouy et al., 1989). LaPatra et al. (1993b) further refined this assay for IHNV and conducted a series of studies that defined the importance of this serum antibody response as a diagnostic indicator of previous exposure to the virus and as a very important and functional immune response that could protect the host. Briefly, the serum samples to be

tested are heated at 45°C for 30 min to inactivate residual complement. A twofold dilution series of the serum is performed and a known quantity of IHNV is mixed with each serum dilution and incubated for 30 min on a rocker platform to allow specific antibody, if present, to bind to the virus. An exogenous source of rainbow trout complement is added as rainbow trout serum and the mixture is incubated for another 30 min with rocking and the complement catalyzes the antibody-mediated virus neutralization reaction. Then each serum, antibody, virus mixture is plaque assayed on preformed monolayers of *Epithelioma papulosum cyprini* (EPC) cells and the neutralization titer is reported as the reciprocal of the serum dilution that caused a 50% reduction in the average number of plaques compared with the negative control. Without the addition of a complement, you only rarely detect neutralization titers. It is very important for the accuracy and reproducibility of this assay that positive and negative control sera are included and that each batch of complement undergoes a quality assurance/quality control testing program. Rainbow trout serum from specific pathogen-free (SPF) fish has been detected with low levels of IHNV- and VHSV-neutralization activity.

Using the complement dependent neutralization assay, it has been clearly shown that susceptible juvenile salmonid species that survive an IHN epizootic will exhibit a range of neutralization titers. In addition, the magnitude of this neutralizing antibody response and the severity of the IHN epizootic appear to be directly correlated; that is, the higher the virus-induced mortality, the higher the prevalence of antibody-positive fish and the higher the neutralization titers detected. The kinetics of antibody production are temperature dependent, and at 14.5°C, a significant neutralizing antibody response cannot be detected until approximately 200 degree days after exposure to IHNV, but again this is highly variable and depends at least on the life stage of the fish, the temperature of the water they are being reared in along with the virus exposure concentration and duration (LaPatra et al., 1993b). Various titers of neutralizing antibodies have been examined in passive immunization tests using 1 g rainbow trout that were injected with 50 µL of rainbow trout serum. Passively immunized fish are rested for 24 h and then waterborne challenged with a virulent strain of IHNV using a standardized procedure. Fish injected with titers as low as 20–40 exhibited a relative protection of 67–82% compared with fish injected with negative control sera, emphasizing the importance of this neutralizing antibody response for protection. Injection of serum with increased titers resulted in even greater protection. This led to the development of a passive immunotherapy strategy that appeared to have some potential application if the IHNV infection could be detected soon enough in a susceptible group of fish (LaPatra et al., 1994a). This serum neutralization assay can also be used for risk assessment of juvenile populations. If there is any detectable IHNV-neutralization activity in the serum of a susceptible group of juvenile salmonids the risk of clinical disease manifesting appears to be much lower. This assay can also very effectively be used to determine vaccine efficacy. If a potential vaccine is able to elicit a neutralizing antibody response by 400 degree days postvaccination the vaccinated fish should be significantly less susceptible to IHNV (LaPatra, 1996). An enzyme-linked immunosorbent assay (ELISA) was also developed for the detection of antibodies against IHNV and strongly correlated with the complement-dependent neutralization assay, but purified virus was required. In addition, a Western blot assay was used in the same study to determine the reactivity of the test sera to specific proteins of the virus (Ristow et al., 1993).

Using the complement-dependent neutralization assay it has also been clearly shown that adult salmonids have a detectable IHNV neutralization response even though they are not susceptible to clinical disease at this life stage. Adult salmonids are asymptomatic carriers of the virus, but high concentrations of IHNV can be detected at spawning in the fluid that surrounds the eggs and also in internal tissues, such as the kidney and spleen. Interestingly, adult salmonids with detectable neutralization activity in their serum at spawning are generally not carriers of IHNV, which significantly lowers the risk of egg-associated transmission of the virus. Virus neutralization activity is also detectable in end life-stage salmon (eg, Chinook) at spawning even though the dogma at that life stage is that the immune system is shutting down because the fish are going to die (LaPatra, 1996). We have also attempted to hyperimmunize 1.5-year-old rainbow trout at monthly intervals that were 6 months from spawning with the goal of enhancing the maternal transfer of immunity. While binding antibody appeared to be detectable in eyed-eggs from hyper-immunized adults, we were unable to demonstrate any difference in the protection of these fry to an IHNV challenge compared with fry that came from mock-immunized adults (LaPatra, unpublished results).

18.2.5 Disease Diagnostics

In order to prevent the spread and occasional outbreaks of the disease, several methods have been developed for diagnosis and detection of IHNV (OIE, 2015a). A preliminary diagnosis of the disease can be made based on fish displaying increased mortality and clinical signs of the disease described previously. It is very important to keep in mind the different ways in which IHN can manifest and to take samples for testing accordingly. If only kidney and spleen tissues are sampled, you may miss the neurotropic and epitheliotropic forms of IHN. A useful and quick diagnostic test for hematopoietic IHN is to look for "foamy" macrophages and necrotic debris in anterior kidney organ imprints that are stained with a differential

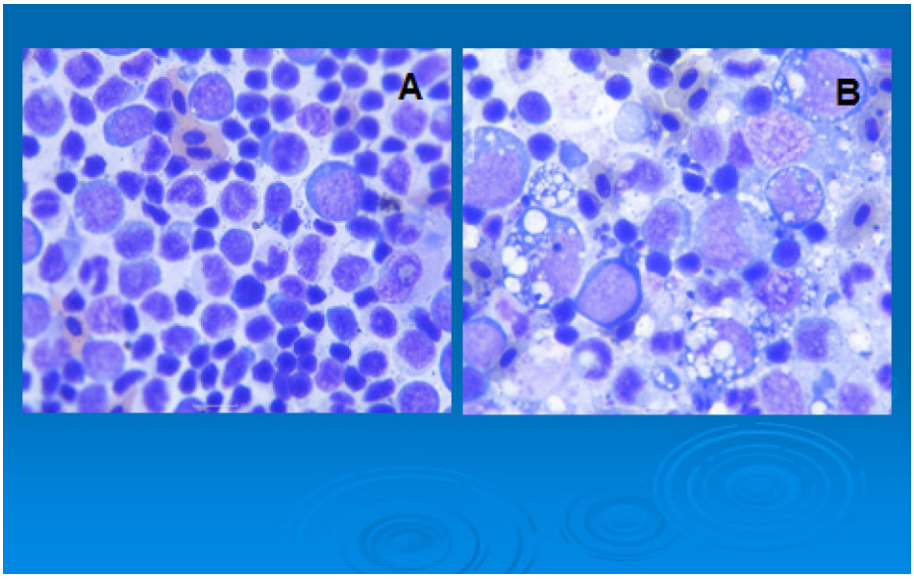

FIGURE 18.4 A useful and quick diagnostic test for hematopoietic IHN is to look for "foamy" macrophages and necrotic debris in anterior kidney organ imprints done on glass slides that are stained with a differential stain; (A) Normal appearing anterior kidney organ imprint; (B) Anterior kidney imprint from a rainbow trout affected by acute IHN.

stain (Fig. 18.4). This is also an excellent method for detecting mixed infections that may occur, such as IHN and bacterial coldwater disease, which is caused by *Flavobacterium psychrophilum*. These two pathogens together can cause significant mortality impacts in young fish that are much more severe than either pathogen alone.

Stained smears of peripheral blood and anterior kidney imprints have been examined from fish naturally and experimentally infected with IHNV. The most distinct and consistent changes included macrophages with cytoplasmic vacuolation and ingested cellular debris, nuclear pyknosis of hematopoietic cells and the presence of degenerate nuclear and cytoplasmic debris. In addition, the occurrence of bilobed erythrocytes, in addition to the changes described previously, appear to be pathognomonic for IHN. The results showed that IHNV concentrations of 10^4 to 10^5 pfu/g of tissue were required before these cellular changes could be observed. A presumptive diagnosis of IHN can be made rapidly using this methodology. Serological confirmation of IHNV can also be obtained by using a fluorescent antibody test on blood smears and organ imprints that were obtained from clinically infected juvenile fish.

Isolation of the virus from suspect fish using established cell lines, followed by confirmation using a serum neutralization assay is considered the gold standard for detection of IHNV (OIE, 2015a; Winton, 1991). However, cell culture for virus isolation and confirmation can be time-consuming if only low concentrations of IHNV are present, such as in a subclinical infection. When high concentrations of IHNV are present, as described previously, typical cytopathic effect (CPE) can be observed by 48–72 h postinoculation. The more time consuming portion is confirmation by serum neutralization, which can take 7–10 days. More rapid identification of the virus can also be performed by immunofluorescence (IFA; LaPatra et al., 1989a), ELISA (Ristow and Arnzen de Avila, 1991), in situ hybridization using biotinylated probe (Deering et al., 1991), immunohistochemical and immunogold labeling (Drolet et al., 1995), reverse transcriptase-polymerase chain reaction (RT-PCR) (Arakawa et al., 1990; Barlič-Maganja et al., 2002; Chiou et al., 1995) and real-time RT-PCR (Dhar et al., 2008; Overturf et al., 2001; Purcell et al., 2006).

Antibody-based IHNV detection methods, such as the serum neutralization test, have been optimized and approved by the OIE, as well as regulatory organizations in the United States and Canada (OIE, 2015a). An antigen detection assay, based on ELISA using MAbs against the N protein or polyclonal antibodies for the detection of IHNV in cell culture, has also been approved by OIE. The assay requires less than 24 h with a limit of detection of 10^3 pfu/mL (Ristow and Arnzen de Avila, 1991).

Among the different methods of IHNV detection, molecular methods are the most rapid and sensitive. However, unlike cell culture-based assays, molecular methods cannot distinguish between infectious and noninfectious virus. These methods are also extremely sensitive so proper protocols must be in place so that false positive signals do not occur. Other methods include molecular padlock probe and loop-mediated isothermal amplification (LAMP). Nonlethal sampling for IHNV can also be accomplished by examining cutaneous mucus or fin clips by cell culture or RT-PCR (Dhar et al., 2008; LaPatra et al., 1989b).

18.2.6 Prevention and Control

Present control strategies for IHNV rely upon aquaculture practices that avoid the presence of the virus at the rearing facility. Eggs and fry are taken from stocks with no previous history of the virus and reared in SPF water. When egg supplies from IHNV-negative fish are limited, the eggs from fish stocks with a history of IHNV are treated with an iodine-containing surfactant to inactivate virus on the eggs' outer surface (Amend and Pietsch, 1972). Iodophor (100 mg/L) egg disinfection destroys at least 99.98% of the virus on the surface of green and eyed eggs (Goldes and Mead, 1995). In most cases this treatment has been successful in preventing the transmission of IHNV to progeny. However, occasional outbreaks of disease are still observed in progeny after treatment with iodophor and egg incubation and fish rearing in SPF water. This has suggested that vertical transmission may occur (Meyers et al., 1990). While it is impossible to prove that true intra-ovum transmission of IHNV does not occur, the results of laboratory and field tests and empirical observations from the field support that this risk can be managed. The use of iodophor on newly fertilized eggs during the water-hardening step and the surface disinfection of eyed eggs prior to hatching have been shown to be effective management strategies. However, the most important component is the use of virus-free water supplies for the hatching and rearing of the young and most IHNV-susceptible life stages of fish. Use of these management strategies on eggs obtained from IHNV-positive stocks of salmonids can significantly reduce the risk of egg-associated transmission of IHNV and the subsequent disease impacts due to IHN that can approach 100% mortality (Engelking et al., 1991).

Controlling the spread of IHNV by disinfection of hatchery water supplies has had some limited success. Methods such as ozonation, ultraviolet light, chlorination-dechlorination and the addition of elemental iodine or other germicides such as ethanol, phenol and cresol soap solution or methanol have all been shown to be effective in laboratory settings (Winton, 1991). In field trials, however, problems have arisen because of mechanical and electrical failures that occur routinely with equipment that must deliver a compound at a virus-killing dose that is nontoxic to the fish. Nevertheless, the approach has sufficient promise that early life stage rearing facilities with limited or no SPF water are beginning to incorporate water disinfection equipment into the facilities (Jim Winton, personal communication).

To date, DNA vaccines appear most effective at controlling IHN, but only one has been licensed for use in the United States and Canada. However, this vaccine is only being routinely used in the Atlantic salmon industry in British Columbia, Canada. The IHNV DNA vaccines that are highly effective at protecting against disease have been thoroughly described in the published literature and are the only IHNV vaccine type that is commercially available. These vaccines contain the viral surface glycoprotein gene (*G*-gene) inserted into a plasmid vector and synthesized in *Escherichia coli* (Corbeil et al., 1999). There is a well-characterized DNA vaccine designated pIHNwG (pWG) that utilizes the DNA encoding for the *G*-gene of the IHNV reference strain WRAC, which is highly protective against disease under a variety of conditions (Kurath et al., 2007).

In numerous published vaccine trials, single small doses of 0.01–10 µg of the pWG vaccine injected intramuscularly into juvenile rainbow trout resulted in the stimulation of a strong protective response. In rainbow trout fry (mean weight, 0.8–1.8 g) doses of 0.01–1 µg resulted in relative percent protection (RPS) compared with mock-vaccinated control fish of 83–100%. In subyearling rainbow trout (mean weight, 110–120 g) 100-fold higher doses were tested ranging from 1.0 to 10.0 µg because of the 100-fold increase in the body mass of the fish and resulted in RPS values of 100%. A single study in these fish examined a 0.1 µg DNA vaccine dose and there was no protection (LaPatra et al., 2000). The pWG vaccine was also tested in rainbow trout against a variety of isolates of IHNV that originated from different species and different geographic areas of the world and significant protection was demonstrated against all the IHNV isolates tested (Corbeil et al., 2000a). Another important vaccine parameter that was characterized was the duration of protection. In this study, rainbow trout (mean weight: 2.5 g) were vaccinated with a 0.1-µg dose of the pWG DNA vaccine and challenge evaluated at 3, 6, 12 and 24 months postimmunization. At 3 months, there was 100% protection; however, at 6, 12 and 24 months the RPS was 69%, 65% and 66%, respectively (Kurath et al., 2006). Although the protection observed was significant at each time point tested, perhaps using a 10-fold higher dose (0.1–1.0 µg) of the pWG vaccine, which is still extremely small, could have boosted the protective effect of the vaccine even higher. One of the unique characteristics of this vaccine was its capability to provide protection as soon as 4 days postvaccination (60 degree days) (LaPatra et al., 2001a). LaPatra and Lorenzen and their research groups then began to examine not only the onset of protection, but specificity of protection after vaccination. They characterized what is now termed the *early antiviral response (EAVR)*, which has rapid onset (4–14 days at 14.5°C) and low specificity; that is, fish vaccinated with the IHNV DNA vaccine were protected against both VHSV and IHNV and fish vaccinated with the VHSV DNA vaccine were protected against both IHNV and VHSV. However, this early protective response that was elicited with the DNA vaccines had limits and did not cross-protect against bacterial pathogens (Lorenzen et al., 2002b). Later, others characterized the EAVR and suggested that it was an interferon-mediated defense mechanism, hence the rapid and nonspecific nature of the response. The EAVR then gave rise to the specific antiviral response that

was characterized by a slower onset (14–28 days at 14.5°C) and a high degree of specificity; that is, there was no cross-protection against heterologous viruses. These results supported the dogma that the key to eliciting a potent and long duration adaptive immune response is to elicit a strong and early innate response, which is exactly what these vaccines did very well and probably the reason they are so protective (Lorenzen et al., 2002a). Interestingly, in the results of the duration of protection study where significant protection was observed through 24 months postvaccination in 2.5-g rainbow trout that received a single 0.1-μg dose, there were detectable IHNV neutralization titers in the sera of unchallenged fish at 3, 6 and 12 months. However, by 24 months no virus neutralization activity was detected in the serum of the vaccinated fish, yet significant protection was again observed (RPS, 66%). This possibly suggests a third phase in the immune response that is elicited by this vaccine that could be termed the *long duration antiviral response.*

The efficacy of the pWG vaccine was also examined in Atlantic salmon pre-smolts (freshwater) and smolts (salt water) and in juvenile Chinook and sockeye salmon and shown to be equally efficacious as had been repeatedly demonstrated in rainbow trout (Garver et al., 2005; Traxler et al., 1999). A major limitation to the field usage of this vaccine is that is must be administered by intramuscular injection (Corbeil et al., 2000b). This is very labor intensive and expensive for large numbers of fish. Mass DNA vaccine delivery methods are being researched that would likely significantly expand vaccine usage in North America. However, the difficulty associated with licensing due to the perceived safety issues has been one of the major impediments hindering the use of DNA vaccines in aquaculture (Lorenzen and LaPatra, 2005). This has been cited over and over again for years without any definitive evidence of potentially harmful safety issues. The DNA vaccine against IHN virus that is delivered by intramuscular injection has been in use in British Columbia, Canada since it was approved in 2007 and has and is being used extensively in the marine net-pen salmon industry. Virtually all the salmon that are transported to marine net pens are vaccinated with this vaccine while still in the freshwater-rearing phase (mean weight, 50–100 g). In fact, in 2012, it was estimated that more than 28 million doses (10-μg/dose) of the APEX IHN vaccine had been injected into fish that ultimately had been reared in net pens in the Pacific Ocean (Kyle Garver, personal communication). All those fish are monitored and were processed for human consumption and there has not been one negative report regarding the use of this vaccine. In fact from an animal welfare standpoint, the vaccine has been very beneficial and the only IHN epizootics that have been reported since the vaccine was approved and put into use were in unvaccinated fish. Now virtually the entire industry in British Columbia is using this vaccine because of this success and after 8 years and countless doses of vaccine, there again has not been one negative report regarding the safety of the vaccine. Based on these results, the future for controlling impacts due to IHN appears bright once alternative mass-immunization strategies have been developed for delivery of this very efficacious IHNV vaccine.

18.3 VIRAL HEMORRHAGIC SEPTICEMIA VIRUS

18.3.1 Introduction and Disease

Historically, VHSV has had a devastating effect on rainbow trout (*O. mykiss*) aquaculture in Europe. The annual losses of rainbow trout in Europe since the first appearance of VHSV on fish farms have been estimated to exceed 40 million pounds per year (Gregory, 2008); however, the true economic cost of viral hemorrhagic septicemia (VHS), similar to many fish diseases, is not well characterized. VHS remains one of the most important diseases of rainbow trout in northern and central Europe. The disease was described by Schäperclaus (1938), who was the first to describe many important fish diseases and the etiology of VHS was first identified as Egtved virus in 1963 (Jensen, 1963). A notable characteristic and part of the reason for the importance of this virus in so many countries in the world is the very large and continually increasing number of fish species that can be infected with clinical consequences (reviewed in Skall et al., 2005, OIE, 2015c; Smail and Snow, 2011; International Database on Aquatic Animal Disease http://www.cefas.defra.gov.uk/idaad/) (Table 18.1). This is in direct contrast to the very similar virus, IHNV, the impacts of which are largely restricted to salmonids, as previously described.

18.3.2 Epidemiology

Olesen et al. (1993) recognized three serogroups (I–III) of VHSV that originated from 127 genotype I isolates, identified cross-reactivity depending on the MAb used, but did identify a shift from serogroup I to II isolates in Europe. Additional isolates of VHSV have also been categorized serologically (Basurco and Benmansour, 1995; Benmansour et al., 1997; Fregeneda-Grandes and Olesen, 2007; Raja-Halli et al., 2006); however, serogroups do not correlate with genotypes and have been supplanted by the latter. These studies have also demonstrated that North American VHSV isolates cannot be discriminated from European isolates, whether using monoclonal or polyclonal antibodies.

VHSV can be classified genetically, based on *N* and *G* gene sequences, into four genotypes that correspond with the broad geographical origins of isolates (Nishizawa et al., 2006a). Freshwater and marine isolates in continental Europe belong to genotype I; members of this genotype are subdivided into Ia, which are of freshwater in origin derived from rainbow trout, and Ib, isolated mainly from marine fish species in the North and Baltic seas and contiguous waters (Einer-Jensen et al., 2004; Snow et al., 1999, 2004). Additional subgenotypes Ic and Id are historical or minor groupings (Raja-Halli et al., 2006) and Ie are found in free-living turbot in the Black Sea (Nishizawa et al., 2006b). Several marine isolates identified as genotype II were found in the Eastern Gotland Basin and near Bornholm Island in Denmark (Einer-Jensen et al., 2005; Snow et al., 1999, 2004). Marine isolates from the North Sea and Atlantic are included in genotype III. This includes VHSV isolates obtained from farmed turbot (*Scophthalmus maximus*) and other species (Duesund et al., 2010). Genotype III viruses have also been isolated from fish in the waters surrounding Skagerrak in Denmark, as well as from European eels in the Loire River estuary in France (Einer-Jensen et al., 2005; Snow and Smail, 1999) and from Norway (Dale et al., 2009). Marine isolates from the Pacific Ocean, including the waters surrounding Japan and North America belong to genotype IV (Novoa et al., 2006). Genotype IV was recently subdivided into isolates from the Pacific (IVa) and those from the western Atlantic (Gagne et al., 2007) and Great Lakes (IVb) (Elsayed et al., 2006; Lumsden et al., 2007). The global distribution of VHSV remains restricted to the temperate Northern Hemisphere.

The genotype has a profound influence on host impact. A VHSV Ib (marine) isolate, caused mortality ranging from 20% to 70% by immersion, but up to 100% by intraperitoneal (i.p.) injection in turbot (Brudeseth et al., 2008). The identical isolate was unable to infect a primary rainbow trout gill cell line and was nonpathogenic when rainbow trout were exposed by immersion (Brudeseth et al., 2008). The authors suggested that the inability of the virus to replicate in rainbow trout gill epithelium was a factor in the lack of virulence. Numerous studies have also demonstrated that isolates of VHSV Ib are nonpathogenic or produce low mortality among rainbow trout following waterborne infection (Campbell et al., 2009; Nishizawa et al., 2006b; Skall et al., 2004). A similar pattern regarding the limited virulence of marine isolates in freshwater species is applicable to genotype IVa as well. A genotype IVa isolate was highly virulent in waterborne exposures of Pacific herring (*Clupea pallasii pallasii*) (Kocan et al., 1997), but a similar isolate was avirulent in rainbow trout (Meyers and Winton, 1995) and had limited or no ability to replicate in vitro in rainbow trout fin cell line or primary gill cell lines (Yamamoto et al., 1992).

Five in vitro passages attenuated infectious pancreatic necrosis virus in brook trout (McAllister and Owens, 1986), however, sequential in vivo passages of an isolate of VHSV led to increased virulence. A marine genotype III VHSV isolate that was serially passaged five times in RT without intervening isolation using cell culture had greater virulence than the original isolate before passage (Snow and Cunningham, 2000). Although the authors were unable to determine the factors responsible for increased virus virulence, their study was important since it demonstrated that adaptation to trout by a nontrout-derived strain of the virus could increase virulence.

While there is evidence that the gene that encodes the G protein is important in the virulence of VHSV isolates (Gaudin et al., 1999), the increase in virulence for rainbow trout of the genotype III isolate of VHSV described previously was not matched by alterations in the sequence of the *G* gene (Snow and Cunningham, 2000). Nucleotide alterations that would produce only very few amino acid changes had occurred after the sequential passages and these were concluded not to be important (Snow and Cunningham, 2000). However, a comparison of nucleotide sequences between two genotype Ib isolates revealed that minor substitutions in nucleotide sequences of the *G* gene might alter the virulence of VHSV in juvenile rainbow trout (Campbell et al., 2009). One isolate caused 75% mortality in rainbow trout by the i.p. route and 54.5% mortality following immersion. whereas a very similar isolate caused 35% and 2% mortality in i.p. and bath-immersion groups, respectively (Campbell et al., 2009).

The identification and spread of VHSV in North America over the past 30 years illustrates the poor state of knowledge of natural virus reservoirs outside of Europe and the need for monitoring of not just cultured but wild fish populations. The lesson in Europe and North America is the same, however; that institution of regulatory and basic sanitation measures, along with continued monitoring, can reduce the impact of virus on commercial operations to an acceptable degree. VHSV was first detected in North America in spawning Chinook and coho salmon (Brunson et al., 1989; Hopper, 1989). This identification resulted in a much greater effort to determine the geographical range of the virus and the species of fish infected. By the end of the 20th century, VHSV (now genotype IVa) had been identified from dead pilchards (*Sardinops sagax*), shiner perch (*Cymatogaster aggregate*) and stickleback (*Gasterosteus aculeatus*) from Vancouver Island (Kent et al., 1998) and from mackerel (*Scomber japonicus*), eulachon (*Thaleichthys pacificus*) and surf smelt (*Hypomesus pretiosus*) (Gagne et al., 2007; Hedrick et al., 2003). In the same period, mortality events associated with VHSV in Alaskan waters were documented in Pacific herring, Pacific hake (*Merluccius productus*) and walleye pollock (*Theragra chalcogramma*) (Marty et al., 1998; Meyers et al., 1999).

The identification and spread of VHSV genotype IVb in the Great Lakes is the most recent large-scale example of the incursion of VHSV to freshwater (Elsayed et al., 2006; Lumsden et al., 2007). Detected retrospectively as early as 2003 from frozen muskellunge (*Esox masquinongy*) (Elsayed et al., 2006), mortality events in spring 2005 affecting muskellunge and freshwater drum (*Aplodinotus grunniens*) in Lake Ontario, Canada was the first indication of clinical impact (Lumsden et al., 2007). Previous mortality events had likely occurred, but were not investigated promptly because screening of disease in wild fish is often a low priority. By 2007, the virus had spread to all four southern Great Lakes, freshwater lakes outside the Great Lakes proper (USDA, 2009) and was detected in the Mississippi watershed in 2008. Finally, in 2010, the virus was detected using quantitative RT-PCR (qRT-PCR) from clinically normal fish in Lake Superior (USGS, 2007). The total number of fish species infected with subgenotypes IVa and IVb of VHSV within North America was more than 40 in 2006 (USDA, 2006) and the number continues to rise. After the initial and quite substantial financial impacts due to the presence of an OIE-listed virus in the Great Lakes, introduction of screening and regulatory measures have effectively limited further impacts. Mortality events have continued to occur at lesser frequencies (eg, Freshwater drum, Bay of Quinte, 2014) since then. The origin of VHSV in the Great Lakes is considered to be the Atlantic Ocean where the virus was first detected from several species (Gagne et al., 2007) and the very limited lack of genetic diversity in IVb isolates obtained from the Great Lakes since 2003 (Thompson et al., 2011). Isolates from the Atlantic have been suggested to occupy a separate subgroup (Gagne et al., 2007), however, this has not gained widespread acceptance and most researchers consider these isolates to be included in IVb.

It seems reasonable that VHSV historically originated in seawater and has been widely distributed in marine fish species since, and that the virus has moved to the freshwater environment (Einer-Jensen et al., 2005; Raja-Halli et al., 2006; Snow and Cunningham, 2000). In European aquaculture, a major route of introduction of VHSV Ia to freshwater systems in the past was thought to be from using marine fish as a component of commercial aquaculture feeds or by using baitfish infected with VHSV (Einer-Jensen et al., 2005). Modern practices using commercial feeds that are free of infectious agents have largely eliminated this route. Direct transmission from wild fish can occur and a marine isolate of VHSV genotype III was found for the first time to be the cause of a mortality event in net-pen cultured Atlantic salmon in Norway (Dale et al., 2009). It has also been speculated that ingestion of VHSV-IVa-infected herring was the primary source of transmission to wild Chinook and coho salmon (Amos et al., 1998; Meyers and Winton, 1995). These salmonids, which are inherently relatively resistant to this genotype of VHSV, can be subclinically infected at spawning leading to detection and subsequent eradication of affected fish. Sporadic mortality events due to IVa in cultured net-pen Atlantic salmon have been documented (Garver et al., 2013; Lovy et al., 2013), however, the virus is not maintained in the cultured population and the overall economic impact is limited. The exchange of virus between wild and farmed fish was raised as a potential for adaptation of the virus with greater impacts on farmed fish (Dale et al., 2009; Garver et al., 2013).

Although most VHS outbreaks occur between late winter and spring, VHSV infection cannot be ruled out in other seasons (Ghittino, 1965). The water temperature at which VHSV causes mortality in the field is broad, ranging from 3°C to 15°C. Mortality is greatest at 3°C and lower at 15°C (McAllister, 1990) and mortalities are unlikely to occur above 20°C (Sano et al., 2009). The most important route of transmission in epizootics of VHS is horizontal; with the virus being shed into the environment from infected fish. Horizontal transmission of the virus can be either by direct contact with diseased fish or indirect contact via contaminated water and fomites (Dale et al., 2009; Einer-Jensen et al., 2004; Gastric and Kinkelin, 1980; Kocan et al., 2001; Meyers and Winton, 1995; Oidtmann et al., 2011; Pham et al., 2012).

The gills and the skin are considered in most situations to be the most important tissues for uptake and replication of VHSV (Lorenzen and Lapatra, 1999) with spread to other tissues via blood. It has been speculated that the primary rounds of replication of the virus might take place in the gill lamellar epithelium prior to dissemination (Montero et al., 2011; Neukirch, 1984). VHSV can be detected by immunohistochemistry (IHC) in branchial epithelial cells in the early stages of viral infection in vivo (Brudeseth et al., 2005) and primary gill epithelial cells from rainbow trout were permissive for replication of VHSV IVa (Brudeseth et al., 2008). Gills, however, are not only suitable for entry because rainbow trout experimentally infected with IVb had necrosis of gill cells that contained virus for up to 38 days after infection (Al-Hussinee et al., 2010), indicating continued replication and shedding. The normal epidermis of most fish is poorly vascularized and would likely be a less efficient route compared with the gill, although IHNV has been shown to invade via the epidermis at fin bases (Harmache et al., 2006), an area of skin that overlies a better vascularized dermis. In addition, skin explants of rainbow trout were suitable for replication of VHSV Ia and IVa, and virus production peaked at 2 days postinfection (p.i.), with titers of 10^9, and 10^4 pfu/g, respectively (Yamamoto et al., 1992).

After a primary round of VHSV replication in the gill or dermal tissues, replicating virus in the acute phase of disease can be detected in large numbers in many tissues, but most consistently in renal, splenic and cardiac tissues (Wolf, 1988). The earliest detection of VHSV by qRT-PCR in pooled tissues (head kidney and spleen) was at 2.5 days p.i. in rainbow trout in comparison with detection by virus isolation at 7 days (Chico et al., 2006). At 3 days p.i., VHSV genotype I was detected

in pooled tissues (kidney and spleen) of RT by antigen-capture ELISA (Mourton et al., 1992). VHSV genotype III was identified by IHC in the heart and kidney 7 days postexperimental infection in turbot (Brudeseth et al., 2005). In subacute or chronic phases of the disease, the virus might also be present in high titer in the brain (Hershberger et al., 2010; Wolf, 1988). Hematopoietic tissue are often targeted by VHSV and replication occurred in primary tissue cultures of head kidney leukocytes (Estepa et al., 1992; Tafalla et al., 1998) and viral antigen was detected within leukocytes of experimentally infected fish for VHSV genotype I and III in rainbow trout or turbot, respectively (Brudeseth et al., 2005; Evensen et al., 1994).

A large array of factors, some mentioned previously, play a role in the length of incubation and detection of VHSV in tissues postexposure. The incubation period of VHSV is water temperature-dependent, ranging from 7 to 14 days at 12°C while at 1°C, incubation can be 28 days (Center for Food Security and Public Health, 2003; Ghittino, 1965) or longer. Fish infected with VHSV shed the virus through the urine or feces (Ghittino, 1965; Meyers and Winton, 1995) or reproductive fluids (Jorgensen, 1970) and likely via gills (Al-Hussinee et al., 2010). In two separate studies, Pacific herring began to shed virus by 2 days p.i., following waterborne exposure to VHSV IVa with maximum virus shedding occurred between 6 and 8 days p.i. (Hershberger et al., 2010; Kocan et al., 1997). The interval between infection and shedding and the amount of virus shed will vary with the fish species, genotype of virus used and the viral dose and route (Hershberger et al., 2010).

Evidence to date suggests that vertical transmission does not occur clinically with VHSV. While the virus is present in ovarian fluid and milt (Eaton et al., 1991), VHSV did not adsorb to Chinook salmon sperm, unlike IHNV (Mulcahy and Pascho, 1984). Naturally infected turbot did not produce infected fry as determined by virus isolation or RT-PCR (Nishizawa et al., 2006b); similarly the fry of naturally infected trout were free of VHSV using virus isolation (Jorgensen, 1970). There is evidence that the virus can be isolated from eggs shortly after fertilization (Jorgensen, 1970). Viral antigen and/or genome was also found in ovaries and testes from muskellunge and ovaries from freshwater drum collected from Great Lakes mortality events (Al-Hussinee et al., 2011; Al-Hussinee and Lumsden, 2011) and from experimentally infected fathead minnow (*Pimephales promelas*) and rainbow trout (Al-Hussinee et al., 2010). Despite this evidence, disinfection of reproductive products is a practical method to prevent transmission to progeny and any virus present in eggs may be inhibited by intra-oocyte antiviral factors (Wang et al., 2008). The parasitic leech (*Myzobdella lugubris*) collected from the Great Lakes contained VHSV IVb, but experimental transmission of the virus to fish by infected leeches was not demonstrated (Faisal and Schulz, 2009).

18.3.3 Pathology and Immunity

Fish that are clinically infected with VHSV might alter their behavior during the acute phase of infection to accumulate near the outlet of the pond, at the sides or on the bottom of tank. Fish are often anorexic, and then become lethargic (OIE, 2015b). Mortalities typically start a few days after the initial clinical signs begin, but the mortality rates vary markedly according to fish species, age, environmental conditions and the virulence of virus isolate. In juvenile fish of a susceptible species infected with a virulent isolate, mortality can reach 100% (CFSPH, 2003).

The clinical signs of classical VHS can be divided into three forms; acute, chronic and nervous forms (Wolf, 1988). The typical pathological lesions seen during the acute phase are pronounced multifocal external and internal hemorrhages. The external hemorrhagic lesions can progress to ulcerations and are more common around the eyes, at fin bases and less common on other body surfaces. The internal hemorrhages are most commonly present as petechiae on the serosal surface of most visceral organs. Infected fish that experience severe hemorrhage may also be anemic (Bowden, 2003). The prominent clinical signs of the chronic phase of infection are exophthalmia (Einer-Jensen et al., 2006; King et al., 2001) and serosanguinous ascites (Skall et al., 2004), although these lesions can also develop acutely (Hedrick et al., 2003). Dark discoloration of the skin may also be noticeable during this phase of infection, but this depends on the species of fish. Clinical signs attributed to the nervous system include aberrant swimming or position, inability to right, marked lethargy and are said to be prominent during the late (or nervous) form of infection, if the fish survives.

Hemorrhages, exophthalmia and ascites are common lesions that have been noted in naturally and experimentally infected fish (Einer-Jensen et al., 2006; King et al., 2001; Skall et al., 2005), whereas generalized paleness was the main finding in infected round goby (*Neogobius melanostomus*) (Groocock et al., 2007). Even though VHSV IVa caused significant mortalities in Pacific herring, Pacific hake and walleye pollock during the summer of 1998, no obvious gross pathological lesions were documented (Meyers et al., 1999). Given the numerous genotypes and the very large number of fish species affected, it is not surprising that there can be a substantial variation in the range and severity of lesions noted. The degree of hemorrhage varies markedly even with the same genotype of VHSV; freshwater drum displayed marked hemorrhage in most tissues (Lumsden et al., 2007) while muskellunge had virtually none (Elsayed et al., 2006); both were infected and died due to VHSV IVb.

Severe multifocal to confluent necrosis in the kidney and spleen (lymphopoietic and hematopoietic tissues) and varying degrees of vasculitis are the classic histological lesions described in rainbow trout infected with VHSV genotype Ia isolates (Evensen et al., 1994; Nishizawa et al., 2006b; Wolf, 1988). In contrast, marine 1b isolates commonly cause lesions in the heart, liver and brain and affect hematopoietic tissues to a lesser extent (Brudeseth et al., 2005; Evensen et al., 1994; Isshiki et al., 2001). The microscopic heart lesions in freshwater drum first presented during the mortality event in 2005 from Lake Ontario and were spectacular, with almost complete confluence of necrosis (Lumsden et al., 2007). Transmission electron microscopy of these heart lesions revealed numerous characteristically shaped virions (Fig. 18.5). Lesions in hematopoietic tissues are still commonly present in most affected species regardless of isolate source and genotype, but may be subtle as for Pacific hake, hirame (*Paralichthys olivaceus*), freshwater drum and round goby in natural infections with genotype IVa and IVb isolates (Estepa et al., 1999; Groocock et al., 2007; Isshiki et al., 2001; Kim and Faisal, 2010; Lumsden et al., 2007; Meyers et al., 1999). Vasculitis, sometimes florid and fibrinoid, was prominent in many tissues including the brain of naturally infected freshwater drum (Lumsden et al., 2007) (Fig. 18.6).

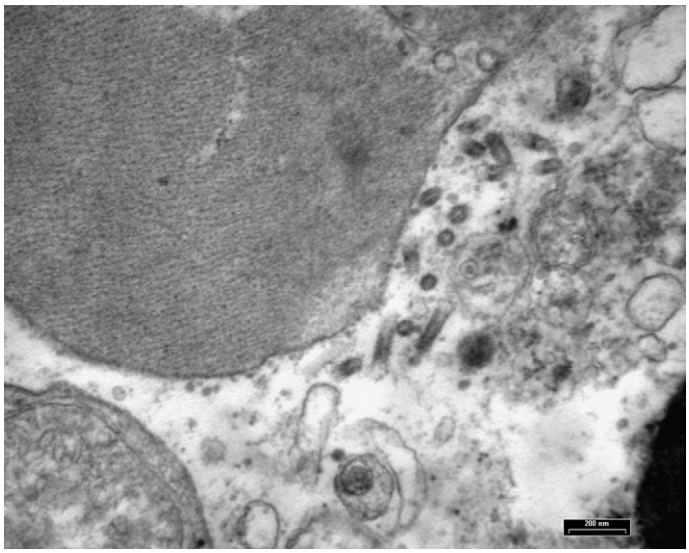

FIGURE 18.5 Numerous bullet-shaped virions associated with necrotic organelles from the heart of freshwater drum naturally infected with VHSV IVb.

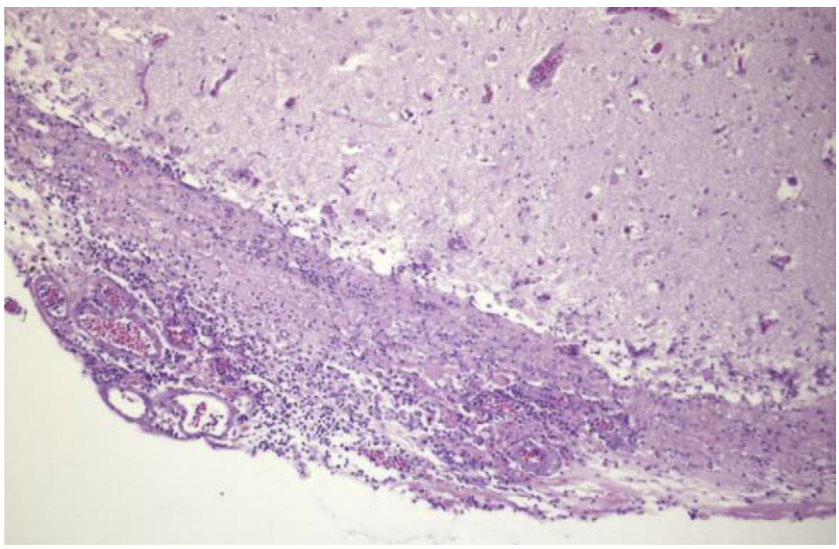

FIGURE 18.6 A florid meningitis from a freshwater drum infected with VHSV IVb. Note the numerous dilated meningeal vessels with prominent fibrinoid vasculitis.

Characteristic renal histological lesions are hemorrhages, which are usually accompanied by focal to multifocal degeneration and necrosis of renal tubules (Snow and Cunningham, 2000) and of the interstitium (Brudeseth et al., 2005; Evensen et al., 1994; Kocan et al., 1997; Meyers et al., 1999). Fibrin accumulation in the interstitium and sinusoids was also noted by Kim and Faisal (2010). Renal interstitial leukocyte infiltrations were seen mainly associated with areas of necrosis, which were also immunopositive for VHSV(Brudeseth et al., 2005). Experimentally infected trout and naturally infected freshwater drum and other species were prominently immunopositive for VHSV in the apical epithelium of mostly intact renal tubules (Al-Hussinee et al., 2010, 2011), potentially supporting excretion of virus in urine. Focal to multifocal degeneration and necrosis of splenic ellipsoids has also been detected in infected fish (Kim and Faisal, 2010; Kocan et al., 1997; Stone et al., 2008). Hepatocellular necrosis (Al-Hussinee et al., 2011; Brudeseth et al., 2005) as well as aggregation of erythrocytes in sinusoids (correlated with a hyperemic liver grossly) have been described (Ghittino, 1965; Kocan et al., 1997; Stone et al., 2008) and were the most prominent lesions in naturally infected crappie (*Pomoxis nigromaculatus*) and smallmouth bass (*Micropterus dolomieu*) (Al-Hussinee et al., 2011).

In the CNS, hemorrhages were found mainly in the meninges (Dale et al., 2009; Lumsden et al., 2007; Skall et al., 2004), which agreed with the presence of multifocal meningeal hemorrhages and necrotic foci in the neuropil seen histologically (Dale et al., 2009). Scattered multifocal necrosis was also present in the brain, medulla and spinal cord associated with gliosis and leukocyte infiltrations (Dale et al., 2009; Lumsden et al., 2007). Fish that had experienced nervous signs were confirmed infected with VHSV by virus isolation (Gastric and Kinkelin, 1980; Meyers et al., 1999; Neukirch, 1984; Nishizawa et al., 2006b; Skall et al., 2004) and/or by IHC or RT-PCR (Dale et al., 2009). Despite the common occurrence of exophthalmia in VHSV-infected fish, lesions of the eye are uncommonly described. Exophthalmia has been associated with proteinaceous fluid accumulations of the anterior chamber of the eye and edema and hemorrhages in the rete, choroid and iris (Lumsden et al., 2007).

Susceptibility to infection as discussed can be related to fish species, age or immune status of the fish (Hershberger et al., 1999, 2007; Lorenzen and Lapatra, 1999). Rainbow trout are very susceptible to VHSV genotype Ia and are less susceptible to genotype Ib or North American genotypes IVa (Winton, 1991; Follett et al., 1997) and more recently to IVb (Al-Hussinee et al., 2010; Kim and Faisal, 2010). The variation is thought to be primarily due to viral genotype virulence differences; however, host variation could play a substantial role (Dorson et al., 1995).

Nutritional factors (Bain et al., 2010), fish density (Hershberger et al., 1999; Kane-Sutton et al., 2010), rough handling of fish (Bain et al., 2010; Elston and Meyers, 2009; Kane-Sutton et al., 2010), water quality (Meyers and Winton, 1995) or infection with other pathogens (Bain et al., 2010) are postulated or considered to be the most common stressors that are likely predisposing factors for a VHS outbreak. These complex relationships are difficult to investigate, however, and the precipitating factors are often not conclusively determined, particularly for outbreaks in the wild.

Summarizing the large effort regarding the use of VHSV as a tool to study teleost immunity is a task beyond the scope of this chapter and previous publications should be examined (Lorenzen and LaPatra, 1999, 2005; Lorenzen et al., 1999; Smail and Snow, 2011). External mucosal layers, such as the gill and skin play an important role in protection of the host against the majority of pathogens, including viruses (Aoki et al., 2008; Bols et al., 2001; Ellis, 2001). Interferon (IFN) is one of the main innate immune factors that is active against most viral infections (Bols et al., 2001; Ellis, 2001). After physical barriers, a type 1 IFN response is most critical to limit virus replication (Bols et al., 2001; Ellis, 2001). Increased expression of IFN 1-like genes was detected by microarray during the acute phase of VHSV infection, following VHSV genotype I-DNA vaccine in rainbow trout (Tafalla et al., 2008). Several heterogenous proteins (Mx1, Mx2 and Mx3) were often identified after IFN production (Das et al., 2008). These proteins can be induced by many stimuli, but most experiments have used polyinosinic: polycytidylic acid (poly IC, synthetic double-stranded RNA) either in vitro (RTG-2 cell line) or in vivo (Tafalla et al., 1998, 2007, 2008). These proteins were also detected at mucosal tissues (eg, skin or gill tissues) (Montero et al., 2011).

Macrophages appear to be important in limiting replication of the virus via extrinsic and intrinsic mechanisms (Stohlman et al., 1982). Production of IFN (mainly type I) by infected macrophages protects other cells from infection (extrinsic) in contrast to the ability of a macrophage to limit internal viral replication, for example by nitric oxide (NO) production (intrinsic) (Stohlman et al., 1982; Tafalla et al., 1998, 2008). NO production was detected in turbot kidney macrophage cell lines during VHSV infection and NO production was correlated with a reduction in viral replication (Tafalla et al., 2001).

Age influences VHSV susceptibility; mortality of Pacific herring associated with VHSV IVb occurs in younger fish and the prevalence of infection in older fish is greatly reduced (Hershberger et al., 1999). The authors naturally postulated that immunity to VHSV would occur with increasing age; however, neutralizing antibodies have not yet been detected in adult herring that are refractory to the disease. Rainbow trout fry from 0.3 to 3.0 g infected with genotype Ia isolates experience up to 100% mortality with virulent strains at 9–12°C. At larger sizes mortality is lower, in the range of 10–15% (Smail and Snow, 2011).

Acquired immunity in teleosts against pathogens can be achieved by the host following natural infection or through immunization (Lorenzen and Lapatra, 1999; Lorenzen et al., 2002b). The low virulence of VHSV (strain F1) for brown trout allows a limited infection that can induce enough acquired immunity to resist infection (Enzmann et al., 1992). Increased mortality in juvenile versus adults in naïve populations of herring experiencing a VHSV-induced mortality event is thought to be due, in part, to a dose effect with adult herring surviving the low waterborne viral doses spread within a school long enough to acquire immunity to resist the infection (Hershberger et al., 2007; King et al., 2001).

The G protein of VHSV, similar to other viruses in the family, is the main target of neutralizing antibodies, which are critical to reduce viral titers in infected fish (Lorenzen et al., 1990, 2002a). Neutralizing antibodies were detected after 8 weeks in fish experimentally infected with VHSV (Olesen and Jørgensen, 1986). The successful development of neutralizing antibody is dependent on temperature with sustained viral proliferation, occurring preferentially at low temperatures (5–10°C), stimulating higher titers (Vestergård Jørgensen, 1982).

18.3.4 Diagnostic Methods

The use of clinical signs for a presumptive diagnosis of VHS can be difficult unless one is familiar with the particular species in question and the variable impacts of the viral genotypes endemic to the region. Clinical signs may be absent, but in many cases behavioral alterations will be present including lethargy and abnormal swimming with involvement of the CNS. Other common clinical signs are darkening, hemorrhages in many tissues including the base of the fins and eyes, exophthalmia and an enlarged abdomen with ascites (OIE, 2015b).

Diagnosis of VHS to OIE standards presently requires isolation of the virus in cell culture followed by serum neutralization. A wide range of cells can be used to isolate VHSV, including bluegill fry (BF-2), rainbow trout gonad (RTG-2), EPC, fathead minnow (FHM) and Chinook salmon embryo (CHSE-214) are most commonly used (Wolf, 1988); the OIE recommends BF-2, EPC or FHM depending on the genotype in question. Recent efforts, spurred by the emergence of genotype IVb in the Great Lakes, have included the development of numerous cell lines from walleye including an endothelial cell line from bulbous arteriosus (Vo et al., 2015). Confirmatory techniques include indirect fluorescent antibody test (IFAT) and ELISA or RT-PCR (OIE, 2015b). Multiplex qRT-PCR is replacing these techniques for screening in many countries; however virus isolation and confirmation are required for approved VHS-free status. Antibodies in fish can be detected using ELISA, IFAT, plaque neutralization or Western blot (Olesen et al., 1991; Fregeneda-Grandes and Olesen, 2007). Variable specificity of antibody-based detection methods and issues illustrated previously with serotyping of VHSV isolates can make these tests problematic.

RT-PCR has been widely used in the past decade and the method of Snow et al. (2004) is currently recommended (OIE, 2015b). Quantitative RT-PCRs, some multiplexed for all VSHV genotypes (Garver et al., 2011) or more than one group of viruses, are now used more commonly and have the advantage of high sensitivity, rapidity and high-throughput (Hope et al., 2010; Matejusova et al., 2008), making it most attractive for surveillance. There is no differentiation of live from dead virus so PCR techniques are still used in combination with virus isolation. The greater sensitivity of PCR techniques, however, can often lead to suspect positives that are not confirmed with virus isolation. Detection of VHSV using LAMP combines reverse transcription and DNA amplification in a single tube is simple and cost-effective (Soliman and El-Matbouli, 2006) and would be appropriately used in regions with limited technological capability.

18.3.5 Prevention and Control

Strict biosecurity measures are the best safeguard against infection of stocks with VHSV, including avoidance of surface water. This is not always possible however, and ultraviolet inactivation of incoming water can be effective (Huber et al., 2010; Oye and Rimstad, 2001), but is expensive and limited to farms with relatively low throughput of water or those on partial recirculation. Where the virus is endemic in the wild, risk-based methodology is most useful (VHSV Expert Panel and Working Group 2010; Thrush and Peeler, 2013).

Selection of animals for resistance to pathogenic organisms is a controversial method, but can have a place in disease management where one agent is of overwhelming importance and few other strategies are available. A program for generation of rainbow trout with increased resistance to VHSV infection was started in mid-1980s (Chevassus and Dorson, 1990). The program was based on selection of male and female rainbow trout that were resistant to experimental infection with VHSV genotype Ia. Resistance to VHSV infection increased significantly by the third generation of trout compared with the first generation (Dorson et al., 1995). Use of these fish as brood stock might provide offspring with increased resistance to VHSV (Lorenzen and Lapatra, 1999), however, this example of genetic selection likely has a limited management role in areas where eradication of infected fish is the main method of disease control. This would also apply to the use of brown (*Salmo trutta*) and rainbow trout hybrids that have enhanced resistance to VHSV (De Kinkelin, 1988).

Sanitation programs to control the spread of VHSV in rainbow trout in Denmark were established in the 1960s. In areas with infected ponds (the predominant aquaculture set-up) this program consisted of three main strategies; draining, disinfection and restocking (Gregory, 2008). Once warmer water temperatures were reached (with decreased viral replication), eradication of all fish in the pond was followed by draining and disinfection. For net-pen systems, all structures must be taken out of the lake or river and disinfected on land. Disinfection of ponds requires quicklime, while all farm equipment must be cleaned and disinfected using formalin or iodine. Restocked fish must be from certified virus-free populations. These strategies resulted in a reduction in the incidence of VHS outbreaks in rainbow trout farms from 500 to only 26 within 27 years (Gregory, 2008). However, similar sanitation programs might not be helpful especially for situations where the marine environment directly connects with the freshwater environment. A somewhat similar situation now exists with the net-pen aquaculture operations in Northern Ontario in Lake Huron and Georgian Bay, which now contains VHSV IVb. Fortunately, VHSV has not been identified in farmed rainbow trout in Ontario and any infections would likely remain subclinical and potentially undetected because trout are resistant to IVb (Al-Hussinee et al., 2010).

At least four types of vaccines have been trialed experimentally including live attenuated, killed, recombinant (Byon et al., 2006; Lecocq-Xhonneux et al., 1994) and DNA-based vaccines (De Kinkelin et al., 1995). Despite the extensive research and the demonstrated efficacy of experimental vaccines there are no commercial vaccines available for VHS (Lorenzen et al., 2002a). Live-attenuated vaccines were developed for rainbow trout with varyingly successful protection (De Kinkelin, 1988; Jorgensen, 1982; Kinkelin and Bearzotti, 1980); however, licensing of live vaccines was not given regulatory approval (Smail and Snow, 2011). Subunit vaccines focusing on recombinant G protein in a variety of expression systems (Lecocq-Xhonneux et al., 1994; Lorenzen et al., 1993) led to neutralizing antibody and protection, but only when delivered by i.p. injection. Oral vaccination using a gastric acid protection coating and an attenuated VHSV strain led to high relative protection of vaccinated trout when delivered either orally or i.p. Antibody response was variable, but cell mediated immunity was stimulated by oral administration as measured by upregulation of CD4 (Adelmann et al., 2008). This type of approach that is amenable for mass oral vaccination and use in small fish but doesn't employ live virus is presently the only realistic strategy toward a commercial vaccine for VHSV.

A DNA vaccine for VHS was found to be highly immunogenic in vaccinated fish and offered good protection from experimental infection (Cuesta and Tafalla, 2009). The *G* or *N* genes of VHSV have been used as DNA vaccines and sequences from both genes were able to induce protective immunity (Cuesta and Tafalla, 2009; Lecocq-Xhonneux et al., 1994; Lorenzen and LaPatra, 2005). The potential efficacy of DNA vaccines appears very promising. Commercialization of DNA vaccines for VHS will remain a challenge, however, due to size and per unit value of the primary aquaculture species of interest; smaller trout, the expense and production costs of DNA vaccines and the primary reliance for control of disease on the slaughter of affected fish and maintenance of virus-free facilities/regions where possible.

18.4 SPRING VIREMIA OF CARP VIRUS

18.4.1 Introduction and Disease

Infectious dropsy of carp (IDC), hemorrhagic septicemia, red contagious disease or rubella are terms that were used to describe a contagious disease of carp caused by a complex of bacterial fish pathogens (Schäperclaus, 1969). IDC usually manifested in two distinct forms; chronic ulcerative and acute ascitic (dropsy). The latter was associated with a cytopathic agent that was isolated on a kidney cell line (Osadchaya, 1964) ultimately leading to identification of SVCV as rhabdovirus (Fijan, 1972; Fijan et al., 1971). The name is descriptive of a viremia primarily affecting yearling common carp (*Cyprinus carpio carpio*) especially in the springtime. The disease is economically important and can cause serious mortality exceeding 50% particularly in yearling common carp (Fijan, 1976). Spring viremia of carp (SVC) is notifiable to the OIE in addition to other eight infectious diseases of fish. A disease is included on this list because it causes significant multinational production losses, has a negative impact on wild fish populations and can be spread internationally via live fish or fish products (OIE, 2010, 2015c).

18.4.2 SVCV Distribution and Host Range

SVCV was first reported in the former Yugoslavia (Fijan et al., 1971) and was rapidly reported from other European countries including France (Tuffery, 1972—unpublished; Fijan, 1976), Czechoslovakia (Macura et al., 1973), West Germany (Bachmann and Ahne, 1973) and the former Soviet Union (Rudikov et al., 1975). Subsequently it has been reported in most other European countries (Ahne et al., 2002); however, some countries are notably SVCV-free including Denmark, Finland, Ireland and Sweden, in addition to SVCV-free regions in England including Northern Ireland, the Isle of Man, Jersey and

Guernsey (Bovo, 2005). Hawaii was the first locale to report the isolation of SVCV from outside Europe and Asia as well as the first report of SVCV in an invertebrate host. Affected fish were from ponds containing cultured shrimp and that also contained a mixed fish population (Johnson et al., 1999). SVCV has also been reported from Korea (Oh et al., 2001), China (Gao et al., 2004), the Middle East (Perelberg et al., 2003; Soliman et al., 2008), the continental United States (Dikkeboom et al., 2004) and recently in Canada (Garver et al., 2007).

SVCV naturally infects carp and other cyprinids (reviewed in Sano et al., 2011) including common carp (Fijan et al., 1971), koi (*Cyprinus carpio koi*) (Goodwin, 2002), grass carp (*Ctenopharyngodon idella*) (Ahne, 1975), goldfish (*Carassius auratus*) (Ahne, 1973), orfe (*Leuciscus idus*) (Dixon et al., 1994), tench (*Tinca tinca*) (Ahne, 1982) and more recently emerald shiner (*Notropis atherinoides*) (Cipriano et al., 2011). Infected noncyprinid species include rainbow trout (Jeremić et al., 2006), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) (Cipriano et al., 2011), Siberian sturgeon (*Ascipenser baerii*) and pike (*Esox lucius*) (Vicenova et al., 2011) and penaeid shrimp (Johnson et al., 1999) (Table 18.1). Species that were successfully experimentally infected but are not known to have been naturally infected include roach (*Rutilus rutilus*) (Haenen and Davidse, 1993), zebrafish (*Danio rerio*) (Sanders et al., 2003), northern pike (*Esox lucius*) (Ahne, 1985b), guppy (*Lebistes reticulatus*) (Bachmann and Ahne, 1974), fathead minnow and white sucker (*Catostomus commersonii*) (Misk et al., 2015).

18.4.3 Epidemiology

A phylogenetic analysis of SVCV and pike fry rhabdovirus (PFRV) glycoprotein gene of different isolates obtained from tench, grass carp, roach, bream and false harlequin, sheath fish and orfe indicated four genogroups (I, II, III, IV). All SVCV isolates were assigned to genogroup I. Moreover, the analysis of a 550-bp sequence of the G gene performed on the 15 SVCV isolates recognized four geographically restricted subgroups (Ia–d) (Stone et al., 2003). Genogroup Ia are isolates from Asia, the United States and Canada; genogroup Ib and Ic are isolates from Moldova, the Ukraine and Russia, and genogroup Id are isolates from the United Kingdom (Dikkeboom et al., 2004; Garver et al., 2007; Stone et al., 2003).

An epidemiological study on 43 worldwide SVCV isolates (35 were Ia) was performed to consider the diversity of nucleotide sequences of an 898-bp segment of the phosphoprotein gene. The study identified two distinct subgroups Iai and Iaii. There was also high nucleotide sequence similarity (97.75–100% and 95.5–100%) in the *P* gene and *G* gene (426 bp) in all Ia genogroup isolates from North America and the United Kingdom (Miller et al., 2007). A separate analysis of Austrian SVCV isolates, again based on phylogenic analysis of a 630-bp segment of the *G* gene indicated 91.1–99.4% nucleotide similarity within the Id group and further classified Id into three genoclusters: Id1, Id2 and Id3 (Basic et al., 2009).

A study of the steps of viral entry and multiplication in common carp after experimental waterborne infection at 13°C revealed that the entry point and primary site of multiplication of SVCV was the gills; from which the virus could be isolated within 2 h p.i. on the fathead minnow cell line. SVCV then spread via the blood stream causing primary and secondary phases of viremia at the 6th and 11th day p.i., respectively (Ahne, 1978). At this point, or a couple of days earlier depending on the individual study, virus is detectable in the kidney, liver and spleen in high titers indicating that these organs represent the primary tissue targets for virus replication (Ahne, 1978; Baudouy et al., 1980; Fijan et al., 1971). Virus shedding occurred via feces starting from the 11th day p.i. and the virus was viable in feces for several days (Ahne, 1978).

SVCV is transmitted horizontally. It is shed through infected fish feces and possibly through mucous casts, infected scale pockets and skin ulcers (Ahne, 1978). Water transmission is the primary source of horizontal transmission and SVCV survived in 10°C tap water and 4°C mud (pH 7.4) for 6 weeks. In stream water at 10°C, SVCV retained infectivity for up to 2 weeks. Dried virus (at temperatures from 4°C to 20°C) survived for more than 3 weeks (Ahne, 1982). Vertical transmission has not been documented, but the virus can be present in reproductive fluids; a survey of Hungarian carp hatcheries collected 491 ovarian and 211 seminal fluid samples and yielded only three positive ovarian samples (Bekesi and Csontos, 1985). In addition, the fish louse, (*Argulus foliaceus* L.) (Pfeil-Putzien, 1978), and the leech, (*Piscicola geometra* L.) (Ahne, 1978), can act only as mechanical vectors (Ahne, 1985a). Moreover fish-eating birds can aid in spreading the infection. SVCV was recovered from heron (*Ardea cinerea*) regurgitant 120-min postfeeding on infected fish (Peters and Neukirch, 1986).

SVCV can replicate in cell cultures at a wide temperature range; from 4°C to 31°C (Ahne, 1973; Clark and Soriano, 1974). The optimum temperature for virus replication and virion-associated transcriptase activity is between 20°C and 25°C (Roy and Clewley, 1978).

In contrast to replication in cell culture, the temperature at which SVCV infects and replicates in fish is restricted by the immune/inflammatory response. Mortality mostly occurs at water temperatures between 10°C and 17°C and did not occur over 20°C under experimental conditions (Ahne, 1986; Baudouy et al., 1980). Following waterborne exposure to SVCV

at different temperature profiles, the results were as follows: above 18°C, increasing from 11°C to 18°C and below 10°C, mortality in common carp was the highest below 10°C, reduced between 11°C and 18°C and absent above 18°C (Baudouy et al., 1980). However, in less than 1-month-old common carp and sheatfish (*Silurus glanis*) infection can occur at 23°C and this was interpreted to be due to their immature immune systems (Pasco et al., 1987). Excretion and survival of virus during the winter at lower temperatures has been proposed to be the most important factor for transmission (Sano et al., 2011). There is surprising little direct evidence for shedding of SVCV from infected fish and only a few instances where virus has been isolated from clinically unaffected fish (Bekesi and Csontos, 1985; Garver et al., 2007; Warg et al., 2007). Koi, emerald shiner and fathead minnow that survived experimental injection with SVCV were detectably infected after mortality had ceased and the intestine and renal tubules were strongly immunopositive for virus, suggesting shedding from these tissues (Misk et al., 2015).

18.4.4 Pathology and Immunity

Pathology and mortality may vary with different SVCV isolates and water temperature will affect the immune response of common carp and other fish to SVCV, which in turn will affect the manifestation of disease (Ahne, 1986). Clinical signs of fish infected with SVCV are aggregation at the pond banks, side swimming, sluggish movement, resting in normal or abnormal positions and loss of balance. External lesions include exophthalmia, abdominal distension, redness and swelling (protrusion) of the vent, which may shed white-yellow thick mucoid fluid due to catarrhal enteritis, pale gills with petechial hemorrhages and hemorrhage anywhere on the skin and fins. Internal gross lesions include ascites, peritonitis, edematous and enlarged kidney, an enlarged spleen with hemorrhage and sometimes infarction, catarrhal (or sometimes hemorrhagic) enteritis, and petechial hemorrhages in the epithelium and wall of the swim bladder (Bachmann and Ahne, 1974; Dikkeboom et al., 2004; Fijan, 1972, 1976; Fijan et al., 1971; Negele, 1977).

Lethal osmoregulatory imbalance was postulated to be a possible cause of death due to multiplication of virus in hematopoietic and excretory kidney tissue (Ahne et al., 2002), although evidence for osmotic impact has not been provided to date. In SVCV, experimentally infected sheatfish had lowered hematocrit (Jeney et al., 1990) and an increase in circulating neutrophils, monocytes, eosinophils and basophils was noted. In addition, plasma from SVCV-infected common carp had lower Ca^{++}, inorganic phosphate, alkaline phosphatase, total protein and cholesterol and a marked increase in total bilirubin, alanine aminotransferase, lactic acid dehydrogenase and α-hydroxybutyryl dehydrogenase (Řehulka, 1996).

In carp, histopathological lesions can be observed in all major organs. SVCV multiplication in blood vessel endothelium often produces a vasculitis and Ahne et al. (2002) and others have suggested that this is the most probable cause of both external and internal hemorrhages. In the kidney, there is tubular degeneration and nephrosis with luminal casts, in addition to necrosis and interstitial nephritis (Negele, 1977). Multifocal hepatic necrosis, necrotizing vasculitis and an infiltration of lymphocytes and histiocytes was present in the liver. There was splenic hyperplasia of the reticuloendothelium, enlarged melanomacrophage centers and hemorrhage. In the intestine, there was a cell-poor enteritis and perivascular inflammation with necrosis and sloughing of the epithelium. In the heart, there was a pericarditis, endocarditis and myocarditis along with focal myocardial necrosis. In the swim bladder, a "disrupted multilayer" was described; presumably, this referred to epithelial erosion/ulceration with areas of hyperplasia and dysplasia. In addition, there was submucosal hemorrhage and dilated blood vessels. There can also be peritonitis (Negele, 1977). Additional histopathological lesions described include an encephalitis, although few details were given (Osadcaja and Rudenko, 1981).

Few histopathological lesions have been described for other infected fish species. In the roach, lesions include exocrine pancreatic necrosis. In rainbow trout, no lesions were noted following an experimental waterborne infection (Haenen and Davidse, 1993). In zebrafish, the lesions described were gill necrosis and inflammation; multifocal hepatic and splenic necrosis and increased numbers of melanomacrophages in gills, liver and kidney (Sanders et al., 2003). In Nile tilapia (*Oreochromis niloticus*), lesions included an encephalitis and congestion of the internal organs (Soliman et al., 2008); however, once again the description was not detailed. Lesions in experimentally infected koi, fathead minnow and emerald shiner were similar and necrotizing lesions in the kidney, intestine and spleen, with co-localized immunopositive staining for SVCV was noted. White sucker and rainbow trout had few lesions and rare immunopositive cells (Misk et al., 2015).

From published micrographs of fish infected with SVCV, the author's impression is that the renal tubular and gastrointestinal lesions often seem to be overstated and are partly confounded by autolysis. Perhaps not surprising because these two highly enzymatically active tissues undergo autolysis quickly. Studies should employ IHC more often to substantiate lesion association with virus presence. SVCV was detected in cryosections of gill, kidney, heart, liver, spleen and intestine of experimentally infected crucian carp using a MAb (Chen et al., 2008) and immunopositive staining was demonstrated in necrotic hepatocytes and in renal tubules of common carp (Gaafar, 2011).

SVCV was found to induce apoptosis in carp cell lines (Miest et al., 2015). RNA sequence analysis of the transcriptome of EPC cells after infection with SVCV revealed that many regulated genes were associated with apoptosis pathways and with oxidative stress (Yang et al., 2014). SVCV infection of EPC cells and common carp resulted in downregulation of heme oxygenase-1, leading to speculation that host oxidative stress contributes to cell death during infection (Yuan et al., 2012). Further study of the mechanisms of cell death in different tissues would be worthwhile.

The carp immune response to SVCV is temperature dependent. The development of neutralizing antibody at 20°C is faster (7 days) and protective, while it is delayed (7 weeks) or doesn't develop at all at 10°C, depending on the route of infection (Ahne, 1986). Reduced immunity of carp, at temperatures at or below 10°C, helps explain the common environmental conditions associated with mortality events and the detection of neutralizing antibodies after outbreaks at lower temperatures. Common carp that survived infection at 13°C developed neutralizing antibodies that persisted up to 4 months p.i. (Baudouy, 1978). The inconsistent development of neutralizing antibody is highly likely due to variation in exposure or vaccination route and this is one factor limiting the efficacy of some vaccines. Common carp immersion-exposed to SVCV (4×10^4 pfu/mL at 13°C) did not mount an antibody response up to 30 days p.i. (Ahne, 1978) and less than 10% of fish orally vaccinated developed neutralizing antibody (Fijan, 1976).

With the recent appearance of SVCV in locations outside Europe, renewed interest in the virus has occurred. This is particularly evident in the subject of interaction of SVCV with host immunity. Some of these papers are selectively included here. Zebrafish, which are susceptible to the virus, have featured prominently as a relevant immune model (Sanders et al., 2003). Zebrafish pathway-targeted microarrays enriched for immune genes were recently developed and identified Toll-like and retinoic acid-inducible gene-I (RIG-I)-like receptors associated with SVCV infection (Encinas et al., 2013). The glycoprotein of SVCV was found to induce autophagy in EPC cells, facilitating replication; however, autophagy also promoted the survival of SVCV-infected cells (Liu et al., 2015). SVCV infection induced type 1 IFN(s) and their induction can be protective (Adamek et al., 2012; Chen et al., 2015; Encinas et al., 2013). The intracellular pattern recognition receptor, RIG-I and melanoma differentiation-associated gene 5 (MDA5), recognize cytosolic viral nucleic acids and their induction resulted in production of type 1 IFNs. RIG-I genes were upregulated following infection of zebrafish with SVCV (Zou et al., 2015), as was MDA5 in cell lines after infection (Zou et al., 2014). Finally, proteomic analysis or microRNA profiles of EPC cells following SVCV infection revealed numerous regulated genes (Liu et al., 2013) and microRNA sequences that directly interacted with the SVCV genome (Wu et al., 2015). Studies similar to these may allow improved strategies for modifying host response to infection.

18.4.5 Diagnostic Methods

Commonly, the history of yearling dead carp in water temperature below 20°C in addition to gross lesions including abdominal distension, pale gills and petechial hemorrhage would increase the expectations for a diagnosis of SVC and would require necropsy and virological examination. The disease can be presumptively diagnosed by history, behavioral signs and gross external and internal changes while a confirmatory diagnosis is obtained by virus isolation followed by a number of different techniques covered next, although RT-PCR followed sequence analysis of the product is now the most commonly used technique (OIE, 2015c).

The OIE recommends the use of *Epithelioma papulosum cyprini* (EPC) and FHM cell line for the isolation of SVCV. Both cell lines produce high SVCV titer when infected. EPC produces a titer of greater than 10^8 pfu/mL (Fijan et al., 1983) and in FHM the virus titer should be at least 10^6 pfu/mL (Clark and Soriano, 1974). The optimum in vitro growth temperature depends on the type of the cell line used; in EPC and FHM cell lines, the optimum temperature is 20–25°C and the CPE usually develops within 24 h and up to 3 days (Fijan et al., 1983; Gravell and Malsberger, 1965).

An incomplete list of the numerous other cell lines of piscine origin that are permissive for replication of SVCV includes: rainbow trout gonad (RTG) (Clark and Soriano, 1974), northern pike gonad (PG) (Wolf and Mann, 1980), rainbow trout embryo (RTE & RTE2), rainbow trout tail (RTT), sake embryo head (*Oncorhynchus keta*) (SEH), epithelioma papulosum goldfish (EPG), wakasagi fin (*Hypomesus nipponensis*) (WF-1), snakehead heart (SHH), snakehead muscle (Fernandez et al., 1993a), heart and swim bladder cell lines (Zhao et al., 2003), Japanese striped knife jaw gonads (JSKG), embryos of a hybrid of kelp (*Epinephelus moara*) and red spotted grouper (*Epinephelus akaara*) (KRE), a cell line derived from the skin of greater amberjack, (*Seriola dumerili*) (PAS) (Fernandez et al., 1993b), eel ovary (EO-2) and ovary of grass carp (CO) (Gao et al., 2004), heart cell line of the giant grouper (*Epinephelus lanceolatus*) (ELGH) (Guo et al., 2013), skin cell line from the red-spotted grouper (Lei et al., 2012), skin cells from Ussuri catfish (*Pseudobagrus ussuriensis*) (Ou et al., 2014), muscle and fin cells of the bluefin trevally (*Caranx melampygus*) (Zhao and Lu, 2006). Medaka (*Oryzias latipes*) haploid embryonic stem cells were also sensitive to SVCV (Yuan et al., 2013) and will provide a unique model for host factor-virus interaction. In addition, nonpiscine cell lines, such as human diploid cell strain (WI38), hamster cell line (BKH21), green

monkey cell line (VERO), viper cell lines (VSW & VH2), gecko lung (GL1) and terrapene heart (TH1) (Clark and Soriano, 1974) have also been used to grow the virus and produced titers of SVCV varying from 10^7 to 10^8 pfu/mL.

Identification of SVCV can be virus- or antibody-based and can be done either after virus isolation or directly on homogenates of tissues. A definitive diagnosis requires isolation of SVCV followed by virus neutralization test, IFAT, ELISA and RT-PCR followed by sequencing of the PCR product (OIE, 2015c).

The use of ELISA, fluorescent antibody (FAT) and immunoperoxidase (IPT) tests provide a faster presumptive diagnosis of SVCV. FAT and IPT have a similar sensitivity ($10^{2.8}$ $TCID_{50}$/g tissue) and in certain occasions where there are few virions or few viable virions in tissues FAT and IPT can detect virus where virus isolation on cell lines may not (Faisal and Ahne, 1983) and both FAT and IPT can be performed on either cell culture or directly on infected fish, but IPT has the additional advantage that slides can be preserved indefinitely, observed on an ordinary light microscope, and has less background (Faisal and Ahne, 1980). Bio-X IFAT, which uses a mouse MAb, reacted only with SVCV subgenogroup Id, illustrating the reliance of antibody-based tests on antibody specificity (Dixon and Longshaw, 2005). ELISA is in greater widespread use than either IPT or FAT, provides detection of SVCV in cell culture, fluid or tissues and can detect as little as $10^{2.8}$–$10^{3.5}$ $TCID_{50}$/0.1 mL (Rodak et al., 1993; Way, 1991). However low antibody titers (1: 3500 dilution to produce a 50% reduction in plaque titer) can lead to false negatives (Dixon and Hill, 1984). The TestLine ELISA kit, which uses a rabbit polyclonal is broadly reactive to all four genogroups of piscine vesiculovirus (Dixon and Longshaw, 2005).

Most fish rhabdoviruses are antigenically distinct and neutralization tests therefore are mostly specific (Frerichs, 1989), however, SVCV shares antigenic determinants in the G and N proteins with PFRV and other fish rhabdoviruses and serological cross-reactivity occurs with polyclonal antibodies in both ELISA (Dixon and Hill, 1984; Way, 1991) and IFAT (reviewed by Ahne et al., 2002, Jorgensen et al., 1989). Any antisera used therefore needs to be properly validated (OIE, 2015c). Interestingly, some MAbs and neutralizing mouse antibodies developed to SVCV (isolate 0504) recognized three structural proteins (G, N and P) in Western blots suggesting that the antibody may identify a common epitope in these proteins (Chen et al., 2008). Screening for neutralizing antibodies in fish serum is possible (Dixon et al., 1994), however, serology is not yet a reliable method for diagnosis due to the variability of serological responses of fish to viral antigens (OIE, 2015c), particularly when viral exposure occurs at the low end of the species thermo-neutral range (Fijan et al., 1977; Kennedy-Stoskopf, 1993).

A variety of nucleic acid-based methods can be used to detect SVCV. The ribonuclease protection assay differentiates SVCV from PFRV (Ahne et al., 1998). RT-PCR targeting the *G* gene is highly sensitive and can identify all four genogroups of vesiculoviruses and the four subgenogroups of SVCV (Stone et al., 2003). The OIE recommends that the RT-PCR product is sequenced to confirm a diagnosis of SVCV (OIE, 2015c). However, a macroarray assay using digoxigenin (DIG)–labeled amplicons that are mixed with immobilized genogroup specific probes on a nylon membrane can also be used in laboratories without access to sequencing. The assay was able to identify the major four genogroups of vesiculoviruses, as well as the two subgenogroups of SVCV Ia (Sheppard et al., 2007). Combining reverse transcription and DNA amplification in a single tube using the LAMP technique is simple and cost-effective (Liu et al., 2008b).

Several quantitative real-time RT-PCR assays targeting the *G* gene have been developed (Misk et al., 2015; Shimahara et al., 2015; Yue et al., 2008) as well as multiplex assays for all three major rhabdoviruses (Liu et al., 2008a). Advantages over conventional RT-PCR are often: increased sensitivity, high-throughput with reduced contamination and quantification of template (Yue et al., 2008). Gold nanoparticles adsorb single- or double-stranded nucleotides allowing very rapid detection of SVCV in a variety of samples without amplification and sensitivity was comparable to RT-PCR (Saleh et al., 2012).

18.4.6 Prevention and Control

Where the virus is not endemic, fish infected with SVCV are eradicated. In some countries (eg, the United Kingdom) eradication is practiced even in regions where the virus is in wild fish and/or water. Surface water containing SVCV in Eastern Europe is common and exposure of farmed fish is often unavoidable except if a spring-fed water supply is available (Ahne et al., 2002). Reduction in the impact of disease requires a variety of biosecurity and management measures typically used where an infectious agent is endemic. Prophylactic disinfection of eggs with iodophor treatment is effective (Ahne and Held, 1980). The Krasnodar strain of carp that was produced after several generations of selection is relatively resistant to infection (Kirpichnikov et al., 1993).

A review of RNA viruses affecting European aquaculture and the vaccines for these viruses was recently published (Gomez-Casado et al., 2011). Several inactivated virus vaccines have been used experimentally and in the field in Eastern Europe (Fijan and Matasin, 1980; Fijan et al., 1977; Kölbl, 1980). These include those given by injection (Fijan et al., 1977; Kölbl, 1980), immersion (Fijan and Matasin, 1980) and oral (Kölbl, 1990, from Sano et al., 2011). Some injection vaccines provided variable protection for up to 11 months (Fijan et al., 1977); however, none of these vaccines are available commercially.

Similar to VHSV and IHNV, DNA vaccination for SVCV has been developed and can be effective experimentally (Emmenegger and Kurath, 2008; Kanellos et al., 2006). DNA vaccination offers great insight into immunological mechanisms of protection in fish but also illustrates the technological advance versus cost mismatch common in aquaculture. DNA vaccination for SVCV, however, is not likely to be commercially available soon, even in potentially high-value fish such as koi. The bulk of the fish affected by SVCV are only moderately individually valued fish and DNA vaccination carries a high manufacturing and regulatory cost (Gomez-Casado et al., 2011). Even high-value koi in wealthy markets would provide limited economic incentive for commercial vaccine production. There are few examples similar to IHNV, where DNA vaccination is commercially available and used in British Columbia, Canada, where the potential loss of very high-value Atlantic salmon and a sufficiently sized industry, justifies the cost. DNA molecular adjuvants may be one way to increase the efficacy of vaccines should they be economically justified. Sequence from two amino acid segments of the glycoprotein G of VHSV that were found to induce type 1 IFNs were used in combination with an SVCV DNA vaccination in zebrafish and provided improved survival and duration of protection using one-tenth the vaccine dose (Martinez-Lopez et al., 2014). Tandem synthetic-interfering RNA molecules to the *N* and *P* genes inhibited SVCV replication in EPC (Gotesman et al., 2015) and a similar technology has been used in vivo for rabies; however, application of this technology faces the technology/cost mismatch common to many fish diseases.

A risk-based model using approximate lake temperatures in the United Kingdom and the mean number of days per year that water temperature satisfied optimal conditions for SVCV and KHV was developed to predict areas in which outbreaks might occur and allow efficient use of resources (Thrush and Peeler, 2013). A risk framework to examine the influence of climate change on outbreaks of viral disease in the United Kingdom predicted decreased occurrence of SVCV, IHNV and VHSV with warming waters and increased KHV mortality (Marcos-Lopez et al., 2010).

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Chapter 19

Orthomyxoviruses of Fish

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19.1 INTRODUCTION

The name of the family *Orthomyxoviridae* is derived from *Orthos* and *myxa,* the Greek words for *correct* and *mucus*, respectively. The term was coined to distinguish the orthomyxoviruses from the paramyxoviruses. The family is well known for containing influenza viruses. The word *influenza* is derived from *influentia*, the Latin word for *epidemic*, and it was originally used because epidemics were thought to be due to astrological or other occult influences (McCauley et al., 2012). This family also contains infectious salmon anemia virus (ISAV) and tilapia lake virus (TiLV), which are unique orthomyxoviruses that infect fish. ISAV has been characterized only recently (Falk et al., 1997), and is now classified in the family *Orthomyxoviridae* (Krossøy et al., 1999), genus *Isavirus* (McCauley et al., 2012). There are excellent reviews available on the subjects of ISA and ISAV (Kibenge et al., 2004; Cottet et al., 2011; Rimstad et al., 2011; Aamelfot et al., 2014). Even more recently, TiLV that causes mass die-offs of tilapia in Israel (Eyngor et al., 2014), Ecuador (Ferguson et al., 2014) and Colombia has been identified; the virus has 10 genome segments, with 5′ and 3′ noncoding regions characteristic of influenza viruses, and it likely represents a new genus in the family *Orthomyxoviridae* (Bacharach et al., 2016).

19.1.1 Orthomyxovirus Classification and Virion Properties

Orthomyxovirus particles are enveloped and highly pleomorphic (may be spherical, oval, filamentous, etc.) with a diameter of 80–120 nm in spherical form and >300 nm long in filamentous form and with characteristic large surface glycoprotein (GP) spikes (Fig. 19.1). In influenza virus A, the prototype of the family, the envelope projections consist of rod-shaped hemagglutinin (HA) and mushroom-shaped neuraminidase (NA) GPs, in a ratio of approximately 4:1 (Palese and Shaw, 2007), which carry subtype-specific antigenic determinants, the basis for antigenic grouping of influenza virus A. Depending on the genus, other viral envelope proteins include M2, NB, BM2 or CM2. M2 and BM2 make up the ion channels in the virus envelope and mammalian cells. The envelope proteins overlay a matrix of M1 protein, which plays an important role in determining virion morphology. The presence of the envelope makes orthomyxoviruses very labile under ordinary environmental conditions; the virions are sensitive to heat, extremes of pH and dryness, lipid solvents such as ether and chloroform and detergents such as sodium deoxycholate (SDC) and sodium dodecyl sulfate (SDS).

The viral genome is segmented, linear, negative-sense, single-stranded RNA requiring an RNA-dependent RNA polymerase of viral origin for replication (see Table 2.3 in Chapter "Classification and Identification of Aquatic Animal Viruses" of this book). Depending on genus, virions contain a precise number of segments with a total genome size of 10–15 kb. Each segment occurs in the enveloped particle as a nucleocapsid with helical symmetry and the ends of each viral RNA (vRNA) segment forming a helical hairpin bound by the heterotrimeric RNA polymerase complex (PB2, PB1, PA), as depicted in Fig. 19.1B. The RNA molecules possess conserved and partially complementary 5'- and 3'-end sequences characteristic of the genus (Desselberger et al., 1980; Presti et al., 2009; Kulshreshtha et al., 2010; Hause et al., 2013; Bacharach et al., 2016), which function as the promoter for vRNA replication and transcription by the vRNA polymerase complex. The noncoding regions also include the messenger RNA (mRNA) polyadenylation signal and part of the packaging signals for virus assembly (Palese and Shaw, 2007). To date, the family *Orthomyxoviridae* is made up of seven genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Isavirus*, *Quaranjavirus* and the newly proposed genus *Influenzavirus D* (Table 19.1). The novel orthomyxo-like virus Tilapia lake virus (TiLV), with 10 genome segments, likely belongs to a new unnamed eighth genus in *Orthomyxoviridae* (Bacharach et al., 2016). Phylogenetic relationships within the family are illustrated in Fig. 19.2.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00019-X
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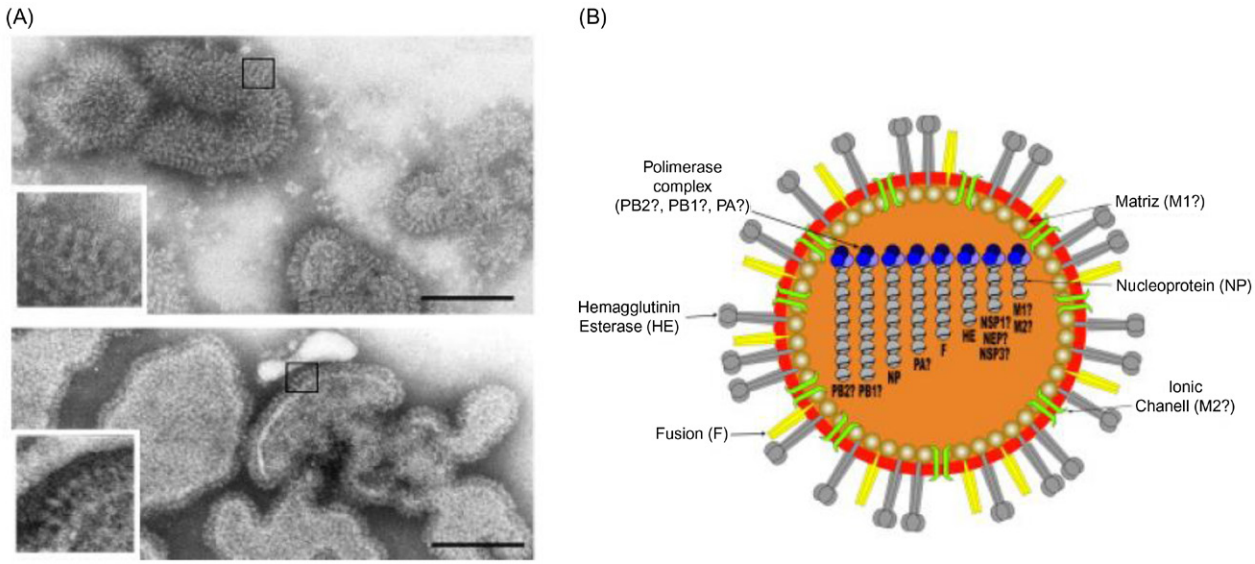

FIGURE 19.1 Influenza virus A and ISAV. (Panel A) Electron micrograph of influenza virus A/PR/8/34 (top) and ISAV (below), at a magnification of 150,000×. The squares in the photomicrographs show the amplified region that appears in the lower-left corner, at a magnification of 400,000×. Bars represent 100 nm. (Panel B) Schematic representation of the ISAV; the arrows indicate the major structural proteins that make up the viral particle and the proteins that form ribonucleoprotein complexes and also indicate the putative proteins generated by each segment of the viral genome. *From Cottet, L., Rivas-Aravena, A., Martin, M., C.-S., Sandino, A. M., Spencer, E., 2011. Infectious salmon anaemia virus – genetics and pathogenesis. Virus Res. 155, 10–19. Figure 1. Copyright © Elsevier B.V. (2010), with permission.*

19.1.2 Orthomyxovirus Genome Organization

Orthomyxoviruses exploit various splicing strategies to express their viral proteins to maximize the genome coding capacity. The orthomyxovirus genome encodes at least 12 proteins (see Table 19.2). The first five genome segments encode one protein each, except for *Influenzavirus A*, where in some strains, segment 2 encodes a second protein PB1-F2, varying in length from 11 to 90 amino acids (Chen et al., 2010), and a third protein PB1 N40, 718 amino acids long. The PB1-F2 protein has various lengths, amino acid sequences, cellular localizations and functions (promotes apoptosis, increases inflammation and regulates viral polymerase activity) in different strains, which result in strain-specific pathogenicity (Chen et al., 2010). The smaller genome segments encode 1–3 proteins each via spliced and bicistronic mRNAs (or leaky scanning) (Lamb and Horvath, 1991; Garcia-Rosado et al., 2008). The functions of the orthomyxovirus proteins are summarized in Table 19.3, using genus *Isavirus* as an example.

19.1.3 Orthomyxovirus Replication

In orthomyxoviruses, depending on the genus, the first step in virus replication, virus attachment to cells occurs via a hemagglutinin (HA, HE, HEF or GP) binding to *N*-acetylneuraminic (sialic) acid on the host cell surface. In the case of the influenza A virus, different cells have different linkages of the carbon-2 of the terminal sialic acid to the carbon-3 or carbon-6 of a galactose residue, forming α-2,3- or α-2,6-linkages, which in turn determine the host range (Bouvier and Palese, 2008). The virus enters by receptor-mediated endocytosis and uncoating occurs through low pH-dependent fusion between the viral envelope and cell membranes (Sieczkarski and Whittaker, 2005; Pinto et al., 1992). Virus replication of orthomyxoviruses is unique in that the viral nucleocapsids are transported to the nucleus, where transcription by viral transcriptase (PB1 and PB2) occurs following capture of host heterologous nuclear RNA (8–15 nucleotides long) as primer (also referred to as "cap snatching" (Krug, 1991)). *Thogotovirus* differs from the other orthomyxoviruses in having capped viral mRNA without host-derived sequences at the 5' end (Weber et al., 1996; McCauley et al., 2012). Assembly of nucleocapsids occurs in the nucleus. Virus matures in cytoplasm by budding from the plasma membrane. When budding is complete, HA spikes continue to bind the virions to the sialic acid on the cell surface until virus particles are actively released by the receptor destroying enzyme (or sialidase) activity of the neuraminidase (NA) or esterase (HE, HEF). The NA also removes sialic acid residues from the virus envelope itself, which prevents viral particle aggregation to enhance infectivity (Palese and Compans, 1976).

Defective interfering particles (DIPs) appear following infection at high multiplicity of infection (m.o.i.); the so-called *von Magnus phenomenon* (von Magnus, 1954). The genome of DIPs is defective, and they interfere with the replication of

TABLE 19.1 Classification and Biological Features of Orthomyxoviruses

Genus	Virus	Number of Genome Segments (Negative-Sense ssRNA)	Envelope Proteins	Target Host and (Reservoir Host)	Target Organ/Cells	Disease
Influenzavirus A	Influenza A virus (18 subgroups of HA and 11 subgroups of NA)	8	HA	Avian and Mammalian species (wild aquatic birds)	Lung (epithelial cells and alveolar septa) in mammals	Respiratory disease in mammals
			NA		Lung and intestinal tract in birds	Hemorrhagic, systemic in poultry
			M2 ion channel			
Influenzavirus B		8	HA	Humans and seals		Respiratory disease
			NA			
			NB; BM2 ion channel			
Influenzavirus C		7	HA-esterase-fusion (HEF)	Human, swine and dogs (humans)		Mild respiratory disease
			CM2			
Influenzavirus D[a]		7	HEF	Swine and cattle (cattle)	Nasal, pharyngeal and lung epithelial cells	Respiratory disease
			CM2			
Thogotovirus		6 or 7	Glycoprotein (GP)	Ticks, mosquitoes, humans and other mammals, as well as waterfowl		Febrile illness and encephalitis in humans
Quaranjavirus[b]		6	Glycoprotein (GP)	Ticks and birds		
Isavirus	Infectious salmon anemia virus	8	Hemagglutinin-esterase (HE);	Farmed Atlantic salmon (wild Atlantic salmon and brown trout)	Endothelial cells of heart, liver and kidneys, and macrophages	Hemorrhagic, systemic in farmed Atlantic salmon
			Fusion (F)			
Unassigned	Tilapia lake virus[c]	10		Farmed tilapia	Brain and liver	Ocular disease and hepatitis, haemorrhagic, systemic in farmed tilapia

[a]*Proposed in 2014 (Hause et al., 2014).*
[b]*A new genus approved in 2013 (Presti et al., 2009; Adams, M.J., King, A.M.Q., Carstens, E.B., 2013. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2013). Arch. Virol. 158, 2023–2030).*
[c]*Tilapia lake virus (TiLV) is a novel orthomyxo-like virus causing mass die-offs of tilapia in Israel and Ecuador, representing a new unnamed genus in the family Orthomyxoviridae (Bacharach et al., 2016).*

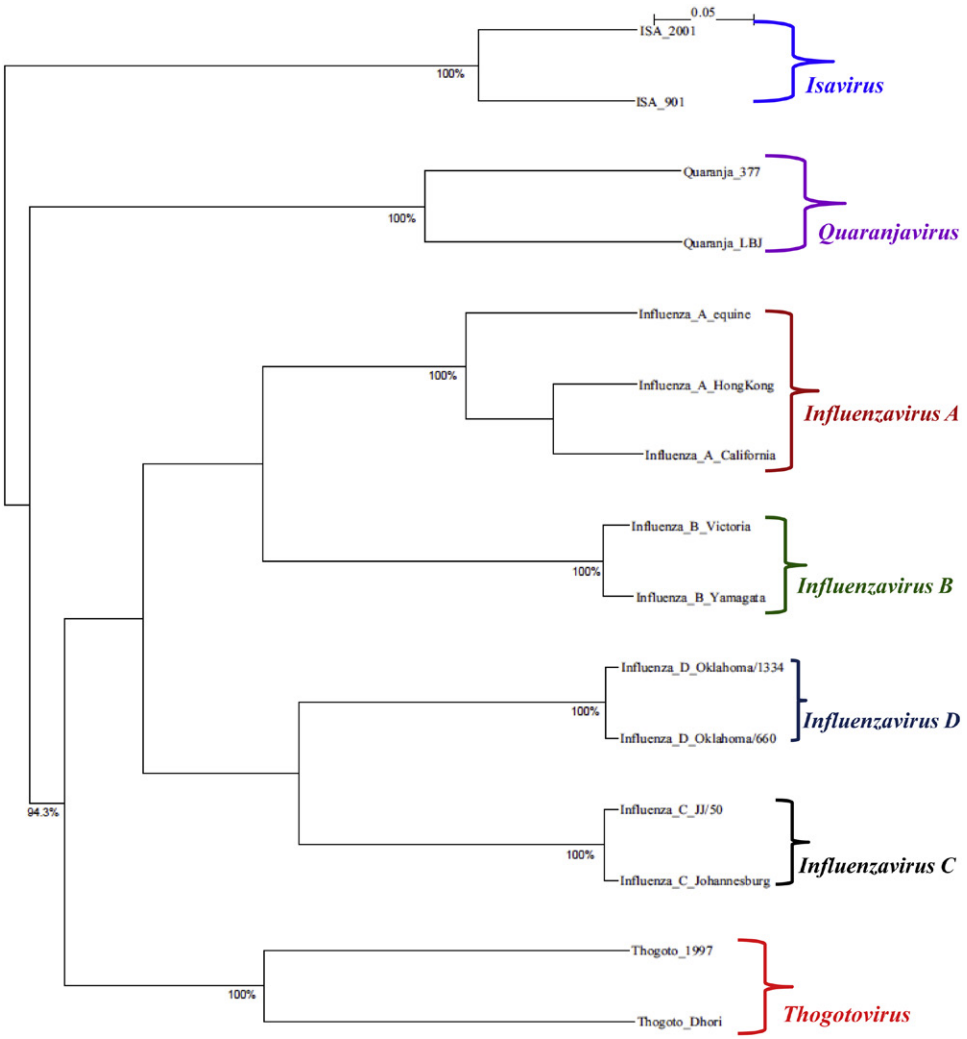

FIGURE 19.2 Phylogenetic tree showing the relationships within the family *Orthomyxoviridae*. Nucleotide sequences of polymerase basic 1 (PB1) genes of selected members in the seven genera of family *Orthomyxoviridae* were used to generate a phylogenetic tree. The GenBank accession numbers for the sequences used for comparison were *Isavirus*: AF404346, GU830904; *Quaranjavirus*: FJ861695, FJ861697; *Influenzavirus A*: GU053121, CY044267, FJ966080; *Influenzavirus B*: CY018763, CY018771; *Influenzavirus D*: JQ922306; KF425660; *Influenzavirus C*: M28060, AF170575; *Thogotovirus*: AF004985, M65866. Tilapia lake virus segment 1 sequence KU751814, with weak homology to the PB1 subunit of influenza C virus, was also included as a novel orthomyxolike virus (Bacharach et al., 2016). Sequences were processed using ClustalX 2.0 (Larkin et al., 2007). The multiple sequence alignment was manually verified and adjusted to achieve high-quality alignment. The phylogenetic tree was constructed by maximum likelihood using the neighbor-joining method and Tamura-Nei genetic distances (Saitou and Nei 1987). The L protein of the *Vesicular Stomatitis* virus (M20166) was used as an outgroup sequence to determine the root of the tree, but the outgroup itself was not included in the tree. Bootstrapping was performed 1000 times. Bootstrap supports of topology are shown below the nodes. The scale bar represents the number of substitutions per site. *(Courtesy of Dr. Yingwei Wang, School of Mathematical and Computational Sciences, University of Prince Edward Island, Canada).*

the complete orthomyxovirus particles (Nayak et al., 1978; Tobita et al., 1986), resulting in reduced virus titers and possibly in virus persistence. Genomic segment reassortment occurs during mixed infections involving virus of the same species, but not between viruses of different types (eg, influenza A and influenza B) or those of different genera. Reassortment of HA and NA genes of human, swine and avian influenza viruses produces antigenic shift, resulting in new virus subtypes to which the human population is immunologically naïve, which then cause pandemics (Ahmed et al., 2007). Antigenic diversity in influenza virus A in nature also occurs through accumulation of single-base mutations in HA and NA segments which produce antigenic drift, resulting in new virus strains that cause epidemics or epizootics. Reverse genetic systems have been established for *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* (reviewed by Neumann and Kawaoka, 2001; Hoffmann et al., 2002) and *Isavirus* (Toro-Ascuy et al., 2015), allowing the generation of genetically engineered orthomyxoviruses entirely from cloned complementary DNA (cDNA).

TABLE 19.2 Orthomyxovirus Genome Coding Assignments

Genome Segment[a]	Gene Products[b] for the Respective Genus in the Family *Orthomyxoviridae*							
	Influenzavirus A	*Influenzavirus B*	*Influenzavirus C*	*Influenzavirus D*[c]	*Thogotovirus*[d]	*Quaranjavirus*[e]	*Isavirus*	Unnamed (TiLV)[f]
1	PB2 (87 kDa)[g]	PB2	PB2 (87.8 kDa)	PB2	PB2 (88 kDa)	PB2	PB2 (79.5 kDa)	PB1 (57.107 kDa)
2	PB1 (96 kDa)	PB1	PB1 (86 kDa)	PB1	PB1 (81 kDa)	PA	PB1 (80.5 kDa)	Unknown (51.227 kDa)
	PB1-F2**							
	PB1 N40**							
3	PA (85 kDa)	PA	P3 (81.9 kDa)	P3	PA (71.5 kDa)	PB1	NP (66–74 kDa)	Unknown (47.708 kDa)
4	HA_1 (48 kDa);	HA_1	HEF(88 kDa)	HEF	GP (65–75 kDa)	unknown (527 aa)	PA (65.3 kDa)	Unknown (38.625 kDa)
	HA_2 (29 kDa)	HA_2						
5	NP (50–60 kDa)	NP	NP (63.5 kDa)	NP	NP (52–54 kDa)	GP	F (50–53 kDa)	Unknown (38.058 kDa)
6	NA (48–63 kDa)	NA	P42 (42 kDa)	P42	ML (32 kDa) and	Unknown (266 aa)	HE (38–46 kDa)	Unknown (36.381 kDa)
				CM2	M (29 kDa) – THOV or			
				M1′	M1 (30 kDa) and			
		NB (18 kDa)	CM2 (18 kDa)	M1	M2 (15 kDa) - DHOV			
			M1′ (31 kDa)					
			M1 (27 kDa)					
7	M1 (25 kDa)	M1 (25 kDa)	NS1 (27.7 kDa)	NS1	Unknown—DHOV	Absent	NS1 (35 kDa)	Unknown (21.834 kDa)
	M2 (15 kDa)	BM2	NEP/NS2 (21 kDa)	NEP/NS2			NEP (18 kDa)	
							NS3 (10.6 kDa)	
8	NS1 (25 kDa)	NS1B	Absent	Absent	Absent	Absent	M1 (22–24 kDa)	Unknown (19.474 kDa)
	NEP/NS2 (12 kDa)	NEP/NS2					M2 (26.4 kDa)	
9	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Unknown (13.486 kDa)
10	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Unknown (12.732 kDa)

[a]*Genome segments are numbered in the order of decreasing length.*
[b]*Gene products: PB2, polymerase basic protein 2; PB1, polymerase basic protein 1, **(a second protein PB1-F2 varies in length from 11 to 90 amino acids; a third protein PB1 N40 is 718 amino acids long in some strains Influenzavirus A); PA, polymerase acidic protein (P3 in Influenzavirus C and D as it lacks the acidic features at neutral pH (Yamshita, M., Krystal, M., Palese, P., 1989. Comparison of the three large polymerase proteins of influenza A, B, and C viruses. Virology 171, 458–466)); NP, nucleoprotein; HA, hemagglutinin in two subunits in Influenzavirus A and B; HEF, hemagglutinin-esterase-fusion in Influenzavirus C (Herrler, G., Durkop, I., Becht, H., Klenk, H.-D., 1988. The GP of influenza C virus is the hemmagglutinin, esterase, and fusion factor. J. Gen. Virol. 69, 839–846) and Influenzavirus D (Hause et al., 2014); F, fusion protein in Isavirus (Aspehaug et al., 2005); HE, hemagglutinin-esterase in Isavirus (Falk et al., 2004); NA, neuraminidase; M1, matrix protein 1; M2, matrix protein 2/ion channel protein; NS1, nonstructural protein 1; NS2, nonstructural protein 2 (is also known as nuclear exporting protein, NEP, and is present in purified viral preparations); NS3, nonstructural protein 3 in Isavirus (Kibenge et al., 2007a,b).*
[c]*Influenzavirus D is a new genus proposed in 2014 (Hause et al., 2014).*
[d]*Thogotovirus has two species, Thogoto virus (THOV) with 6 segments and Dhori virus (DHOV) with 7 segments (McCauley et al., 2012).*
[e]*Quaranjavirus is a new genus approved in 2013 (Presti et al., 2009; Adams, M.J., King, A.M.Q., Carstens, E.B., 2013. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2013). Arch. Virol. 158, 2023–2030).*
[f]*TiLV, Tilapia lake virus, with 10 genome segments, is a novel orthomyxolike virus that likely represents a new, unnamed genus in the family Orthomyxoviridae. The estimated protein molecular weights correspond to the genome segment number only, and except for segment 1, which predicts a protein with weak homology to the PB1 subunit of Influenzavirus C, the remaining nine segments have no recognizable homology to other known sequences (Bacharach et al., 2016).*
[g]*Numbers in parenthesis are either the observed molecular weights (protein mass) or are estimations based on amino acid sequences.*

TABLE 19.3 ISAV Genome Coding Assignments and Protein Characteristics

Genome Segment	Molecular Size (kb)[a]	5′UTR (nt)[b]	3′UTR (nt)[b]	Protein Name[c]	Protein Size (aa)[c]	Protein Mass (kDa)[c]	Predicted Function[d]
1	2.3–2.4	8	89–91[15-4]	PB2	722	79.5	Polymerase subunit; mRNA cap recognition
2	2.3–2.4	24	98[14-4]	PB1[e]	709	80.5	Polymerase subunit; RNA elongation, endonuclease activity
3	2.2	48	147–150[14-4]	NP	616	66–72	RNA-binding protein; nuclear import regulation
4	1.9–2.0	22–23	67[13-5]	PA	579	65.3	Polymerase subunit; protease activity
5	1.6–1.7	8	142–144[15-3]	F	444	47–50	Surface GP; fusion activity
6	1.5	5–7	131–138[14-4]	HE	389	42	Surface GP; major antigen, receptor binding and esterase activities, virus release
7	1.3	21	99-100[14-4]	NS1	301	35	Interferon antagonistic protein
				NEP	159	18	Nuclear export of RNA
				NS3	81	10.6	Unknown function
8	1.0	21	294–295	M1	197	22–25	Matrix protein; vRNA interaction, RNA nuclear export regulation, viral budding
		35	144–145[13-5]	M2	242	26.4	Ion channel; virus uncoating and assembly (Interferon modulation in ISAV)

[a]Values obtained by Northern blotting (Mjaaland et al., 1997; Clouthier et al., 2002).
[b]Values obtained from cDNA of genomic segments of six genetically distinct ISAV isolates: NBISA01 and ADL-ISAV-07 belonging to two different genotypes of ISAV (Kulshreshtha et al., 2010), 390/98 Scotland, 982/08 Scotland, Glaesver 2/90 Norway and RPC/NB 98-049 Canada (Fourrier et al., 2011), and Glaesver 2/90 Norway (Mérour et al., 2011). The 5′ and 3′ untranslated regions (UTRs) are written for the positive sense (cRNA), and are equivalent to the respective 3′ and 5′ noncoding regions (NCRs) of the negative sense RNA segments (vRNA). The total length includes the range between the shortest and longest 5′ and 3′ UTRs for each segment among the six ISAV isolates (Kulshreshtha et al., 2010; Fourrier et al., 2011). The 3′ UTR does not include the stop codon. Numbers in brackets denote # nucleotides to polyadenylation signal, and length of polyadenylation signal.
[c]Protein nomenclature and data used by Cottet et al. (2011) and Mérour et al. (2011) for ISAV. See also Table 19.2 for homologs of orthomyxovirus proteins and Figure 19.3 for expression of different orthomyxovirus genes.
[d]Protein function inferred from Cottet et al. (2011) for ISAV and from Bouvier and Palese (2008) for influenza A virus.
[e]A novel orthomyxolike virus Tilapia lake virus (TiLV), with 10 genome segments with no recognizable homology to other known sequences, except for segment 1, which predicts a protein with weak homology to the PB1 subunit of Influenzavirus C, was recently described (Bacharach et al., 2016). A complete sequence of the PB1 *gene of a putative koi carp orthomyxovirus was obtained from koi carp in California with 43% amino acid sequence identity with ISAV (Anonymous, 2013).*

19.2 GENUS *ISAVIRUS*

19.2.1 Disease Occurrence and Significance

The clinical disease, infectious salmon anemia (ISA), in farmed Atlantic salmon (*Salmo salar*) was first recognized in Atlantic salmon in 1984 on the southwestern coast of Norway (Thorud and Djupvik, 1988), although the virus might have been present in Norwegian salmon farms as early as 1977 or 1978 (Nylund et al., 1995a; Devold et al., 2001). The situation developed into an epidemic, which peaked in 1990, forcing the Norwegian authorities to impose a series of biosecurity measures that significantly reduced but not eliminated disease outbreaks (Håstein et al., 1999). The World Organization for Animal Health (OIE) recognized the viral disease in 1990 and named it ISA, and it has been an OIE-notifiable disease ever since. The causative agent, ISAV, was first detected by electron microscopy and virus isolation from tissues of infected fish in 1995 (Dannevig et al., 1995a,b), which allowed its molecular characterization (Falk et al., 1997) and subsequent classification to the family *Orthomyxoviridae* (Krossøy et al., 1999), genus *Isavirus* (McCauley et al., 2012). The disease was first reported outside Norway in 1996, in New Brunswick, Canada (Byrne et al., 1998; Mullins et al., 1998), although there is anecdotal evidence indicating that the virus was present in the Bay of Fundy by 1995. Subsequently, ISA outbreaks were reported in Scotland, in the Faroe Islands, Denmark, in Maine, and in Chile in 2007 (reviewed by Cottet et al., 2011). The economic impact of the disease was probably most apparent in Chile, where losses during the 2007–11 ISA outbreaks were estimated at about $1 billion (ie, 50% of the economic value of the industry) (Alvial et al., 2014). More recently, ISA outbreaks have occurred in Nova Scotia and Newfoundland, Canada (CFIA, 2014). ISAV is now endemic in fish populations in Norway, New Brunswick, Canada, Scotland, Faroe Islands, Maine and Chile, and virulent strains of the virus have been replaced by low-pathogenic variant ISAVs called *ISAV-HPR0 viruses.* The field disease has only been found in farmed Atlantic salmon and is characterized by high mortality with exophthalmia, pale gills, ascites and hemorrhagic liver necrosis, renal interstitial hemorrhage and tubular nephrosis. No natural outbreaks of ISA have been reported in fish other than farmed Atlantic salmon, although the virus can be detected by reverse transcription polymerase chain reaction (RT-PCR) in feral fish (Kibenge et al., 2004; Plarre et al., 2005; EFSA, 2012). Laboratory data on tissues and sera from farmed coho salmon (*Oncorhynchus kisutch*) indicated the existence of ISAV in Chile as early as 2000 (Kibenge et al., 2001a, 2002) with some fish showing jaundice, although no ISA outbreak was detected in neighboring Atlantic salmon farms (Smith et al., 2006). The virus has also been detected in apparently healthy farmed rainbow trout (*Oncorhynchus mykiss*) in Ireland in 2002 (Anonymous, 2002).

19.2.2 Virus Characteristics

19.2.2.1 Structure and Composition of Virus

ISAV is an economically important pathogen in marine aquaculture. It is the only species of the genus *Isavirus*, one of the seven genera of the family *Orthomyxoviridae* (Table 19.1; Fig. 19.2). However, a complete sequence of PB1 gene of a putative koi carp orthomyxovirus was obtained from koi carp in California with 43% amino acid sequence identity with ISAV (Anonymous, 2013), and there is also an independent reference to an orthomyxovirus from koi carp (Granzow et al., 2014). Most recently, the complete genome sequence of a novel orthomyxo-like virus Tilapia lake virus (TiLV) with 10 genome segments was reported (Bacharach et al., 2016), and likely belongs to a new unnamed eighth genus in *Orthomyxoviridae*.

Viruses in the genus *Isavirus* are enveloped particles with a diameter of 90–140 nm (Dannevig et al., 1995a,b; Nylund et al., 1995a,b), with 13–15-nm-long, mushroom-shaped surface projections consisting of a combined receptor-binding HA and receptor-destroying enzyme activity demonstrated to be an esterase, designated as HE (Falk et al., 2004; Hellebø et al., 2004) and fusion GP (Aspehaug et al., 2005), designated as F. This arrangement of the functional activities of the surface proteins in ISAV is in contrast to other orthomyxoviruses (which have hemagglutinating and fusion activities on one virus protein and the receptor-destroying enzyme activity on a separate virus protein), but is similar to most paramyxoviruses (reviewed by Aamelfot et al., 2014). The genome is composed of eight segments of linear, single-stranded negative sense RNA ranging in length from 1.0 to 2.4 kb (see Table 19.3 above) with a total molecular size of approximately 14.3 kb (Clouthier et al., 2002). The buoyant density of the complete virus particles in sucrose and cesium chloride gradients is 1.18 g/mL (Falk et al., 1997).

19.2.2.2 ISAV Genome and Coding Assignments

All eight RNA segments of ISAV have been sequenced (Mjaaland et al., 1997; Krossøy et al., 1999, 2001; Rimstad et al., 2001; Ritchie et al., 2001, 2002; Cunningham and Snow, 2000; Clouthier et al., 2002; Snow et al., 2003). Comparison

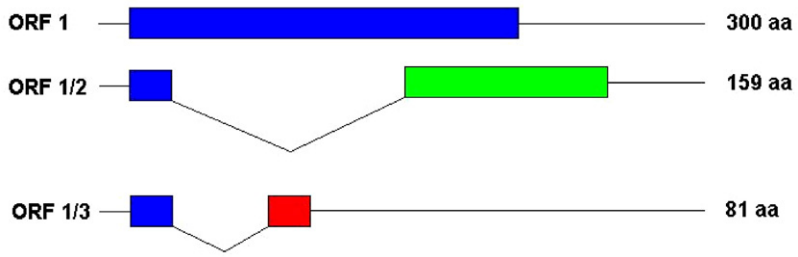

FIGURE 19.3 New gene expression model for segment 7 of ISAV of North American genotype. There are three ORFs, consisting of the primary transcript (7-ORF1) which is 300 amino acids long, and 7-ORF1/2 and 7ORF1/3, each with an intron removed from 7-ORF1 and consisting of 159 and 81 amino acids, respectively. *From Kibenge, F.S.B., Xu, H., Kibenge, M.J.T., Qian, B., Joseph, T., 2007a. Characterization of gene expression on genomic segment 7 of infectious salmon anaemia virus. Virol. J. 4, 34.*

of the ISAV proteins with those of other orthomyxoviruses revealed low amino acid identity values, between <13% and <25% (Krossøy et al., 1999; Kibenge et al., 2001b; Snow and Cunningham 2001; Ritchie et al., 2002). The most conserved proteins between ISAV and influenza virus A/PR/8/34 are PB1 proteins, although they share only 31% amino acid similarity (Mérour et al., 2011). Based on the initial demonstration of two 5'-coterminal mRNA transcripts by RT-PCR, ISAV genomic segment 7 was suggested to share a similar coding strategy with segment 7 of influenza A virus, encoding two proteins (Ritchie et al., 2002; Lamb et al., 1981). In a subsequent study, Kibenge et al. (2007a) showed that ISAV segment 7 encodes three proteins with estimated molecular masses of 32, 18 and 9.5 kDa. The 18-kDa and 9.5-kDa products are based on the removal of an intron each from the primary transcript (7-ORF1) so that the translation continues in the +2 and +3 reading frames, respectively (Fig. 19.3). The segment 7-ORF1/3 product is variably truncated in the sequence of ISAV isolates of the European genotype. All three proteins are recognized by rabbit antiserum against the 32-kDa product of the primary transcript, as they all share the N-terminal 22 amino acids (Kibenge et al., 2007a).

The functions of the orthomyxovirus proteins are summarized in Table 19.3, using genus *Isavirus* as an example.

Genetic reassortment in ISAV has been indicated by molecular and phylogenetic sequence analyses (Cottet et al., 2010; Devold et al., 2006; Markussen et al., 2008). The F protein of some ISAV strains contains insertions near the putative proteolytic cleavage site, acquired through nonhomologous recombination (Devold et al., 2006; Markussen et al., 2008; Kibenge et al., 2009). The virulent strains of ISAV have deletions in a highly polymorphic region (HPR) in the stem of the HE protein (designated as *ISAV-HPRΔ*). The low pathogenic or nonvirulent strains of ISAV (designated as *ISAV-HPR0*) have a full-length sequence (35 amino acids) of HPR (Cunningham et al., 2002) and are considered to have an ancestral relationship with ISAV-HPRΔ (EFSA, 2012). The deletions in HPR probably occur through homologous recombination (copy-choice recombination, presumably because of strand-switching by the vRNA polymerase (Castro-Nallar et al., 2011) during negative RNA strand synthesis from one nucleic acid template of one virus to another (which has been shown to occur in picornaviruses, coronaviruses, influenza virus, alphavirus, rotavirus and orbivirus). The novel ISAV strains (ISAV-HPRΔ) were suggested to be produced during intensive aquacultural practices (Mjaaland et al., 2005).

19.2.2.2.1 ISAV Conserved Terminal Nucleotide Sequences

RNA molecules of members of the family *Orthomyxoviridae* possess conserved and partially complementary 5'- and 3'-end sequences characteristic of each genus. These conserved terminal nucleotide sequences are therefore useful for orthomyxovirus classification. In *Influenzavirus A*, the first 12 nucleotides at the 3' end and the first 13 nucleotides at the 5' end of noncoding regions (NCRs) in all the vRNA segments are highly conserved (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980; de Wit et al., 2007; Bouvier and Palese, 2008; Wang and Lee, 2009). These partially complementary termini base pair form terminal panhandle structures (Hsu et al., 1987), which function as promoters by interacting with the viral polymerase complex during replication and transcription of vRNA (de Wit et al., 2007; Li and Palese, 1994; Hagen et al., 1994; Lee et al., 2003; Fodor et al., 1995; Luyjtes et al., 1989; Tchatalbachev et al., 2001). Moreover, the segment-specific NCR sequences may play important roles in virus virulence (Zheng et al., 1996) and in the rescue of influenza virus using the reverse genetics system (de Wit et al., 2007; Jackson et al., 2002). The sequences for the genomic ends of ISAV RNA have been reported only recently (Kulshreshtha et al., 2010; Mérour et al., 2011; Fourrier et al., 2011). To assist with comparison between different reports, we have adopted the following terminology: 5' and 3' untranslated regions (UTRs) when referring to the positive sense (cRNA), which correspond to 3' and 5' NCRs of the negative sense RNA segments

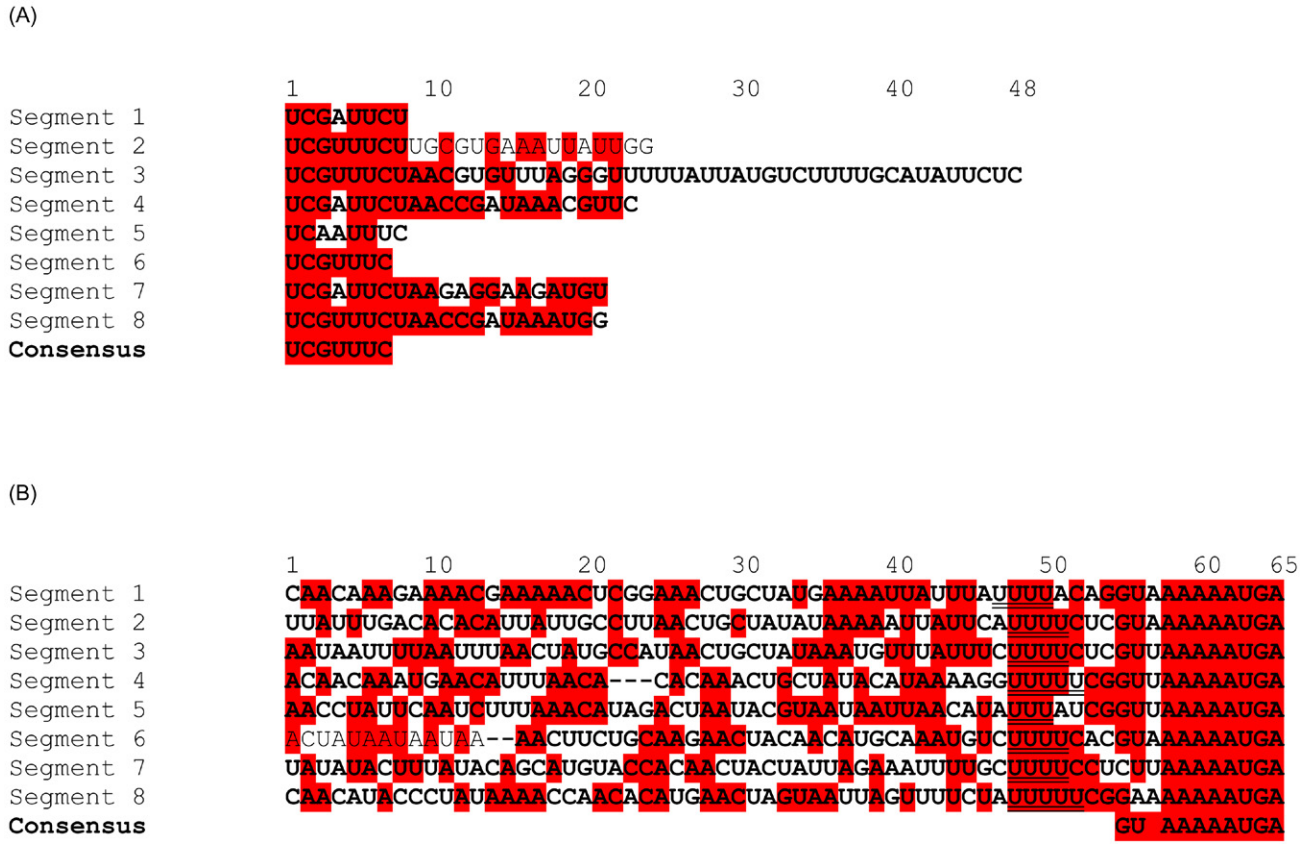

FIGURE 19.4 Alignment of the 3' and 5' noncoding regions of the eight genomic segments of the ISAV strain NBISA01 (GenBank Accession numbers HQ011270-HQ011277). Genome segment sequences were aligned manually. Sequences are in 3' to 5' orientation of vRNA. (Panel A) 3' end NCR. Only the noncoding sequences are shown up to the start codons (not shown). Sequences conserved between genome segments are in bold red (gray in print versions). Note the variation in length between different genome segments of the same virus (Kulshreshtha et al., 2010). (Panel B) 5' end NCR. Only the last 65 noncoding sequences are shown. Sequences conserved between genome segments are in bold red (gray in print versions). The polyadenylation signal sequences are double underlined.

(vRNA). The length of the 5' NCR in all eight ISAV segments is significantly longer than the corresponding 3' NCR. It is also variable in the different genome segments of the same ISAV strain and on the same genome segment in different ISAV strains, ranging from 67 nucleotides in segment 4 to 150 nucleotides in segment 3 (Table 19.3). Moreover, Kulshreshtha et al. (2010) was able to document the presence of intrasegment ISAV *quasispecies* based on sequence variation in the NCR sequences of the transcripts. However, the 3' terminal 7 nucleotides and the 5' terminal 11 nucleotides are highly conserved among the eight genomic segments in the different ISAV strains (3′-UCG$^{U}/_{A}$UUC--------GU$^{U}/_{A}$AAAA$^{A}/_{U}$UGA-5') (Fig. 19.4). In the case of TiLV, the conserved NCR sequences of its 10 genome segments (Bacharach et al., 2016; see Fig. 19.7) differ from those of ISAV, which is consistent with both viruses from different genera. Of note is the strict conservation of the terminal 3 and 4 nucleotides at the 3' and 5' ends, respectively, of vRNA in all orthomyxoviruses, which is indicative of a shared mode of replication requiring interaction of the viral ends with the polymerase complex (Fourrier et al., 2011).

19.2.2.2.2 ISAV Reverse Genetics System

Toro-Ascuy et al. (2015) reported for the first time the successful establishment of a plasmid-based reverse genetics system for the rescue of ISAV. The system corresponds to the 8+4 plasmid system for the reverse-genetics system for influenza A virus (Neumann et al., 1999). Thus, the full-length viral cDNA clones (having the entire coding and noncoding regions) of all eight ISAV RNA genomic segments were individually constructed into pUC57 under the control of the Atlantic salmon internal transcribed spacer region 1 (ITS-1) as a promoter flanked by the sequences of the hammerhead ribozyme in the 5' end and the hepatitis δ virus ribozyme in the 3' end, followed by the transcription terminator of the rabbit β-globin in

pSS-URG (for plasmid for *Salmo salar* universal reverse genetics) plasmid, creating eight genomic plasmids. In addition, four plasmids expressing the four proteins (PB2, PB1, PA and NP) of the ISAV ribonucleoprotein complex under the control of the cytomegalovirus (CMV) promoter were constructed. Transfection of ASK cells with the 12 plasmids resulted in the rescue of infectious recombinant ISAV (rISAV) 7 days posttransfection (Toro-Ascuy et al., 2015).

19.2.2.2.3 ISAV Strain Variations

The concept of ISAV strain variation has gained wide acceptance since Blake et al. (1999) and Inglis et al. (2000) first reported partial genomic sequence data of segments 2 and 8, showing significant differences between Canadian and Norwegian isolates (Kibenge et al., 2004). It is now well established that sequence analysis of all eight genomic segments from different ISAV isolates consistently reveals two genotypes designated according to their geographic origin as European (Genotype I) and North American (Genotype II) (Godoy et al., 2008). Genotype I can be further subdivided into Genogroup 1 (European–in-North America, EU-in-NA) for Genotype I strains also found in North America, and Genogroup 2 (Real European, Real-EU) for Genotype I strains only found in Europe and Chile (Kibenge et al., 2009). The ISAV of the European genotype can also be differentiated into three genogroups, EU-G1, EU-G2 and EU-G3; the EU-in-NA strains are placed in the EU-G2 group, and Real-EU strains are divided between the EU-G1 and EU-G3 groups (Nylund et al., 2007).

Because the two ISAV genotypes correlated to antigenic groupings, they were designated as HA subtypes (Kibenge et al., 2001b). The two HA subtypes of ISAV have nucleotide sequence identities of ≤80.4% and amino acid sequence identities of ≤88.2%, whereas within each subtype, both sequence identities are ≥90.7% (Marshall, 2003). For influenza A virus, which has up to 18 different HA subtypes, amino acid sequence identities for isolates belonging to different subtypes can range from 25% to 80% (Nobusawa et al., 1991; Kawaoka, 1991; Wright et al., 1995; Shekel and Wiley, 2000). Thus, the HA protein of ISAV shows less variation than that of the influenza A virus. Limited virus neutralization tests with rabbit antisera to ISAV isolates of Real-EU genogroup (Genotype I, Genogroup 2) and North American genotype (Genotype II) showed at least three HA subtypes of ISAV: (1) North American, (2) Real-EU and (3) EU-in-NA (Kibenge et al., 2004). Alignment of deduced amino acid sequences in the HE protein of selected ISAV isolates identified the putative antigenic motif around the common potential N-glycosylation site at amino acid positions ^{333}NIT^{335} with one mutation site at positions 320 to 323 on one side and a second mutation site at positions 339 to 367, between European and North American genotypes. The HE protein of ISAV isolates of EU-in-NA genogroup has an additional unique potential N-glycosylation site at amino acid positions ^{362}NQT^{364} (Marshall, 2003). The presence of the additional carbohydrate chain in this region may be the basis for a new epitope accounting for EU-in-NA genogroup being antigenically distinct from the Real-EU genogroup (Kibenge et al., 2004).

Strain designation of ISAV is currently based on sequence deletions in the 35-amino acid HPR of the HE protein encoded on segment 6. The virulent strains of ISAV, designated ISAV-HPRΔ, have deletions in HPR of the HE protein and are named numerically from HPR1 to ≥HPR30, with two of them (HPR20 and HPR21) of North American genotype and the rest of them of European genotype (Nylund et al., 2007; Kibenge et al., 2009). The low pathogenic or nonvirulent strains of ISAV designated ISAV-HPR0, have a full-length sequence (35 amino acids) of HPR (Cunningham et al., 2002) and are considered to have an ancestral relationship with ISAV-HPRΔ (EFSA, 2012). Using multiple alignment and phylogenetic analysis of segment 6 sequences from all ISAV-HPR0 viruses reported worldwide, Godoy et al. (2013) identified three genomic clusters, which correlated with three residue patterns of ISAV-HPR0 (^{360}PST^{362}, ^{360}PAN^{362} and ^{360}PAT^{362}) in HPR. The ISAV-HPR0 residue pattern ^{360}PAT^{362} is the only type of ISAV-HPR0 found in Chile. The pathogenic ISAV isolates also have a mutation at the proteolytic cleavage site (^{267}R) of the F protein (Markussen et al., 2008; Kibenge et al., 2009). This mutation, relative to the F protein of ISAV-HPR0, is either a single amino acid change from glutamine (^{226}Q) to lecine (^{226}L) or a peptide insert, named numerically from IN1 to IN5 (Godoy et al., 2014). IN1 to IN3 have been detected in Norwegian isolates (Devold et al., 2006) and IN4 and IN5 only in Chilean isolates (Kibenge et al., 2009; Godoy et al., 2014). Thus, there is still a need to devise an ISAV strain designation system that takes into account both envelope proteins (HE and F), which have been successfully used in genotyping of ISAV (Kibenge et al., 2009). Godoy et al. (2014), used concatenated ISAV F and HE genes of one representative isolate from every geographical region where ISA has been reported (ISAV isolates chosen: 98-049-1 represents North America; 04-085-1 represents EU-in-NA; 810/9/99 represents Norway I; 93/09/2264 represents Norway_II_1; SK779/06 represents Norway_II_2; U24636 represents Chile; and 390/98 represents Scotland) and constructed a phylogenetic tree that revealed the relationship between the different geographical isolates and possible transmission routes among the regions.

ISAV isolates could also be grouped into three phenotypes based on their ability to grow and cause cytopathic effects (CPEs) in CHSE-214 cells (Munir and Kibenge, 2004), but the molecular basis for this phenotypic variation has not yet been determined. The different ISAV isolates also vary in the molecular sizes of their polypeptides (Kibenge et al., 2000; Griffiths et al., 2001), as do influenza viruses (Kendal, 1975).

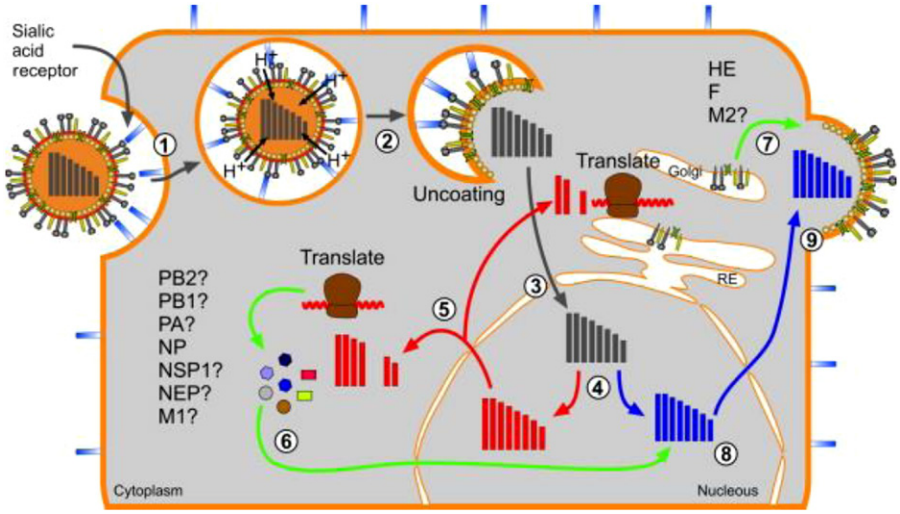

FIGURE 19.5 Schematic representation of the ISAV infection of a cell of fish. They are the most important events associated with this process and also indicate the proteins that are generated during infection. The proteins with a question mark are those in which its putative role is assumed by comparison with influenza. *From Cottet, L., Rivas-Aravena, A., Martin, M., C.-S., Sandino, A.M., Spencer, E., 2011. Infectious salmon anaemia virus – genetics and pathogenesis. Virus Res. 155, 10–19. Figure 3. Copyright © Elsevier B.V. (2010), with permission.*

19.2.2.3 ISAV Replication

19.2.2.3.1 Virus Replication Cycle

The replication strategy of ISAV resembles that of influenza viruses (Kibenge et al., 2004). The influenza viruses attach via their HA protein to host-cell receptors terminating in sialic acids (Zambon, 2001). Hemagglutination assays with erythrocytes from several fish species have indicated that ISAV also uses its HA protein to bind sialic acid receptors on the cell surface (Falk et al., 1997; Eliassen et al., 2000; Kristiansen et al., 2002; Workenhe et al., 2007). Structurally diverse sialoglycoproteins, as well as certain unique sialoglycoproteins, have been reported to occur in salmonid species (Iwasaki et al., 1990). Because ISAV agglutinates horse erythrocytes, and since these contain only N-glycol neuraminic acid, it has been suggested that this may be the cellular receptor for ISAV (Kristiansen et al., 2002). It was subsequently demonstrated that 4-*O*-acetylated sialic acid (Neu4,5Ac$_2$) serves both as a substrate for the receptor destroying enzyme, a sialate-4-*O* esterase, and as a receptor determinant for virus binding (Hellebø et al., 2004); hence, the designation of the ISAV HA protein encoded on segment 6 as a HA-esterase (HE) (Falk et al., 2004). The 4-*O*-acetylated sialic acid was recently shown to be the preferred receptor for ISAV in vivo as well (Aamelfot et al., 2012). Eliassen et al. (2000) and Aspehaug et al. (2005) demonstrated the internalization of ISAV into the endosomes and low pH-dependent fusion of the viral envelope with the endosomal membrane, releasing the viral genome. The subsequent events in the ISAV infection cycle in a fish cell, including translocation of viral ribonucleoprotein complexes to the nucleus, transcription and replication of the viral genome in the nucleus, translation of viral transcripts in the cytoplasm, transport of the proteins to the nucleus and the surface GPs through the secretory pathway from the endoplasmic reticulum, through the Golgi apparatus to the plasma membrane, formation of the ribonucleoprotein complex in the nucleus and subsequent transportation to the budding site of the plasma membrane with the surface GPs (Fig. 19.5), were reviewed by Cottet et al. (2011). ISAV replicates in cell culture without trypsin treatment. Moreover, unlike in influenza viruses, cleavage of the HA protein does not occur in ISAV (Krossøy et al., 2001). In influenza A viruses, amantadine specifically inhibits the M2 ion channel activity, and therefore viral ribonucleoprotein complexes are not released into the cytoplasm. However, amantadine did not have any effect on ISAV replication (Falk et al., 1997).

19.2.2.3.2 In Vitro Virus Replication

Permissive fish cell lines for ISAV include salmon head kidney (SHK-1) cells (Dannevig et al., 1995b), TO cells (Wergeland and Jakobsen, 2001) and Atlantic salmon kidney (ASK-2) cells (Devold et al., 2000) which are macrophagelike cell lines in which virus replicates with production of CPEs. Although all three cell lines are derived from the Atlantic salmon pronephros, they are distinctly different in their growth characteristics (media requirements, split ratio, cell composition), they

display distinctly different viral-induced CPEs and also interact differently with virus-antibody mixtures during the virus neutralization test (Kibenge et al., 2004). The SHK-1 and ASK-2 cell lines are widely used for primary isolation of ISAV; however, at higher passages, they seem to lose sensitivity for ISAV, resulting in poorly defined and slow developing CPEs by some ISAV strains. Some strains of ISAV can also replicate and cause CPEs in the Chinook salmon embryo (CHSE-214) cell line (Bouchard et al., 1999; Kibenge et al., 2000; Griffiths et al., 2001) while others do not replicate in this cell line at all (Munir and Kibenge, 2004), thereby limiting their utility in virus isolation (Kibenge et al., 2000). Moreover, for cytopathic strains, the CPEs in CHSE-214 cells develop slowly, taking up to 17 days p.i. compared to 12 days p.i. in SHK-1 cells, and the virus yields are also lower ($10^{4.5}$ to $10^{6.5}$ $TCID_{50}$/mL) than on the SHK-1 cell line (Kibenge et al., 2004). TO (Wergeland and Jakobsen, 2001) and ASK-2 (Devold et al., 2000) cell lines develop more easily discernible CPEs in a shorter time (by 2–4 days p.i.) than do SHK-1 and CHSE-214 cells and give higher virus yields (as high as $10^{9.1}$ $TCID_{50}$/mL, Wergeland and Jakobsen, 2001; Grant and Smail, 2003). However, they do not recover viruses from some ISAV RT-PCR-positive samples (Kibenge et al., 2001a; Rimstad and Mjaaland, 2002; Mjaaland et al., 2002), indicating that the currently available fish cell lines are not permissive for all ISAV strains. Interestingly, Aamelfot et al. (2012) found the preferred ISAV cellular receptor only in the Atlantic salmon macrophagelike cell lines (ASK-2, SHK-1 and TO), but not in the CHSE-214 cell line. SHK-1 and ASK cell lines can be used in combination to provide an enhanced ability to detect ISAV (Rolland et al., 2005). Virus replication also occurs in Atlantic salmon (AS) (Sanchez et al., 1993; Sommer and Mennen, 1997) and rainbow trout gill (RTgill-W1) cell lines (Bols et al., 1994; Falk et al., 1997); but in these cases, the virus is noncytopathic and is detected by indirect fluorescent antibody testing (IFAT). The virus yield is very poor; the maximum viral titer reported in these cells was 10^3 $TCID_{50}$/mL (Falk et al., 1997). Virus replication on FH-10, BB, EPC and BF-2 cell lines has also been attempted, but these cell lines appear to be refractory to ISAV (Falk et al., 1997; Byrne et al., 1998); the ISAV cellular receptor was not found in the EPC and BF-2 cell lines (Aamelfot et al., 2012).

Two general pathways known to cause CPE and eucaryotic cell death during virus infection are apoptosis and necrosis. In a study using the permissive fish cell lines SHK-1, CHSE-214 and TO, Joseph et al. (2004) observed apoptosis only in ISAV-infected SHK-1 and CHSE-214 cells. ISAV-infected TO cells did not undergo apoptosis but showed leakage of high mobility group 1 (HMGB1) protein from the nucleus, which is characteristic of cells undergoing necrosis (Scaffidi et al., 2002), suggesting that CPEs in these cells are associated with necrosis. ISAV-infected SHK-1 cells did not show leakage of the HMGB1 protein. Infection with two different strains of ISAV showed that the induction of apoptosis correlated with the appearance of CPEs in SHK-1 cells. The ISAV-induced apoptosis was inhibited by a pan-caspase inhibitor Z-VAD-fmk, indicating a caspase activation pathway. The ISAV putative PB2 protein and proteins encoded by RNA segment 7 specifically bound caspase-8 in vitro, suggesting that these viral proteins may have a role in the ISAV-induced apoptosis. These findings demonstrated for the first time that the mechanism of cell death during ISAV infection was dependent on the cell type, which might have implications for ISAV pathogenesis and persistence. It was recently shown that ISAV infection promotes apoptosis of SHK-1 cells through ROS/p38 MAPK/Bad signaling pathway (Olavarría et al., 2015).

19.2.2.3.3 Virulence of ISAV

Several theories have been put forward to explain the geographic and host origin, and therefore the virulence, of ISAV in farmed fish (Nylund et al., 1995b, 1997, 2003). For example, it was suggested that ISAV may have been introduced to Norway with the importation of rainbow trout from North America (Krossøy et al., 2001). It has been suggested that there are natural reservoirs for the virus, probably in fish occurring in the coastal areas where ISA outbreaks frequently occur (Mullins et al., 1999). The virulence of ISAV is intrinsically linked to its emergence as a pathogen in marine-farmed Atlantic salmon. Thus, it is more likely that the emergence of ISAV as a fish pathogen is a reflection of variation in virus-host interactions similar to the situation with avian influenza in poultry. For example, domestic poultry (of the order Galliformes), are not the natural hosts of avian influenza viruses (Perdue et al., 1999; Suarez and Schultz, 2000). However, people have altered the epidemiological variables of avian influenza viruses by creating new ecological niches via bird captivity and domestication, industrial agriculture, national and international commerce and nontraditional raising practices (Swayne, 2000). In these new ecosystems, pathogenic and nonpathogenic microorganisms can be transmitted within and between avian species (or fish species, in the case of ISAV) and adapt to new host species.

For both European and North American genotypes of ISAV, a direct functional relationship can be demonstrated between the length of HE protein stem, ISAV cytopathogenicity in cell culture and pathogenicity for Atlantic salmon (Kibenge et al., 2006, 2007b, 2009). It is now well established that the systemic disease ISA is caused by virulent ISAV strains (designated as *virulent ISAV-HPRΔ*) with deletions in the highly polymorphic region (HPR) spanning residues ^{337}V to M^{372} in the stem of the HE protein; these virus strains also have either an insertion or the ^{266}Q→^{266}L mutation in the *F* gene (Kibenge et al., 2009). ISAV virulence in ISAV-HPRΔ may be attenuated by the absence of the insertion in the *F* gene (Godoy et al., 2014).

However, while the deletion in HPR may be a good genetic marker for differentiation (Ritchie et al., 2009), it is not necessarily the virulence determinant for ISAV. Direct evidence for how the deletion in HPR affects virulence will be possible now that a reverse genetics system for ISAV has been developed (Toro-Ascuy et al., 2015).

Noncultivable, nonpathogenic ISAV detectable only by RT-PCR has full-length HPR sequence (35 amino acids) in the HE-encoding gene and is designated ISAV-HPR0 (Cunningham et al., 2002). ISAV-HPR0 viruses have been variously reported in the research literature (Godoy et al., 2014). All ISAV-HPR0 isolates are characterized by the wild-type F protein with Q^{266} and no sequence insertion (Markussen et al., 2008). ISAV-HPR0 replicates mainly in Atlantic salmon gills, causing only transient subclinical infection (Christiansen et al., 2011). This virus has been detected in apparently healthy wild and farmed Atlantic salmon in most regions with Atlantic salmon aquaculture (Godoy et al., 2013) and is considered to have ancestral relationship with ISAV-HPRΔ (EFSA, 2012). ISAV-HPR0 viruses are detected late during ISA outbreaks and persist long after the disease is contained or eradicated (Kibenge et al., 2012b, 2009; McBeath et al., 2009; Christiansen et al., 2011). Most recently, ISA cases directly linked to the presence of endemic ISAV-HPR0 have been reported (Godoy et al., 2013), supporting the notion that under appropriate conditions, ISAV-HPR0 can mutate to ISAV-HPRΔ.

19.2.3 Clinical Features, Pathology and Epidemiology

19.2.3.1 Clinical Manifestation

The clinical features and epidemiology of ISA were reviewed by Kibenge et al. (2004). Clinical signs of ISA in farmed Atlantic salmon include anorexia, lethargy and anemia. Mortality in a fish cage on a fish farm rises slowly and can vary from 0% to 90% (cumulative mortalities in Atlantic salmon during natural ISA outbreaks and experimental infections range from 0% to100%). The disease course is prolonged with low daily mortality (0.05–0.1%), typically only in a few cages. Virus may be present in a fish cage for up to six months before significant mortality is noted. Stressful events such as handling of fish (eg, during sorting, treatment, splitting or moving of cages) may initiate disease outbreaks on infected farms. The dead fish have exophthalmos (bulging, bloodshot eyes), pale gills due to anemia, blood-tinged fluid in peritoneal and pericardial cavities (ascites), congestion of gut, enlargement of liver and spleen and petechial hemorrhages of the internal organs and membrane lining the abdominal cavity (Fig. 19.6). Histopathology lesions include intestinal mucosal ulceration and hemorrhage, liver hemorrhage and hepatocyte necrosis, erythrophagocytosis in the spleen and kidney, renal interstitial congestion, hemorrhage and tubular necrosis and congestion of branchial lamellar and filamental vessels (Byrne et al., 1998; Mullins et al., 1998; Thorud and Djupvik 1988; Evensen et al., 1991; Godoy et al., 2008).

19.2.3.2 Host Range

ISAV is known to cause overt and fatal systemic infection in farmed Atlantic salmon and asymptomatic infection in feral fish, a situation analogous to avian influenza viruses in domestic poultry and wild birds. The clinical ISA disease can also occur in wild, free-ranging Atlantic salmon, but these fish are less susceptible than the farmed Atlantic salmon either due to genetic variation in the two fishes or due to stress caused by management practices on the salmon farms (Nylund et al., 1995b). Experimental infection of *Oncorhynchus* spp., Arctic char (*Salvelinus alpinus*) and herring (*Clupea harengus*) with ISAV resulted only in subclinical infection (Nylund et al., 1994, 1995b, 1997, 2002; Nylund and Jakobsen 1995; Rolland and Nylund 1999; Devold et al., 2001). The Pacific salmonid species, chum (*Oncorhynchus keta*), steelhead (*Oncorhynchus mykiss*), Chinnook (*Oncorhynchus tsawytscha*) and coho (*Oncorhynchus kisutch*) were also found to be resistant to experimental infection with ISAV, even at doses as high as 10^8 TCD_{50}/mL that induced 98% mortality in Atlantic salmon (Rolland and Winton, 2003). Although attempts to isolate the virus from some of these fishes have not been successful, the virus can be detected by RT-PCR (Kibenge et al., 2004; Plarre et al., 2005). Detection of ISAV by RT-PCR has also been reported in the tissues of pollock (*Pollachius virens*) and Atlantic cod (*Gadus morhua*), but only in fish collected from cages with ISA-diseased Atlantic salmon (Kibenge et al., 2004). Pollock were shown to be able to eliminate the virus within a week of experimental infection (Snow et al., 2002). Kibenge et al. (2006) reported that coho salmon (*O. kisutch*) can remain asymptomatic while being infected with strains of ISAV that are highly pathogenic to Atlantic salmon. Such benign infections in the wild fishery are considered to be the source of virulent strains that cause clinical disease in marine-farmed Atlantic salmon (Murray et al., 2002). The only other fish species conclusively shown to develop clinical disease and die due to ISAV infection are rainbow trout (*O. mykiss*) and Amago trout (*Oncorhynchus masou macrostomus*, an indigenous fish of Japan). In the case of rainbow trout, percentage mortalities of 10–50% were obtained following intraperitoneal (i.p.) injections with highly pathogenic ISAV isolates (NBISA01 and RPC/NB 98-049-1 of North American genotype and 810/9/99 of European genotype) that caused 80–100% mortality in Atlantic salmon (Kibenge et al., 2006; MacWilliams et al., 2007). Of the gross

FIGURE 19.6 Common gross lesions seen at necropsy: (Top panel) Atlantic salmon with exophthalmia and pale gills. (Middle panel) Atlantic salmon with petechial hemorrhages on the abdomen. (Bottom panel) Atlantic salmon with very dark liver and hemorrhages on the visceral adipose tissue. *From Godoy, M., Aedo, A., Kibenge, M., Groman, D., Yason, C., Grothusen, H., Lisperguer, A., Calbucura, M., Avendaño, F., Imilán, M., Jarpa, M., Kibenge, F., 2008. First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. BMC Vet. Res. 4, 28. Figure 2B. Copyright © Panphut et al. (2011), with permission* http://www.biomedcentral.com/download/license.pdf *no changes were made.*

lesions reported for ISA in Atlantic salmon, only visceral adipose petechiation and ascites were commonly observed among the rainbow trout mortality (MacWilliams et al., 2007). Microscopic lesions in these rainbow trout included increased erythrophagia, clusters of cellular degeneration in the hematopoietic portion of the kidney and occasionally epicarditis, endocarditis and myocarditis. These lesions are very different from the typical necrosis in liver and kidney that occur in infected Atlantic salmon, and if they were viewed in rainbow trout under field conditions, it is unlikely that ISA would have been considered as a differential diagnosis (MacWilliams et al., 2007). Biacchesi et al. (2007) obtained 80–100% mortality of genetically susceptible juvenile rainbow trout i.p. injected with ISAV isolate Glesvaer/2/90 of European genotype. The moribund fish displayed typical pathological signs of ISA, including exophthalmos, pale gills and abdominal congestion (Biacchesi et al., 2007). Cumulative mortalities of 95% and 20% occurred in i.p. injected Atlantic salmon and amago trout, respectively, with ISAV isolate Glesvaer/2/90 (Ito et al., 2015). However, there was no evidence of horizontal transmission

of ISAV from moribund amago trout to coreared juvenile Atlantic salmon, although ISAV was detected by RT-PCR in surviving amago trout (Ito et al., 2015).

Aamelfot et al. (2012) demonstrated that only Atlantic salmon macrophagelike cell lines (ASK-2, SHK-1 and TO) have the ISAV cellular receptor, hence, their permissiveness for ISAV replication. These authors further investigated the presence of the ISAV cellular receptor on cells from a large number of marine and freshwater fish species with a view of identifying the ISAV host range in wild fish (Aamelfot et al., 2014). The study found that all salmonids expressed the receptor, but only some of the codlike and perchlike fish did, whereas all flat fish tested negative for the ISAV receptor (Aamelfot et al., 2014). However, the fact that CHSE-214 cells (which are permissive to ISAV isolates of North American genotype, Kibenge et al., 2000) do not have the ISAV receptor (Aamelfot et al., 2012) suggests that ISAV may use more than one cellular receptor (ie, the natural host for ISAV may include both salmonid and nonsalmonid species).

ISAV does not infect humans or other mammals since the virus is inactivated at pH values below 4.5 (and human gastric secretions are at pH 2.0) and does not replicate *in vitro* at temperatures of 25°C or above (Falk et al., 1997), and it does not appear able to replicate in human cells (Anonymous, 2000).

19.2.3.3 Transmission

ISA virus is stable in both freshwater and seawater, and horizontal transmission has been demonstrated by cohabitation of healthy and ISAV-injected fish (Trojans) (Simko et al., 2000; Mikalsen et al., 2001), with the healthy fish becoming infected by coming in contact with biological material contaminated with ISAV because of coprophagy, which in sea farms occurs due to crowding (reviewed by Cottet et al., 2011). Transmission between aquaculture farms can be linked to fish farming practices such as movement of feed boats and well boats carrying fish, and in ballast water, sharing of personnel and equipment (nets, barges, etc.), movement of infected fish, as well as movement of virus through the water column due to proximity to fish slaughterhouse processing plants or other fish farms. Recovered farmed Atlantic salmon can become carriers; 30% of ISA outbreaks in Norway were attributed to other farms in proximity. Aldrin et al. (2011) used seaway distances between farms and genetic distances between ISAV isolates of farms in Norway with ISA between 2003 and summer 2009 to predict the rate of ISA infection of salmon farms. The fitted model predicted that the risk of infection from a neighborhood infectious farm decreased with increasing seaway distance between the two farms. Furthermore, for a given infected farm with a given ISAV genotype, the source of infection was significantly more likely to be ISAV of a small genetic distance than of moderate or large genetic distances. Nearly half of the farms with ISA in the investigated period were predicted to have been infected by an infectious farm in their neighborhood, whereas the remaining half of the infected farms had unknown sources.

Sea lice (*Lepeophtheirus salmonis*) may serve as the mechanical vector (Nylund et al., 1994). Migratory wild salmonids (eg, Atlantic salmon, rainbow trout, brown trout, and sea trout) may be subclinically infected and serve as carriers, particularly in virus spread over long distances (ASF, 2012), similarly to migratory wild birds spreading avian influenza viruses.

In Chile, the involvement of ISAV in a disease outbreak was officially verified in Atlantic salmon in mid-June 2007 (Godoy et al., 2008), where all isolates that were obtained from outbreaks and had their segment 6 sequence belonging to ISAV-HPR7b, similar to isolates from Norway, but had acquired a mutation consisting of a 33 base-pair insert in their segment 5 sequence (Kibenge et al., 2009). Phylogenetic analyses of the Chilean ISAV isolates from different outbreaks suggested that the virus was introduced from Norway in 1996 (Kibenge et al., 2009), probably through fertilized salmon eggs (Vike et al., 2009; Cottet et al., 2010). Vertical transmission of ISAV was recently reported to occur with virulent ISAV-HPRΔ (Marshall et al., 2014), although it may be of little significance in the epidemiology of ISAV infection (Rimstad et al., 2007).

Several reports have described the main risk factors associated with fish farms getting ISA outbreaks (Jarp and Karlsen, 1997; Hammell and Dohoo, 2005; Aldrin et al., 2010; Mardones et al., 2009, 2011).

19.2.4 Pathogenesis and Immunity

It is now well established that in Atlantic salmon, ISAV specifically targets endothelial cells lining the blood vessels in all organs (Falk and Dannevig, 1995; Koren and Nylund, 1997; Aamelfot et al., 2012), macrophages (Moneke et al., 2003) and red blood cells (Workenhe et al., 2008; Aamelfot et al., 2012). Thus, virus replication occurs in several organs particularly the heart, liver, head kidney and spleen, causing a generalized infection of the vascular system. Virus enters primarily via gills (Totland et al., 1996), but it also occurs through the mouth (Mikalsen et al., 2001). Atlantic salmon gill epithelial cells express 4-*O*-acetylated sialic acid, the preferred ISAV receptor, and the virus yield in primary gill epithelial cell cultures is comparable with that of SHK-1 cells (Weli et al., 2013). The most extensive and prolonged virus replication is in heart

tissue (Moneke et al., 2003, 2005). The virus is rapidly disseminated via the circulatory system (Nylund et al., 1995a; Rimstad et al., 1999). The nonpathogenic ISAV-HPR0 replicates mainly in Atlantic salmon gills, causing only transient subclinical infection (Christiansen et al., 2011). In contrast to the virulent ISAV-HPRΔ that targets endothelial cells, ISAV-HPR0 was shown to replicate only in the epithelial cells of Atlantic salmon gills (Aamelfot et al., 2016).

Studies on the pathogenesis of ISA showed that histopathological lesions appeared at about 12–18 days postinoculation in experimentally infected fish, coinciding with onset of mortality (Dannevig and Falk, 1994; Speilberg et al., 1995). Moneke et al. (2005) used *in situ* hybridization (ISH) with a riboprobe targeting ISAV segment 7 mRNA to study the correlation of virus replication in tissues with histologic lesions in Atlantic salmon experimentally infected with ISAV. Severe histopathological lesions were observed in tissues beginning at the onset of mortality and correlated with maximum intensity and frequency of ISH signals, particularly in the liver, kidney and heart. The distribution of ISH signals indicated the presence of a viremia, as signals were observed predominantly in individual blood cells and endothelial cells, and possibly hematopoietic cells of head kidney, but not in the necrotic hepatocytes and renal epithelium. Of the organs sampled, the heart was the first to show ISH signals, and the signals lasted longest in the heart, possibly due to increased activity of the endocardial endothelial cells and the underlining macrophages, which continuously trap and remove circulating virus, and therefore represents the best tissue sample for screening of suspected infected fish (Moneke et al., 2005). Kibenge et al. (2006) compared the infectivity of different ISAV isolates at a dose of 10^6 $TCID_{50}$/0.2 mL/fish given intraperitoneally in three different farmed fishes (Atlantic salmon, coho salmon and rainbow trout) and found that the most virulent strains caused the highest mortalities in Atlantic salmon (>95% mortality) with the shortest duration (9–12 days). These highly pathogenic ISAV strains also caused mortality in rainbow trout with systemic hemorrhagic lesions and an ISH pattern with ISAV riboprobes that were consistent with those typically seen in Atlantic salmon infected with ISAV (MacWilliams et al., 2007).

Bony fish represent a transition point on the phylogenetic spectrum between species possessing only innate immunity (ie, invertebrates) and species depending heavily on adaptive immunity (ie, mammals). Studies of host response to virus infections suggest that mammals and bony fish are more or less similar in the major mechanisms of both innate and adaptive immune responses against viruses (Workenhe et al., 2010). ISAV targets Atlantic salmon vascular endothelial cells and macrophages, is known to induce the innate type I IFN system, as well as adaptive immune response genes of Atlantic salmon (Jørgensen et al., 2008; LeBlanc et al., 2010; Lauscher et al., 2011); and ISAV isolates have strain-specific variations in their ability to induce immune response genes (Workenhe et al., 2009; Svingerud et al., 2013), although the immune responses mounted do not restrict the replication of the virus (Kileng et al., 2007). A luciferase reporter assay utilizing Atlantic salmon Mx protein and minimal IFN promoter and transient expression of ISAV proteins showed that segment 7 ORF1 and segment 8 ORF2 proteins are major- and minor-IFN system antagonizing proteins, respectively (McBeath et al., 2006; Garcia-Rosado et al., 2008). Hetland et al. (2010) showed differences in the distribution of MHC I, MHC II and CD8 cell populations between control and ISAV-infected salmon that support the proposition that ISAV, as with influenza virus, activates CD8-positive T-cell responses.

Atlantic salmon antibody response to ISAV studied by Western blotting revealed that ISAV antibodies bound exclusively to the viral nucleoprotein (Falk and Dale, 1999) and HA protein (Clouthier et al., 2002). The discovery of antibody-mediated uptake and replication of ISAV in macrophagelike fish cell lines (Joseph et al., 2003) suggests that Fc receptor-mediated antibody-dependent enhancement of the ISA virus infection might also occur in vivo, as the virus in Atlantic salmon targets endothelial cells lining blood vessels and macrophagelike cells. However, the immune adaptive response against ISAV following clinical disease appears to provide full protection against reinfection by different ISAV strains (Ritchie et al., 2009). In contrast, it is not known if infection with ISAV-HPR0 viruses offers immunological protection against new infections with virulent ISAV-HPRΔ.

19.2.5 Diagnostic Methods

The laboratory diagnostic assays for ISA include histological examination (Evensen et al., 1991; Speilberg et al., 1995; Byrne et al., 1998) and electron microscopy (Hovland et al., 1994) of fish tissues, the isolation of ISAV in SHK-1 (Dannevig et al., 1995a,b), ASK (Rolland et al., 2005) and CHSE-214 cell lines (Bouchard et al., 1999; Kibenge et al., 2000) and electron microscopic examination of a positive isolate, the use of RT-PCR (Mjaaland et al., 1997; Devold et al., 2000) and IFAT on positive virus isolates or on tissue samples from suspected fish (Falk and Dannevig, 1995; Falk et al., 1998) and ISH on tissue samples from suspected fish (Gregory, 2002; Moneke et al., 2003). In addition, an indirect enzyme-linked immunosorbent assay (ELISA) and indirect competitive ELISA that could detect ISAV-specific antibodies in the sera of infected and vaccinated fish have been described (Kibenge et al., 2002). An ultrasensitive real-time nucleic acid sequence-based amplification (NASBA) method for ISAV was reported by Starkey et al. (2006). A one-tube real-time RT-PCR for ISAV using SYBR Green chemistry was first described by Munir and Kibenge (2004). The assay had a detection limit of 0.006 ng

of ISAV RNA, could be completed in 80 min, and provided the potential to quantitate even noncytopathic virus in cell cultures. Several real-time RT-PCRs using TaqMan chemistry have been reported since then (Plarre et al., 2005; Snow et al., 2006; Workenhe et al., 2008; Kibenge et al., 2011; Christiansen et al., 2011). Godoy et al. (2010) used the real-time RT-PCR with TaqMan chemistry to detect vRNA in formalin-fixed paraffin-embedded tissues. For the ISAV TaqMan assay, the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2014) recommends primer-probe sets targeting ISAV segments 7 and 8 developed by Snow et al. (2006). Most diagnostic laboratories use the Snow et al. (2006) primer-probe set targeting segment 8 (Kibenge et al., 2011, 2012a). Other laboratories and researchers have found the Plarre et al. (2005) primer-probe set targeting segment 7 to be more sensitive. Other researchers have published ISAV segment 8 primer-probe sets that are highly sensitive (Christiansen et al., 2011) but are not included in the OIE Manual (OIE, 2014). Most recently, it was suggested that using ISAV segment 3 real-time RT-PCR may be a more sensitive method of virus detection and quantitation (Valenzuela-Miranda et al., 2014). Routinely, once a clinical sample has been determined to be positive for ISAV, conventional RT-PCR with segment 6 primers (Kibenge et al., 2009) is commonly used to obtain a PCR product that is sequenced in order to determine the HPR type and genotype of the ISAV present.

Single-agent diagnostic assays such as singleplex PCR/RT-PCR that are used for pathogen surveillance/screening programs are severely limited since they are based on known pathogen nucleic acid sequence information. These assays are good in a characteristic disease outbreak or in situations suggestive of infection with one known pathogen but are not ideal in the absence of clinical signs or in situations where a particular disease is not known to occur, such as ISA in Chile prior to June 2007 (Kibenge et al., 2012a).

19.2.6 Prevention and Control

19.2.6.1 Control

The methods used to control the spread of ISAV following an ISA outbreak have improved with increased knowledge about the epidemiology of ISA. For example, in 2005 the Faroe Islands dealt with ISA outbreaks by depopulating and fallowing all seawater sites and restocking under new regulatory management procedures. All countries where ISA has occurred now manage an ISA surveillance program, have restrictions on transportation and movements of farmed fish, have "all in-all out" (year-class separation or generation segregation) fish farming with fallowing periods and zone management, mandatory depopulation and disinfection of infected farms with fallowing; and they also have regulations on disinfection of wastewater from fish slaughterhouses and of intake water to hatcheries (Rimstad et al., 2007, 2011; Alvial et al., 2014; Aamelfot et al., 2014). Smail et al. (2004) reported six commercially available disinfectants efficacious against ISAV for the disinfection of fish farming equipment or fish ova.

19.2.6.2 Regulatory Measures

The OIE, which provides international standards and guidelines for safe trade in animals and their products, has a list of notifiable aquatic animal diseases (those with a risk of spread through trade in aquatic animals and their products) that includes infection with ISAV-HPR-deleted or ISAV-HPR0 (OIE, 2015). Because the discovery of a reportable disease is subjected to OIE trade standards, the presence of either ISA or ISAV infection often leads to expensive eradication or control efforts, and suspect populations may be quarantined until a definitive diagnosis can be made. Good management and increased biosecurity by fish farm managers in combination with early detection of infection through enhanced surveillance and use of rapid and sensitive diagnostic methods are key to preventing ISAV spread. Due to the increase in demand for international trade of live, fresh and frozen fish, areas previously unaffected by ISAV are increasingly exposed to the potential spread of the virus. The OIE currently has three levels of official confirmation of infection with ISAV (OIE, 2014):

- *Confirmation of ISA:* The following criteria should be met: detection of ISAV in tissue preparations by IHC on fixed sections or IFAT on tissue imprints or fixed sections in addition to either (1) isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm or (2) detection of ISAV by RT-PCR by the methods described in the relevant sections of the OIE Manual (OIE, 2014).
- *Confirmation of HPR-deleted ISAV infection:* The following criteria should be met: (1) isolation and identification of ISAV in cell culture from any fish sample on the farm or (2) isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm with corroborating evidence of ISAV in tissue preparations using either RT-PCR or IFAT/IHC.
- *Confirmation of HPR0 ISAV infection:* The following criteria should be met: detection of ISAV by RT-PCR followed by independent amplification and sequencing of the HPR of segment 6 to confirm the presence of HPR0 only.

19.2.6.3 Vaccines and Vaccination

Vaccination against ISAV was reviewed by Kibenge et al. (2012b). Control of ISA by vaccination has been used in North America (since 1999), Faroe Islands (since 2005), in most parts of Norway (since 2009) and in Chile (since 2010), although the currently available vaccines do not seem to offer complete protection in Atlantic salmon (Falk, 2014). It has been shown that the level of protection with ISAV-inactivated vaccines is correlated to the amount of ISAV antigen in the vaccine, and fish immunized with high antigen amounts produce detectable ISAV-specific and neutralizing antibodies and have protection with a relative percent survival (RPS) of as high as 86% (Lauscher et al., 2011). It has also been established that virus is more rapidly eliminated from vaccinated fish than from nonvaccinated fish (reviewed by Kibenge et al., 2012b). The unprecedented 2007–11 spread of ISA in the Atlantic salmon industry in Chile created a high demand for ISA vaccines, which resulted in improved vaccine products being developed and marketed in Chile (Murias, 2012). Up to six different vaccines, five of them being inactivated whole ISAV emulsified with adjuvant and administered by intraperitoneal injection and one oral vaccine (available for the Chilean market) are available commercially (Falk, 2014). However, the efficacy of these vaccines in the presence of the widespread ISAV-HPR0 infections is not known.

The ISAV genome is now completely sequenced, opening up the way to production of recombinant subunit and DNA vaccines against ISAV. This is bound to improve the efficacy of ISA vaccines. To date, there are two commercially available ISAV subunit vaccines (marketed by the Chilean pharmaceutical company Centrovet) based on ISAV HE protein expressed in yeast and delivered either in an oil-adjuvanted injection (monovalent or multivalent with *Piscirickettsia salmonis*, infectious pancreatic necrosis virus and *Vibrio ordalii*) or orally in feed as a bioadhesive cationic polysaccharide formulation (MicroMatrix, Harel, 2009) (monovalent or bivalent with *P. salmonis*) (Falk, 2014). Oral vaccination using a vaccine preparation containing conserved regions of recombinant ISAV HE and F proteins expressed in *Sacharomyces cerevisiae* and encapsulated in MicroMatrix-induced production of IgM-specific antibodies and protected against challenge with a high-virulence Chilean ISAV isolate but was unable to induce expression of the innate antiviral Mx gene (Caruffo et al., 2016). More innovative approaches for generation of novel and improved ISAV vaccines are continuously being undertaken. A salmonid alphavirus (SAV) replicon vaccine expressing ISAV HE was shown to offer protection against ISAV infection (Wolf et al., 2013). Neither the ISAV F nor the ISAV M proteins were found to be protective when similarly expressed (Wolf et al., 2013). When delivered by intramuscular injection, but not by intraperitoneal administration, the replicon vaccine provided high protection against subsequent ISAV challenge in Atlantic salmon and induced a strong innate response locally at the injection site, which may be beneficial and could warrant reduced doses and improved efficacy compared to conventional DNA vaccines (Wolf et al., 2014). Most recently, the development of a novel reverse genetics system for ISAV (Toro-Ascuy et al., 2015) now offers a powerful tool to develop a new generation of ISAV vaccines.

19.2.6.4 Novel Antiviral Strategies

The use of RNA interference (RNAi), a highly conserved gene-silencing mechanism caused by dsRNA in both plants and animals (Hammond et al., 2000), is a promising approach because during the replication cycle, the ISAV genome must be transcribed to mRNA in the cytoplasm (García et al., 2015). Inactivated nonpathogenic *Escherichia coli* (*E.coli*), transformed with a plasmid that expressed a highly conserved region of the ISAV HE gene as dsRNA, which is the precursor of the RNAi mechanism, showed antiviral activity when added to infected ASK cells (García et al., 2015).

19.3 TILAPIA LAKE VIRUS (TILV)

19.3.1 Disease Occurrence and Significance

Tilapia lake virus (TiLV) is a novel orthomyxolike virus that has been shown to be the aetiological agent of a new highly lethal disease of tilapia (Bacharach et al., 2016) also referred to as "syncytial hepatitis of tilapia" (Ferguson et al., 2014). Both wild and farmed lake tilapines (commercial hybrid tilapia *Oreochromis niloticus* x *O. aureus*, wild tilapia *Sarotherodon* (*Tilapia*) *galilaeus* (St. Peter's fish), *T. zilli* (common tilapia), *O. aureus* (Jordan tilapia), and *Tristamella simonis intermedia*) in Israel (Eyngor et al., 2014) and farmed Nile tilapia *O. niloticus* in Ecuador (Ferguson et al., 2014) were affected. The disease, which is characterized by waves of massive mortality in fish farms, appeared in Israel in 2009 and later in Ecuador. Interestingly, in Israel, fish morbidity and mortality are restricted to tilapia; several species reared in the same community as tilapines, including carp (*Cyprinus carpio*), and Gray mullet (*Mugil cephalus*), remained unaffected, even after long-term cohabitation (Eyngor et al., 2014), whereas in Ecuador, only one strain of farm-bred tilapia *Chitralada* was affected (Ferguson et al., 2014). Tilapia is the world's second-most-farmed fish after carp (see Chapter 1, Table 1.2, in this book), with an estimated farmgate value of US$7.5 billion (FAO, 2014), making TiLV a significant threat to aquaculture.

19.3.2 Virus Characteristics

TiLV was isolated using primary tilapia brain cells and E-11 cell lines (cloned subculture of striped snakehead (SSN-1) cell line), with CPE in E-11 cells consisting of syncytia formation (Eyngor et al., 2014). The infectivity was sensitive to ether and chloroform, suggesting the TiLV is enveloped. Sensitivity of the viral genome to RNase I digestion indicated that the TiLV genome is likely ssRNA. Electron microscopic examination of thin sections of the infected E-11 cells with CPE showed enveloped icosahedral particles in the cytoplasm, measuring about 55 to 75 nm in diameter, whereas negative staining of infected cell culture supernatants purified on 25% sucrose cushions revealed virionlike structures of 75–80 nm surrounded by a thick coat (Eyngor et al., 2014). Similarly sized viruslike particles, 60–70 nm in diameter with a 10–15-nm-wide coat with surface projections, were observed ultrathin sections within the cytoplasm of hepatocytes of the infected affected fish (Ferguson et al., 2014).

Bacharach et al. (2016) used next-generation sequencing to assemble 10 contigs of TiLV genomic sequences and designed PCR primers that were used to amplify all 10 contigs from RNA extracts from infected fish and purified virus particles, followed by 5′ and 3′ rapid amplification of the cDNA end (RACE) generate the full sequence of the 10 viral genomic segments. The TiLV genome consists of 10 segments of linear, ssRNA of negative polarity, ranging in length from 465–1641 nt (see Table 19.3) with a total molecular size of 10.323 kb (Bacharach et al., 2016). Each segment has an open reading frame (see Table 19-2), and the NCRs of all 10 segments have been determined; 13 nucleotides at the 5′ termini and 13 nucleotides at the 3′ termini are similar in all segments, an organization similar to other orthomyxoviruses (Bacharach et al., 2016). The 3′ terminal 5 nucleotides and the 5′ terminal 6 nucleotides are highly conserved among the 10 genomic segments (3′-CCAAA$^{U}/_{A/G}$UU$^{A}/_{U}{}^{C}/_{A}{}_{/U}{}^{C}/_{U}{}^{C}/_{U}$ -------G$^{A}/_{G}$G$^{A}/_{U}$AA$^{G}/_{A/U}$AUUUGC-5′) (see Fig. 19.7). In addition, all the 5′ ends of TiLV genomic RNA segments contain a short, uninterrupted uridine stretch (three to five bases long) (Bacharach et al., 2016), which is the polyadenylation signal sequence in orthomyxoviruses (refer to Fig. 19.4).

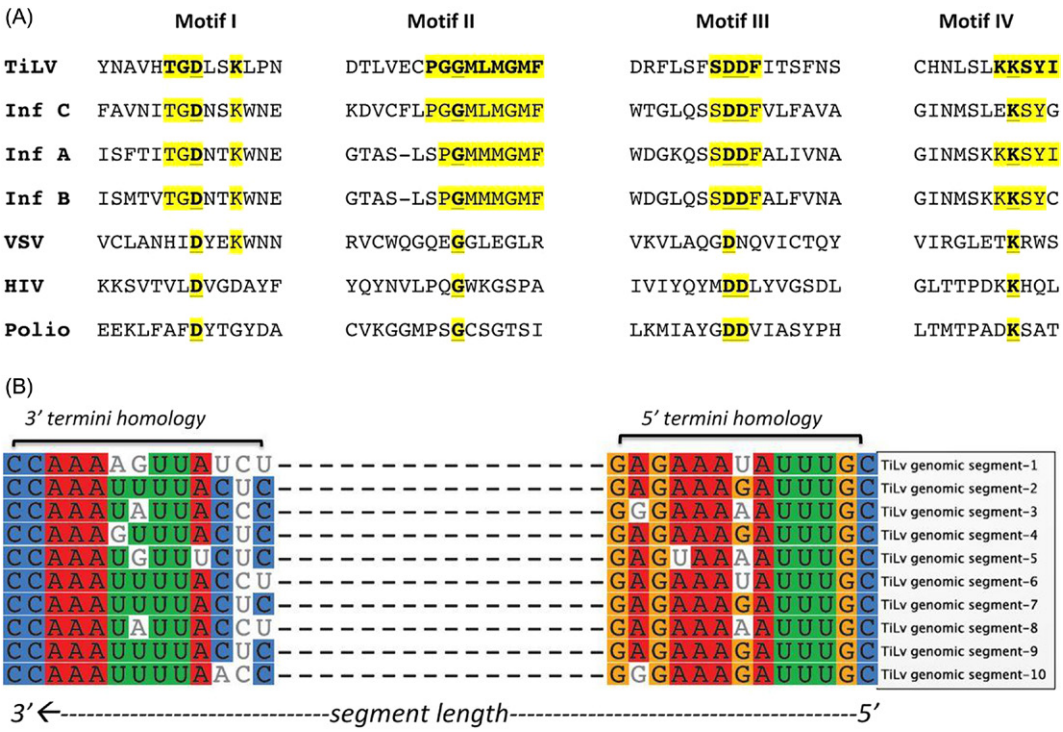

FIGURE 19.7 Genomic characterization of the segments of TiLV isolated from tilapia in Israel. (A) TiLV segment 1 putative protein shows weak homology to motifs conserved in RNA-dependent polymerases. Sequence comparison of TiLV's segment 1 predicted protein with motifs I–IV, conserved in polymerases of influenza virus strains C/JJ/50 (Inf C) (19), A/WSN/33 (Inf A) (34) and B/Lee/40 (Inf B) (35), vesicular stomatitis virus (VSV) (20), human immunodeficiency virus (HIV) (21) and poliovirus (Polio) (22). The relative motif positions are also shown. Invariant sequences in each motif are in boldface and underlined. TiLV sequences that show identity to one of the influenza virus sequences are highlighted in yellow. (B) Genomic segments of TiLV show conserved and homologous features at 5′ and 3′ termini. *From Bacharach, E., Mishra, N., Briese, T., Zody, M.C., Tsofack, J.E.K., Zamostiano, R., et al., 2016. Characterization of a novel orthomyxo-like virus causing mass die-offs of tilapia. mBio 7 (2), e00431–16. Figure 1. Copyright © Bacharach et al., (2016), with permission* http://creativecommons.org/licenses/by/4.0/.

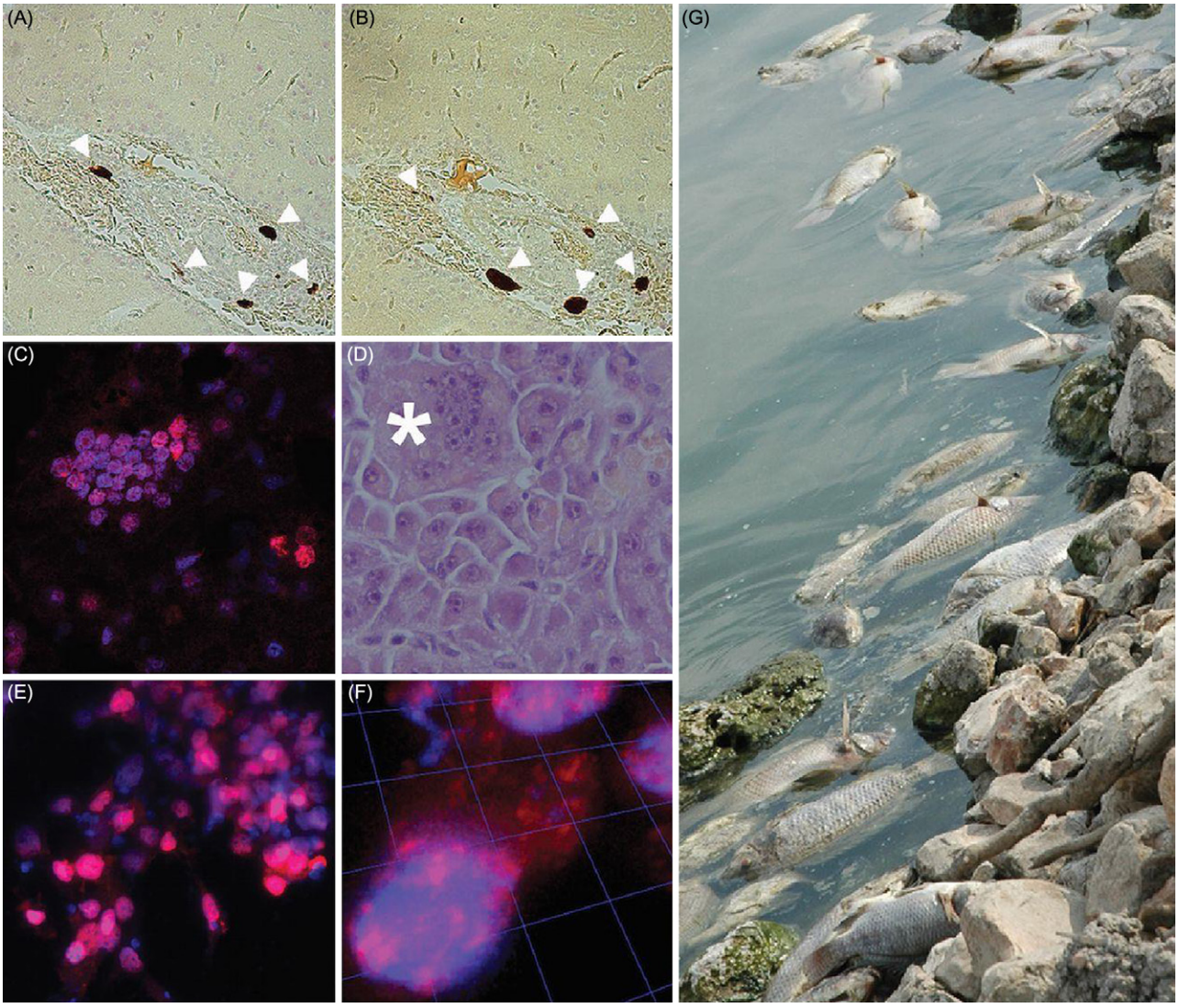

FIGURE 19.8 Detection of TiLV RNA in the brain and liver of infected tilapia and infected E-11 cells by in situ hybridization and image of dead tilapia in Israel. (A and B) Brain sections of infected Nile tilapia hybridized with Affymetrix Cy3-conjugated probes (red (light gray in print versions)) of various polarities to TiLV segment 1 to detect genomic RNA (A) or mRNA (B). White arrowheads indicate hybridization signals. (C) Liver sections hybridized with Cy3-conjugated (red (dark gray in print versions)) *Stellaris* probes to segment 3 to detect mRNA. Nuclei are stained with DAPI (blue (light gray in print versions)). (D) Liver section stained with hematoxylin and eosin reveals multinucleated giant cells (asterisk). (E) TiLV-infected E-11 cells hybridized with Quasar 670-conjugated (red (dark gray in print versions)) Stellaris probe to segment 3 to detect TiLV mRNA. Nuclei are stained with DAPI (blue). (F) Images of confocal sections of cells in panel E were reconstituted into a three-dimensional image. (G) Dead tilapia at a fish farm in Israel. *From Bacharach, E., Mishra, N., Briese, T., Zody, M.C., Tsofack, J.E.K., Zamostiano, R., et al., 2016. Characterization of a novel orthomyxo-like virus causing mass die-offs of tilapia. mBio 7 (2), e00431–16.* Figure 3. *Copyright © Bacharach et al. (2016), with permission* http://creativecommons.org/licenses/by/4.0/.

19.3.3 Clinical Features, Pathology and Epidemiology

In the disease described in Israel, affected tilapines showed pronounced ocular lesions, including opacity of the lens (cataract) advancing to ruptured lenses with uveitis or endophthalmitis, followed by buphthalmia (swelling of the eyeball) and loss of ocular functioning (phthisis bulbi), as well as skin erosions, brain hemorrhages, congestion of spleen and kidney and the presence of augmented melanomacrophage centers (Eyngor et al., 2014). In contrast, the pathology of the disease in South America was focused in the liver and gastrointestinal tract (GIT) (Ferguson et al., 2014) rather than the central nervous system (Fig. 19.8). The natural disease was reproduced experimentally using low-passage TiLV injected intraperitoneally

FIGURE 19.9 Two tilapia showing gross lesions of distended abdomen and exophthalmos. *From Ferguson, H.W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J.A., del Pozo, J., 2014. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. J. Fish Dis. 37, 583–589.* Figure 1 *Copyright © John Wiley & Sons Ltd (2013), with permission.*

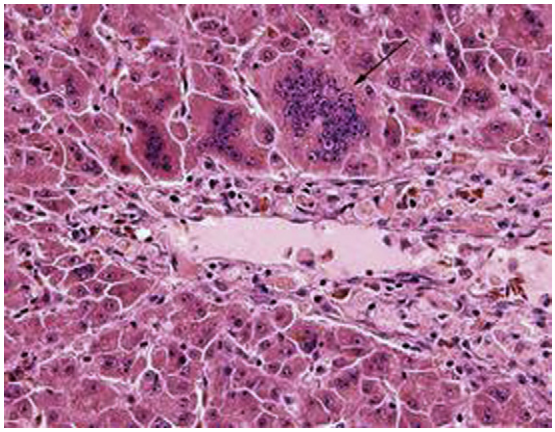

FIGURE 19.10 Liver from moribund tilapia showing syncytial giant cells (arrow), (H&E, x400). *From Ferguson, H.W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J.A., del Pozo, J., 2014. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. J. Fish Dis. 37, 583–589.* Figure 3 *Copyright © John Wiley & Sons Ltd (2013), with permission.*

in tilapia; the experimental disease had a mortality of >80%, and it was 80–100% with cohabitation (Eyngor et al., 2014). Survivors are immune to further TiLV infection (Eyngor et al., 2014; Ferguson et al., 2014).

In the Ecuador case, mortality started 4–7 days posttransfer in fingerlings approximately 3 cm long and weighing 3 g, with less than 20% surviving. Affected fish showed darkening, abdominal distension, scale protrusion and exophthalmia (Fig. 19.9). Histopathology was characterized by necrosis of the GIT and a distinctive hepatocellular syncytial cell formation (Fig. 19.10), hence the name for the new disease, syncytial hepatitis of tilapia (Ferguson et al., 2014).

19.3.4 Diagnostic Methods

Eyngor et al. (2014) prepared a cDNA library of the TiLV genome by random priming and shotgun cloning. One of the clones was extended by 5′ and 3′ RACE reactions, resulting in 1326 bases of a putative TiLV sequence (GenBank accession no. KJ605629), which allowed the design of a PCR-based diagnostic test for monitoring TiLV infection in tilapines. Moreover, virus isolation is also possible using primary tilapia brain cells and E-11 cell line (Eyngor et al., 2014).

19.3.5 Prevention and Control

As for other viral diseases, prevention and control strategies for TiLV disease should include improving biosecurity and stocking practices. Eyngor et al. (2014) and Ferguson et al. (2014) both reported that fish surviving an outbreak which were

subsequently transferred to the grow-out ponds seemed to have better growth and feed conversion data, which could support the idea of using vaccination to prevent this disease. Control through breeding from fish that survive this viral disease also appears feasible (Ferguson et al., 2014).

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Chapter 20

Coronaviruses in Aquatic Organisms

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20.1 TAXONOMY

The *Coronaviridae* are a family of enveloped viruses with a single positive-strand RNA genome, and belong to the order *Nidovirales*. This order was first introduced by the International Committee on Taxonomy of Viruses (ICTV; Pringle, 1996) in 1996, and comprises the families *Arteriviridae* and *Coronaviridae*. The designation is derived from the Latin term *nidus* and reflects the large nested arrangements of subgenomic (sg) messenger RNA (mRNA) (Cavanagh, 1997). The morphological and molecular structures of nidoviruses differ widely, and so the viruses were assigned to the families *Arteriviridae, Coronaviridae, Roniviridae* and *Mesoniviridae*, according to their ultrastructural and genomic characteristics (de Groot et al., 2012a; Lauber et al. 2012; Nga et al., 2011; Zirkel et al., 2013). *Nidovirales* have a unique set of genetic markers: a putative multinuclear zinc-binding domain (ZBD) and a uridylate-specific endoribonuclease (NendoU) domain (Gorbalenya et al., 2006). The classification of *Nidovirales*, including the type species, is summarized in Table 20.1.

The family *Coronaviridae* comprises many genera and species, including important human and veterinary pathogens that cause respiratory, gastrointestinal, cardiovascular and neurological diseases. The main feature of coronaviruses is the coronal structure, which displays prominent projections from the surface of the envelope. Coronaviruses were originally classified according to their antigenic relationships and host species. During recent decades, numerous coronavirus isolates/genomes have been identified, and the classifications have been revised accordingly.

The family *Coronaviridae* is divided into two subfamilies: *Coronavirinae* and *Torovirinae* (González et al., 2003; de Groot et al., 2012b). Members of *Coronaviridae* infect three groups of vertebrates: mammals (genera *Alphacaronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Torovirus*), birds (genera *Gammacoronavirus* and *Deltacoronavirus*) and fish (genus *Bafinivirus*) (de Groot et al., 2012b). A nidovirus recently isolated from an Indian python (python nidovirus [PNV]) probably belongs to the subfamily *Torovirinae* (Bodewes et al., 2014), although it has not been formally classified.

Coronaviruses are classified according to prominent genetic features, which are localized within the replicase polyprotein pp1ab. Viruses showing more than 90% homology (at the amino acid level) within the conserved replicase domains belong to the same species (de Groot et al., 2012b). The following conserved domains and certain motifs of the polyprotein pp1ab are applied as demarcation criteria: 3C-like protease ($3CL^{pro}$), RNA-dependent RNA polymerase (RdRp), ADP-ribose1″-phosphatase (ADRP), 3′-to-5′exoribonuclease (ExoN), superfamily 1 helicase with an N-terminal ZBD, NendoU, a putative ribose-2′-*O*-methyltransferase domain (O-MT) and a ribosomal frame shift signal (RFS) (de Groot et al., 2012b).

20.2 VIRUS STRUCTURE

The name, *Coronaviridae*, is derived from the Latin term *Corona*, and reflects the typical crownlike appearance conferred by the prominent surface projections. Coronaviruses are enveloped particles, which may be spherical (*Coronavirinae*), kidney-shaped (*Torovirus*) or bacilliform (*Bafinivirus*). The average diameter of coronaviruses ranges from 120 to 160 nm. Rod-shaped torovirus particles are 100–140 nm in length and 32–42 nm in width, whereas bafinivirus measures 150–185 × 57–70 nm (Fig. 20.1). The mean relative molecular mass (M_r) of coronavirus particles is 400×10^6. The buoyant density of coronavirus in sucrose is 1.15–1.20 g/cm^3, whereas that of torovirus and bafinivirus is 1.14–1.18 g/cm^3 and 1.17–1.19 g/cm^3, respectively (de Groot et al., 2012a).

Virions comprise an internal nucleocapsid structure, which is covered by an envelope derived from the Golgi apparatus or intermediate compartment of the infected cell. The envelope comprises a unit membrane, which bears club-shaped projections measuring 12–24 nm in length. These projections, which comprise spike (S) glycoprotein trimers, allow the virions to attach to specific receptors expressed on the plasma membrane of the host cell (Delmas and Laude, 1990). A typical

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00020-6
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TABLE 20.1 Classification of *Nidovirales* Accepted by the International Committee on Taxonomy of Viruses (ICTV)

Family	Subfamily	Genus	Type Species
Arteriviridae		*Arterivirus*	Equine arteritis virus (EAV)
Coronaviridae	*Coronavirinae*	*Alphacoronavirus*	Alphacoronavirus 1
		Betacoronavirus	Murine coronavirus
		Gammacoronavirus	Avian coronavirus
		Deltacoronavirus	Bulbul coronavirus HKU11
	Torovirinae	*Torovirus*	Equine torovirus
		Bafinivirus	White bream virus
Mesoniviridae		*Alphamesonivirus*	Alphamesonivirus 1
Roniviridae		*Okavirus*	Gill-associated virus

feature of coronaviruses is the formation of syncytia, which are induced by the S protein. Betacoronaviruses exhibit a second type of surface projection, which comprises a homodimeric hemaglutinin-esterase (HE) glycoprotein; this protein also mediates receptor binding. Viruses expressing the HE protein bind to and agglutinate erythrocytes (Modrow et al., 2010). The matrix (M) glycoprotein is located on the cytoplasmic side of the viral envelope and plays a central role in viral budding and contributes to the stability of the viral core structure (Escors et al., 2001a, b; Kuo and Masters, 2002).

Coronaviruses express a few copies of the small nonglycosylated envelope (E) protein, which is not expressed by *Torovirinae*. The E protein plays an important role in virus assembly and morphogenesis because of its ion channel and/or membrane-permeabilizing activity. Although the E protein is not essential for all coronaviruses, its loss results in a marked reduction in viral titer or inhibited virus maturation, release and spread (Ortego et al., 2002; Fischer et al., 1998; Kuo and Masters, 2003).

The internal ribonucleocapsid has a helical (*Coronavirinae*), tubular (*Torovirus*) or cylindrical (*Bafinivirus*) core structure and comprises a helical viral RNA genome complexed with multiple copies of the RNA-binding nucleocapsid (N) protein. The N protein is responsible for genome encapsidation and packaging. It is also essential for sg RNA synthesis and genome replication (Almazan et al., 2004; Schelle et al., 2005).

20.2.1 Viral Genome

Coronaviruses have the largest genome of all the RNA viruses. The viral genome is infectious and comprises a positive single-stranded RNA encompassing 25.4 kb (eg, Porcine Deltacoronavirus; PDCoV) to 31.8 kb (eg, bottlenose dolphin coronavirus HKU22). The as-yet-unassigned PNV genome comprises >33 kb and is probably the largest known RNA genome.

The capped and polyadenylated viral RNA has untranslated regions (UTR) at the 5′ and 3′ termini. The largest open reading frames (ORFs) are 1a and 1b, which are localized at the 5′ end and make up 60%–75% of the viral genome. These ORFs encode the nonstructural proteins and the viral replicase components. Both ORFs overlap within a small region containing a -1 RFS signal (Gorbalenya et al., 2006). ORFs encoding the smaller structural S, M, N, HE and E proteins are located downstream of ORF 1b. Both the number and the order of encoded structural genes may vary within and between different genera of *Coronaviridae*. In general, the viral genome is organized as follows: 5′-UTR-replicase-S-M-N-UTR-3′ (de Groot et al., 2012b).

20.2.2 Morphogenesis

Detailed information about the replication of nidoviruses isolated from aquatic organisms is lacking. Nevertheless, coronaviruses and toroviruses share a similar genome organization and replication strategy. The principle features of the viral infection cycle, as reviewed by de Vries et al. (1997) and Ruch and Machamer (2012), are described next.

The first step of the infection cycle is attachment of a virion to its cognate cellular receptor. This step is mediated by the S glycoprotein. The conformation of the S glycoprotein changes upon binding, thereby promoting fusion of the viral envelope with the cell membrane (Lewicki and Gallagher, 2002; Belouzard et al., 2012). Some coronaviruses require cleavage of the

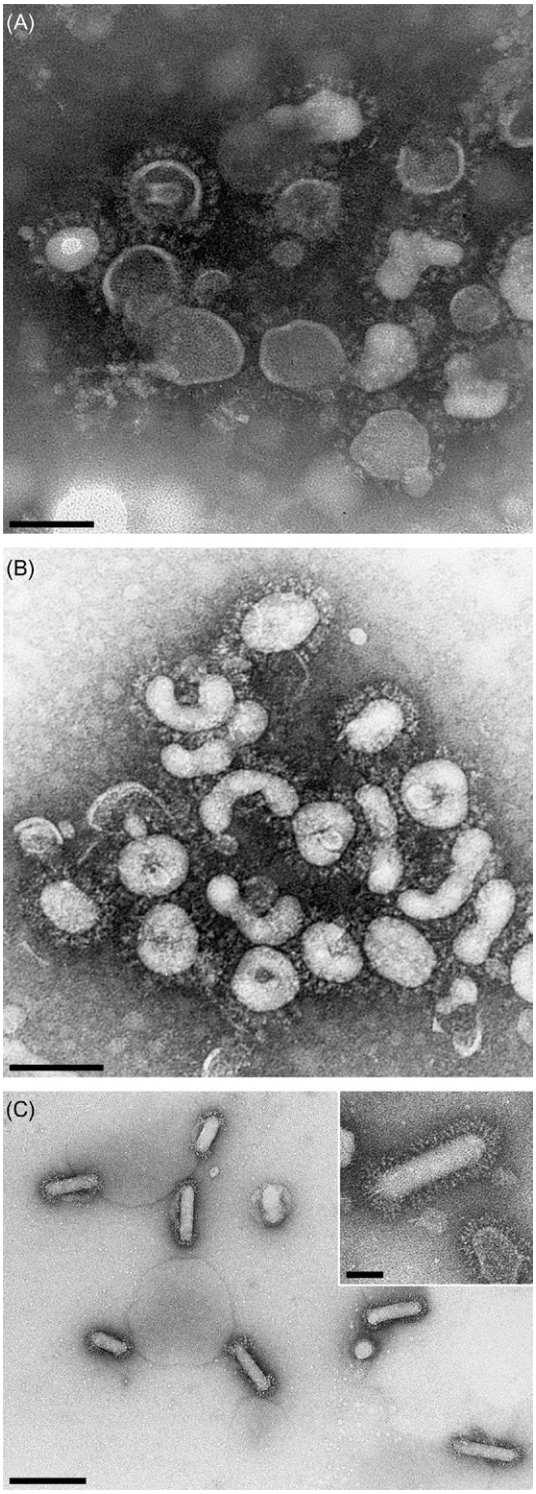

FIGURE 20.1 Electron micrograph showing representative *Coronaviridae* after negative contrast staining. (A) Transmissible gastroenteritis virus (TGEV) (family, *Coronaviridae*; genus, *Alphacoronavirus*). Bar, 100 nm. (B) Equine torovirus (EToV) (subfamily, *Torovirinae*; genus, *Torovirus*). Bar, 100 nm. (C) White bream virus (WBV) (subfamily, *Torovirinae*; genus, *Bafinivirus*). Bar, 200 nm (50 nm in inset). The photographs were kindly provided by Dr. H. Granzow (Federal Research Institute for Animal Health, Insel Riems, Germany).

S glycoprotein for optimal fusion; however, noncleavage does not prevent fusion (Cavanagh, 2005). After fusion of the viral envelope with the cell membrane, the viral genome is released into the cytoplasm. Virus replication takes place entirely within the cytoplasm of the infected cell. The first step involves attachment of the genomic RNA to ribosomes to facilitate synthesis of viral RdRp, which is encoded by ORF 1b. Structural proteins are produced from sg mRNAs. The viral replicase is encoded by two large ORFs, which overlap at the end of ORF 1a and the beginning of ORF 1b. A conserved "slippery" sequence comprising seven nucleotides is located upstream of a pseudoknot structure at the end of ORF 1a. This specific structure facilitates a ribosomal frame shift, resulting in the translation of two long polyproteins named pp1a and pp1ab. These large nonstructural proteins have never been observed in infected cells (de Groot et al., 2012a). Co- and post-translational processing by viral proteinases generates a range of mature nonstructural proteins, including the replicative proteins. These enzymes and proteins are conserved within coronaviruses and are typical features of nidoviruses. The replicase complex produces complementary copies of the genome. The resulting negative-stranded RNA intermediates serve as template for the generation of positive-stranded viral RNA (genome replication) and for the synthesis of a 3′ co-terminal nested set of sg mRNAs (transcription) (Sawicki, 2009). The latter are polyadenylated at the 3′ terminus. With the exception of toroviruses, each sg mRNA carries a short 5′ leader sequence, which is identical to the 5′ end of the viral genome. In coronaviruses and bafiniviruses, transcription of genome templates is regulated by a short AU-rich sequence (transcription regulating sequence; TRS); however, in toroviruses, the process is regulated by putative terminator/promoter elements (TPs). The TRS and TPs are not equivalent in terms of function (de Groot et al., 2012a). Coronaviruses and bafiniviruses generate mRNA via discontinuous synthesis, whereas toroviruses employ a mixed transcription procedure (Cavanagh, 2005; de Groot et al., 2012a,b).

Translation of viral glycoproteins takes place on the rough endoplasmic reticulum. The S, HE and M glycoproteins are then transported to the Golgi complex. The assembly process is initiated by the formation of M protein homomultimers, which accumulate at the membrane of the intermediate compartment between the endoplasmic reticulum and the Golgi (termed the ERGIC) (Neuman et al., 2011; De Haan et al., 1998, 2000). Envelope formation and recruitment of the M homomultimers is promoted by the integral membrane protein E (Ruch and Machamer, 2012; Ye and Hogue, 2007). The viral RNA combines with the N protein to form the RNP; meanwhile, the M and E proteins distort the membrane to form a sphere around the nucleocapsid. The M proteins then recruit the viral core and spike proteins. The newly formed virions bud into the lumen of the ERGIC. Virus containing Golgi vesicles move to the plasma membrane, where virions exit the cell via exocytosis (Siu et al., 2008; Krijnse-Locker et al., 1994).

20.2.3 Recombination

The diversity of nidoviruses is generated via mutation and recombination. The estimated mutation rate for nidoviruses is similar to that of other RNA viruses with smaller genomes; however, the replication rate is associated with a high rate of recombination. Both homologous and heterologous recombination have been demonstrated in tissue culture, in infected animals and in cases of natural infection (Lai, 1990; Cavanagh, 2005; Gorbalenya et al., 2006). It seems probable that the discovery of new nidoviruses will provide new insight into the mechanisms underlying recombination. Such discoveries will also further our understanding of viral evolution and improve taxonomic classification.

20.2.4 Antigenic Components

The S and HE glycoproteins are the major antigens expressed by coronaviruses and both induce the production of neutralizing antibodies and a cell-mediated immune response in the host (Siddell et al., 1983; Lai and Cavanagh, 1997). The N protein may also trigger a protective immune response (Du et al., 2009). In addition, closely related coronaviruses show serological cross-reactivity (Bradburne, 1970) [ie, Human coronavirus 229E (HCoV 229E), Transmissible gastroenteritis virus (TGEV), Porcine respiratory coronavirus ISU-1 (PRCV ISU-1) and Feline infectious peritonitis virus WSU79-1146 (FIPV 79-1146)].

20.3 CORONAVIRUSES IN AQUATIC ORGANISMS

Few reports describe the presence of coronaviruses in aquatic organisms. Coronaviruses have been isolated from marine mammals, including beluga whale (*Delphinapterus leucas*) and bottlenose dolphin (*Tursiops aduncus*), as well as from freshwater cyprinid species such as white bream (*Blicca bjoerkna*) and fathead minnow (*Pimephales promelas*). However, not all of these viruses are officially classified as coronaviruses by the ICTV. Table 20.2 provides an overview of coronaviruses detected in aquatic organisms, along with their classification status. At present, no information is available regarding the ecology, route of infection, cell tropism, mode of transmission and/or putative vectors of coronaviruses that infect aquatic organisms.

TABLE 20.2 Coronaviruses That Infect Aquatic Organisms

Genus	Virus	References	GenBank Accession Number
Alphacoronavirus	Harbor seal coronavirus	Bossart and Schwartz (1990)	FJ766501
Betacoronavirus	n.d.		
Gammacoronavirus	Beluga whale coronavirus SW1	Mihindukulasuriya et al. (2008)	EU111742
	Bottlenose dolphin coronavirus	Woo et al. (2014)	KF793824, KF793825, KF793826
Deltacoronavirus	n.d.		
Torovirus	n.d.		
Bafinivirus	White bream virus	Granzow et al. (2001), Schuetze et al. (2006)	DQ898157
	Fathead minnow virus	Iwanowicz and Goodwin (2002), Batts et al. (2012)	GU002364, GU002365
	Chinook salmon bafinivirus	Lord et al. (2014)	

Viruses officially listed by ICTV are Beluga whale coronavirus SW1 and the White bream virus. To date, no beta-/deltacoronaviruses or toroviruses have been detected.
n.d., not detected.

20.3.1 Alphacoronaviruses

20.3.1.1 Harbor Seal Coronavirus (Unassigned)

The only probable case of coronavirus infection of harbor seals was reported by Bossart and Schwartz (1990). In 1987 three seals (*Phoca vitulina*) housed at the Miami Seaquarium were affected by an acute necrotizing enteritis. Two of the seals died without showing any clinical signs; however, the third exhibited marked leukocytosis, dehydration, hypernatremia and hyperchloremia. Pathological sections from all three seals revealed extensive focal bronchoalveolar hemorrhage and edema, with severe diffuse pulmonary congestion. Moderate-to-severe lymphoid depletion was detected in the spleen and in visceral and peripheral lymphnodes. Coronavirus-specific antigens were detected in the intestinal mucosa. Immunofluorescence staining with antibodies against TGEV, FIPV and canine coronavirus (CCoV) (all alphacoronaviruses) yielded positive results, whereas immunofluorescence staining for bovine coronavirus (BCoV) (a betacoronavirus) was negative. Cross-reactivity among coronaviruses is limited to closely related species (de Groot et al., 2012b). Based on antigenic cross-reactivity, the coronavirus infecting the harbor seals most probably belonged to the genus *Alphacoronavirus*. However, because none of the ICTV demarcation criteria were met, the pathogen was not assigned to the genus.

A short sequence derived from a coronavirus infecting another harbor seal, which encoded the RNA-dependent RNA polymerase, has also been published (NCBI Genbank Acc. No. FJ766501). It is assumed that further studies will confirm the presence of related viruses in harbor seals.

20.3.2 Gammacoronaviruses

20.3.2.1 Beluga Whale Coronavirus SW1 (BWCoV SW1)

The first report about identification of the complete genome from a coronavirus isolated from a marine mammal was published in 2008 (Mihindukulasuriya et al., 2008). A captive-born beluga whale died from acute liver failure after suffering a short generalized pulmonary disease. Histological examination confirmed acute hepatic necrosis. Electron microscopic examination of liver tissue revealed a great number of round viral particles in the cytoplasm of hepatic cells. The estimated particle size (60–80 nm in diameter, with a core of 45–50 nm) differed markedly from that of known coronaviruses. Attempts to isolate the pathogen in noncongeneric cell lines failed, indicating that the virus had a specific host tropism. The viral genome was approximately 31.7 kb in size and encoded nonstructural (replicase; ORF 1a and 1b) and structural (S (ORF 2), E (ORF 3), M (ORF 4) and N (ORF 11)) proteins, which were flanked by UTRs. In addition, eight putative ORFs were localized between the genes coding the M and N proteins. The deduced amino acid sequences showed no similarity to proteins from known coronaviruses. Nevertheless, characteristic molecular features were identified: that is, transmembrane domains (ORFs 5 and 9), putative secretion signal sequences (ORFs 7 and 8) and a uridine kinase motif (ORF 10). Phylogenetic analyses of the structural and replicase proteins revealed that the virus was most closely related to

avian coronaviruses, such as infectious bronchitis virus and turkey coronavirus. Although the homology of the conserved replicase domain (<67%) was markedly lower than the species demarcation threshold (90%), BWCoV SW1 was characterized as a species belonging to the genus *Gammacoronavirus* (de Groot et al., 2012b). Woo et al. (2014) suggested that BWCoV SW1, along with bottlenose dolphin coronaviruses, be listed as a distinct species, *Cetacean coronavirus*, within the genus *Gammacoronavirus*.

20.3.2.2 Bottlenose Dolphin Coronavirus (BdCoV HKU22, Unassigned)

A marine surveillance study conducted in the Ocean Park in Hong Kong between 2008 and 2010 identified a coronavirus in Indo-Pacific bottlenose dolphins (Woo et al., 2014). The coronavirus genome was detected in fecal samples from three apparently healthy animals. In contrast to the isolate from beluga whale, which caused liver damage, the BdCoV did not induce any clinical signs/symptoms, indicating an asymptomatic or mild infection. The viral genome was detected during the infectious stage, whereas N-specific antibodies were detected in serum samples at 4–8 weeks postinfection. The nearly identical viral genomes isolated from the three bottlenose dolphins belonged to the genus *Gammacoronavirus*. Attempts to replicate the viruses in several nonhost-specific cell lines were unsuccessful and information about the ultrastructure of the pathogen is lacking. Therefore, the evidence for BdCoV is based on characterization of the viral genome. Genetic analyses suggested that BdCoV was closely related to BWCoV SW1. Both viruses possess a genome of approximately 31.7 kb and both genomes show similar organization. The N protein and conserved domains within replicase polyprotein pp1ab of BdCoV and BWCoV SW1 show 99.6–99.3% homology; however, they are less homologous with avian coronaviruses (35.4–74.9%). The major difference between the two viruses was detected in the spike protein. Phylogenetic analysis and pairwise amino acid alignments indicated that BWCoV and BdCoV clustered separately within the genus *Gammacoronavirus*. This finding is consistent with the close genetic relationship between the respective hosts, both of whom belong to the order *Cetacea*. Therefore, Woo et al. (2014) proposed a new viral species: *Cetacean coronavirus*.

20.3.3 Bafinivirus

20.3.3.1 White Bream Virus

White bream virus (WBV) is the type species of the genus *Bafinivirus* within the subfamily *Torovirinae*. The name bafinivirus was coined because the virus shows bacilliform morphology (ba-) and is a fish (fi-) nidovirus (ni-). The pathogen was isolated from a healthy white bream (*B. bjoerkna* L.) caught in the Federal State of Saxonia-Anhalt in Germany during a program designed to monitor pathogens in wild freshwater fish (Granzow et al., 2001). The virus replicates at 20–26°C in *Epithelioma papulosum cyprini* (EPC; CCLV Rie 173), Blue gill fry (BF-2; CCLV Rie 290), fathead minnow (FHM; CCLV Rie 057) and carp pituitary (CaPi; CCLV Rie 112) cells and induces cytopathic effects. The pathogen is sensitive to chloroform. Viral particles exhibit ultrastructural features similar to those described for *Roniviridae* [eg, gill-associated virus (GAV-AUS) and yellow head virus (YHV)] isolated from prawns (Chantanachookin et al., 1993; Spann et al., 1997). The mature enveloped virions measure 170–200 nm in length and 75–88 nm in diameter and are surrounded by fuzzy spikes approximately 20–25 nm long. The rigid rod-shaped nucleocapsid measures 120–150 × 19–22 nm and the buoyant density of the virus in sucrose is 1.17–1.19 g/cm^3. The viral genome is 26.6 kb in length and encodes five ORFs, representing the large replicase gene and the structural proteins S, M and N. The virus does not encode a hemagglutinin esterase protein or a homolog of the coronavirus E protein. WBV exhibits molecular features typical of nidoviruses, including a polycistronic genome, the production of sg RNAs during transcription, RFS to express the replicase core domains at the translational level, and post-translational proteolytic processing of viral proteins (Schuetze et al., 2006). Comparative sequence analyses showed that WBV is more closely related to toroviruses than to the other members of the order *Nidovirales*. Although WBV is related to toroviruses, it is different in several important aspects. First, WBV does not encode the structural gene HE. Second, all WBV sg RNAs possess a 5′ leader sequence, whereas three out of the four equine torovirus (strain Berne) sg RNAs lack a 5′ leader. Third, *Bafinivirus* does not encode cyclic nucleotide phosphodiesterase, which is conserved in bovine and equine toroviruses. Fourth, the nonstructural gene products and the structural proteins M, N and S of WBV and torovirus are poorly conserved (Schuetze et al., 2006). Finally, cyprinids are the natural hosts of WBV, whereas mammals are the natural hosts of toroviruses.

Pathogens with similar morphological, physicochemical and genetic features to the WBV have been isolated from other cyprinids, including goldfish (*Carassius a. auratus*), tench (*Tinca tinca*) and grass carp (*Ctenopharyngodon idella*) (Fichtner et al., 2005). Some fish suffer hemorrhage on the gills and skin, or ulceration of the skin. Cross-reactivity between anti-WBV antibodies and viral isolates from goldfish, tench and grass carp confirms a relationship between these

pathogens. Other WBV-like pathogens were isolated from tench and carp after clinical events and fatalities in Germany in 2007 (Fichtner et al., 2009). Infection of specific pathogen-free carp, goldfish and crucian carp (*Carassius carassius*) with a WBV-like pathogen isolated from a dead carp caused nonspecific clinical signs such as exhaustion, reduced food intake, increased mucus production, pseudofeces and petechiae. No mortalities were recorded (Fichtner et al., 2009). Thus coronaviruses appear to be prevalent in cyprinids. Genomic analyses of these pathogens are necessary to confirm a relationship with bafinivirus WBV and to support a clear taxonomic classification of cyprinid coronaviruses.

20.3.4 Unassigned Viruses

20.3.4.1 Fathead Minnow Virus (FHMNV, An Unassigned Member of the Nidovirales)

Fathead Minnow Virus (FHMNV) was first isolated from moribund fathead minnows (*P. promelas*) on a baitfish farm in central Arkansas in 1997 (Iwanowicz and Goodwin, 2002). Affected fish exhibited hemorrhage of the skin and eyes, as well as hepatic, splenic and renal lesions. The virus replicates in EPC, FHM and RTG (rainbow trout gonad) cells between 15°C and 25°C. A unique feature of this pathogen is the production of syncytia in both tissues and cell culture. The bacilliform particles measure 130–185×31–47 nm. The complete genome sequence suggests that the virus belongs to the genus *Torovirus* (Batts et al., 2012). The viral ssRNA is approximately 27 kb in length and displays the same genome organization as WBV, with molecular features typical of a nidovirus genome. Comparison of FHMNV helicase (pp1ab domain), S, M, N and ORF 1ab gene products with those of WBV reveals differing levels of homology, ranging from 15% (S protein) to 70% (helicase). The FHMNV pp1ab sequence is 11% homologous with that of equine arteritis virus, whereas the FHMNV helicase is 35% homologous with that of Equine torovirus (EToV). Phylogenetic analysis of the conserved helicase domain revealed that, although FHMNV is a distinct virus, it is the closest relative of WBV identified to date (Batts et al., 2012).

20.3.4.2 Chinook Salmon Bafinivirus (An Unassigned Member of the Nidovirales)

The first report of a bafinivirus isolated from Chinook salmon (family, *Salmonidae*; genus *Oncorhynchus*) was published in 2014 (Lord et al., 2014). The virus replicates and induces cytopathic effects in RTG-2 and EPC cells at 15°C, 20°C and 25°C. The enveloped particles have a bacilliform structure and are 45 nm in diameter and 120–130 nm in length. The virus possesses a genome of approximately 27 kb, with a gene organization consistent with that of bafiniviruses. Amino acid alignments suggest that this new virus is related to WBV and FHMV.

20.3.4.3 Carp Viruses

Several reports describe the isolation of potential coronaviruses from carp. Next is an overview of other putative coronaviruses reported in the literature. Their relationship to coronaviruses is unclear from an ultrastructural and morphogenetic perspective. Furthermore, it should be noted that these isolates do not fulfill the criteria for taxonomic classification (they are missing genomic and/or morphological data) and are therefore not officially listed by the ICTV.

20.3.4.4 Ctenopharyngodon idella Virus Hungary 33/86 (CIVH 33/86)

In 1986 an unknown virus was isolated from an apparently healthy grass carp (*C. idella*) imported into Germany from Hungary (Ahne et al., 1987). The isolate, CIVH 33/86, multiplies in CAR cells (goldfish [*Carassius auratus*] fin cells), CLC cells (common carp [*Cyprinus carpio*] leukocytes) and FHM cells between 20°C and 25°C. Infected cells fuse and then lyse. The virus is inactivated by chloroform, acid solutions (pH 3) and a temperature of 56°C. Treatment with 5-iododeoxyuridine (IUdR) has no effect on viral replication. Treatment with acridine orange confirmed the presence of a single-stranded viral RNA genome. Electron micrographs revealed enveloped rod-shaped particles measuring 170–220 nm in length and 50–55 nm in width. Finally, the isolate exhibits morphological features consistent with those of toroviruses (Ahne et al., 1987).

20.3.4.5 Carp Coronavirus

Sano et al. (1988) isolated a coronavirus from a common carp that died after suffering an acute infection; clinical signs included erythema on the abdomen and hepatic and renal necrosis. The virus is transmitted to carp fry via the water at a temperature of 20 °C. The pathogen was isolated from the kidney, liver and spleen, and replicates in BF-2, EPC, RTG-2 and FHM cells at 20°C. The enveloped particles measure 60–100 nm in diameter.

20.3.4.6 Carp Viremia-Associated Ana-Aki-Byo

A putative coronavirus, named carp viremia-associated ana-aki-byo, was responsible for high mortality rates in color carp (*C. carpio*) between 1997 and 1998 in Japan (Miyazaki et al., 2000). Necrotic lesions were observed in the visceral organs and virus particles were detected in hematopoietic tissue and spleen. The virus was resistant to IUdR, but sensitive to ether, suggesting that it was enveloped RNA virus. EPC cells were susceptible to infection at 25°C, but EK-1 (eel kidney), FHM, CHSE-214 (chinook salmon embryo) and RTG-2 cells were not. Infected EPC cells displayed karyopyknosis and intracytoplasmic vacuolization. The virus was round and displayed spike projections on the surface. The diameter of the virion ranged from 100 to 180 nm. Tubular structures (diameter, 60–70 nm) of various lengths and crystalline inclusions were observed in the cytoplasm of infected cells.

In summary, studies suggest that coronaviruses are prevalent in cyprinids and salmonids. It is hoped that new analytical tools, methods and technologies will enable the discovery and identification of other coronaviruses and facilitate further research into their molecular biology, phylogeny and evolution. Increased knowledge about the prevalence, distribution and transmission of these viruses, along with further identification of affected host species and ecological factors, will aid efforts to monitor and control infections and to assess the risk of transmission of coronaviruses in aquatic organisms.

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Chapter 21

Picornaviruses of Fish

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21.1 INTRODUCTION

Picornaviruses of fish (Table 21.1) belong to the family *Picornaviridae* which currently consists of 50 species grouped into 29 genera—namely, *Aphthovirus, Aquamavirus, Avihepatovirus, Avisivirus, Cardiovirus, Cosavirus, Dicipivirus, Enterovirus, Erbovirus, Gallivirus, Hepatovirus, Hunnivirus, Kobuvirus, Kunsagivirus, Megrivirus, Mischivirus, Mosavirus, Oscivirus, Parechovirus, Pasivirus, Passerivirus, Rosavirus, Sakobuvirus, Salivirus, Sapelovirus, Senecavirus, Sicinivirus, Teschovirus* and *Tremovirus* (Knowles et al., 2012; Adams et al., 2015; www.picornaviridae.com). The first picorna-like virus was reported in rainbow smelt (*Osmerus mordax*) in 1988 by Moore et al. (1988). Subsequently, many reports appeared on picorna-like viruses in different fish species such as Atlantic salmon (*Salmo salar*) (Hedrick et al., 1990), barramundi (*Lates calcarifer* Bloch) (Glazebrook et al., 1990; Munday et al., 1994), European smelt (*Osmerus eperlanus*) (Ahne et al., 1990), turbot (*Scophthalmus maximus*) (Bloch et al., 1991) and sandbar shiners (*Notropis scepticus*) (Chittick and Stoskopf, 2001; Iwanowicz et al., 2000). Many of these viruses have been identified exclusively using transmission electron microscopy (TEM) of infected fish tissues and/or infected cell culture supernatants. Relatively few studies are available on the biochemical and biophysical properties of these agents.

Since 2013, Sanger sequencing and next-generation sequencing (NGS) have identified several novel fish picornaviruses in many different fish species such as bluegill (*Lepomis macrochirus*), common carp (*Cyprinus carpio*), European glass eel (*Anguilla anguilla*), fathead minnow (*Pimephales promelas*) and golden shiner (*Notemigonus crysoleucas*) (Fichtner et al., 2013; Barbknecht et al., 2014; Lange et al., 2014; Phelps et al., 2014).

21.2 DISEASE CONDITIONS

Clinical symptoms of fish infected with picornaviruses range widely. Picornaviruses have been isolated from apparently healthy fish, including common carp, fathead minnow (FHM) and golden shiner (Lange et al., 2014; Mor et al., unpublished; Phelps et al., 2014). They have also been isolated from bluegill, showing hemorrhages in skin and fins, erythema and pale and swollen internal organs (Barbknecht, 2009; Barbknecht et al., 2014; Fig. 21.1). Picorna-like viruses have also been implicated in neurological signs in juvenile fish, mortality events in smelt and focal hepatitis and mild hematopoietic necrosis in salmonids (McAllister, 1993a, b; Noga, 1996). The picornavirus-affected eel show signs of increased mucus production, mucous ulcers and reddened skin but no hemorrhages (Fichtner et al., 2013). Ahne et al. (1990) speculated that picornalike particles were causing tumorlike skin lesions in adult European smelt (*O. eperlanus* L.) in the North Sea area. Bloch et al. (1991) reported high mortality in larval turbot (*S. maximus*) due to encephalomyelitis caused by picorna-like virus. One of the four lots that tested positive for fathead minnow picornavirus (FHMPV) displayed external clinical pathology including skin lesions and hemorrhaging of the ventral surface and fin bases (McCann, 2012; Phelps et al., 2014; Fig. 21.1). To the best of our knowledge, no name has been assigned to fish diseases associated with picornaviruses.

21.3 VIRUS CHARACTERISTICS

21.3.1 Structure and Composition of Virus

Electron microscopy (EM) is a common approach to detect picorna or picorna-like virus particles from highly infected tissue or cell culture. Several studies using TEM on negative stained extracellular particles have revealed the presence of featureless, nonenveloped virus particles that were round to icosahedral and averaged 30 nm in diameter—criteria similar

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00021-8
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TABLE 21.1 Fish Picornaviruses

Family	Genus	Virus Name	Genome Size (Nucleotides)	Host	Country	References
Picornaviridae		Turbot virus		Larval turbot (*Scophthalmus maximus*)	Norway	Bloch et al. (1991)
				European smelt (*Osmerus eperlanus* L.)	Germany	Ahne et al. (1990)
				Barramundi (*Lates calcarifer*)	Australia	Glazebrook et al. (1990)
				Sandbar shiner (*Notropis scepticus*)	United States	Chittick and Stoskopf (2001); Iwanowicz et al. (2000)
	Lemavirus	Bluegill picornavirus-1 (BGPV-1)	8050	Bluegill (*Lepomis macrochirus*)	United States	Barbknecht et al. (2014)
		Bluegill picornavirus-2 (BGPV-2)		Bluegill and black crappie (*Pomoxis nigromaculatus*)	United States	Mor et al. (unpublished data)
	Limnipivirus	Carp picornavirus (CPV-1)	7632	Common carp (*Cyprinus carpio*)	Germany	Lange et al. (2014)
		Carp picornavirus (CPV-2)	7811	Common carp (*C. carpio*)	United States	Mor et al. (unpublished data)
	Anguavirus	Eel picornavirus (EPV-1)	7496	European glass eel (*Anguilla anguilla*)	Germany	Fichtner et al. (2013)
	Piscesvirus	Fathead minnow picornavirus (FHMPV)	7834	Fathead minnow (*Pimephales promelas*)	United States	Phelps et al. (2014)
		Golden shiner picornavirus (GSPV-1)	7707	Golden shiner (*Notemigonus crysoleucas*)	United States	Mor et al. (unpublished data)

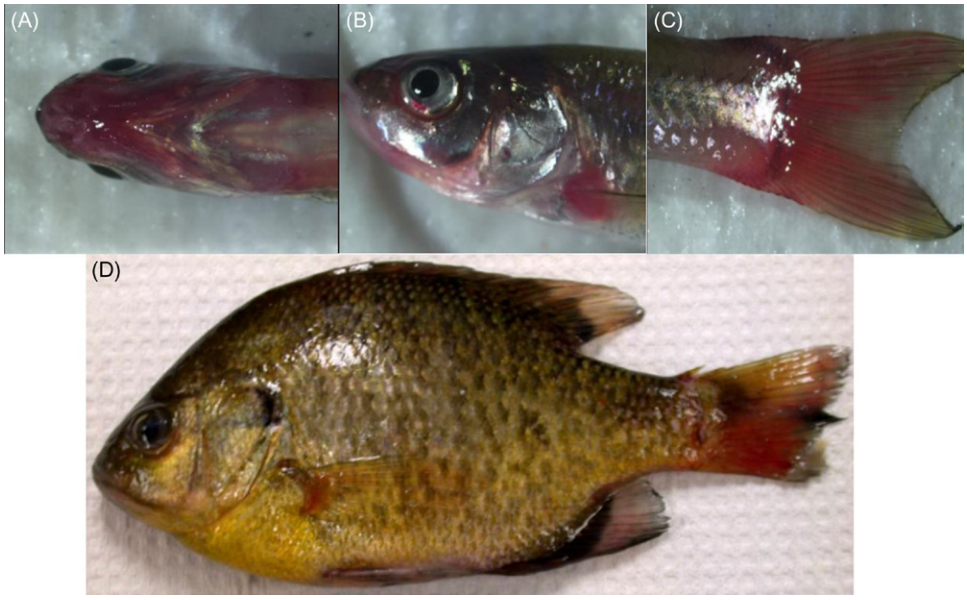

FIGURE 21.1 Clinical signs observed in fathead minnows and bluegills that tested positive for FHMPV and BGPV-1, respectively. (A) Fathead minnow with severe hemorrhages on the ventral surface. (B) Fathead minnow with hemorrhages in the eye and at the base of the pectoral fin. (C) Fathead minnow with severe hemorrhages in the caudal fin. (D) Bluegill with hemorrhages at fin bases. *Reproduced with permission from: (A–C) McCann (2009), (D) Dr. Becky Lasee, USFWS and Barbknecht, M., 2009. Characterization of An Unclassified Virus and Survey for its Presence in Wisconsin Bluegill Populations. M.S. thesis, University of Wisconsin, La Crosse, WI.*

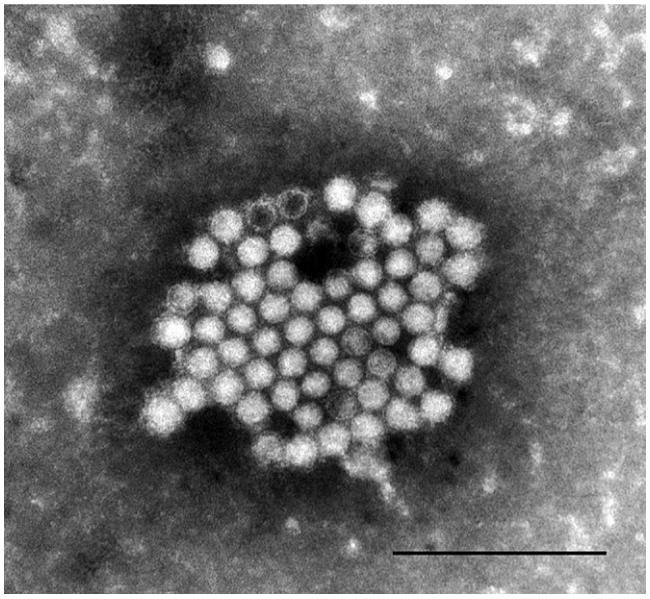

FIGURE 21.2 Negative contrast electron microphotograph of fathead minnow picornavirus (FHMPV) showing aggregation of nonenveloped spherical (about 30–32 nm) virions consistent with viruses of *Picornaviridae*. Bar = 200 nm. *Reproduced with permission from: Phelps, N.B.D., Mor, S.K., Armien, A.G., Batts, W., Goodwin, A.E., Hopper, L., et al., 2014. Isolation and molecular characterization of a novel picornavirus from baitfish in the USA. PLoS ONE 9, e87593.*

to those described for members of the family *Picornaviridae* (Ahne et al., 1990; Bloch et al., 1991; Fichtner et al., 2013; Barbknecht et al., 2014; Lange et al., 2014; Phelps et al., 2014; Fig. 21.2). Bluegill picornavirus-1 (BGPV-1) particles resembled crumpled pieces of paper due to their rough surface appearance (Barbknecht et al., 2014).

Barbknecht et al. (2014) did further work on BGPV-1 using SDS-PAGE and silver staining. The four most prominent bands observed from infected cells were estimated to be 24.6, 32.9, 39.5 and 45.8 kDa. The author summarized that these

proteins could either be viral capsid proteins or virus-associated cellular proteins. However, MALDI-TOF analysis of in-gel digested 24.6-kDa and 32.9-kDa proteins failed to retrieve any database matches despite successful mass spectroscopy. This suggested that these proteins are not of eukaryotic origin and have not been previously sequenced.

21.3.1.1 Inactivation Studies and Nucleic Acid Inhibition Assays

Ahne et al. (1990) treated infectious supernatants of European smelt picorna-like virus from the third passage in Chinook salmon embryo (CHSE) cell line ($10^{8.5}$ $TCID_{50}$/mL) with chloroform ($ChCI_3$, 50%) and CHSE cells pretreated with 5-iodo-2-deoxuridine (IUdR, 50 µg/mL). Neither of the two chemicals affected virus replication indicating that it was a nonenveloped, RNA-containing virus. Lange et al. (2014) conducted a similar study for carp picornavirus-1 (CPV-1); a suspension of F37/06 strain of CPV-1 was treated with 10% chloroform for 4 h at 4°C. After centrifugation at 2000 × *g*, the supernatant was titrated on cell cultures and compared to untreated samples. In both studies, chloroform treatment did not reduce the infectivity of the virus, and treatment with 5-iodo-2-deoxyuridin did not impair virus replication, indicating that the virus was either a chloroform-resistant RNA virus or it was nonenveloped.

21.3.1.2 Nuclease Treatment

Barbknecht et al. (2014) treated extracted viral nucleic acid from BGPV-1 separately with 1 unit of DNase I (New England Biolabs) for 15 min at 37°C and 0.75 µg RNase A (Sigma) for 30 min at 45°C, along with pGEM-3Zf DNA and BF-2 rRNA as controls. After treatment, samples were transferred to a native 1.2% agarose gel, electrophoresed alongside a double-stranded DNA (dsDNA) ladder (Minnesota Molecular) under RNase-free conditions and visualized by ethidium bromide staining. DNase I degraded the pGEM-3Zf control, but failed to digest the BGPV genome while RNase A degraded both the uninfected BF-2 rRNA control and the BGPV-1 genome, confirming the viral genome to be RNA. Extract from concentrated virus particles ran as a single band on the agarose gel. This same band was present in infected cell lysates, along with an additional band that was higher on the gel. Both bands were degraded by RNase A. This second band suggests BGPV-1 may have a double-stranded genome-length RNA replication intermediate within its life cycle as in positive-sense (mRNA-sense) RNA viruses.

21.3.2 Classification and Virus Replication

Fish picornaviruses are classed under Baltimore's viral classification system as group IV viruses because they contain a single stranded, positive-sense RNA genome of between 7.2 and 9.0 kb in length. Fish picornaviruses belong to family *Picornaviridae* with unclassified genus and species. According to criteria proposed by the International Committee on Taxonomy of Viruses (ICTV), members of the same picornavirus genus should share greater than 40%, 40% and 50% amino acid identities for P1, P2 and P3 regions of the genome, respectively. Considering these criteria, all known fish picornavirus sequences seem to form separate genera. Phelps et al. (2014) proposed a new genus (*Piscesvirus*) for FHMPV. Barbknecht et al. (2014) suggested a new genus named *Lemavirus*—from *L. macrochirus*—for BGPV-1. Fichtner et al. (2013) proposed *Anguavirus* for eel picornavirus-1 (EPV-1) and suggested that EPV-1 probably forms a novel picornavirus genus. Similarly, Lange et al. (2014) proposed new genus and species names [*Cyprovirus* (from Latin Cyprinus = carp) and the genus name *Limnipivirus* (from Greek *limne*=lake, picornavirus), respectively] for CPV-1, based on unique genome organization and low sequence similarity with other fish picornaviruses (Fig. 21.3). These studies indicate the need for true classification of fish picornaviruses based upon morphology, genome sequence similarity, species specificity and the type of disease they cause.

21.3.3 Genome Organization

Like picornavirus from other species, fish picornaviruses appear to share some of the common features of the picornavirus genome, but they also contain some distinct features. The genome encodes a single polyprotein flanked by 5′ and 3′ non-translated regions (NTRs). The viral polyprotein is post-translationally cleaved into three regions P1, P2 and P3. These three regions are further processed into 10–12 small viral proteins, including the viral capsid proteins (VP4, VP3, VP2 and VP1), which are encoded by P1, while P2 and P3 encode nonstructural proteins that facilitate protein processing ($2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$) and genome replication [2B, 2 C, 3AB, 3B (VPg), $3CD^{pro}$ and $3D^{pol}$]. The complete genome size varies from the smallest EPV-1 (7496 nt) to the largest BGPV-1 (8050 nt) but the ORF is larger in FHMPV (7933 nt) than in BGPV-1 (7008 nt) (Table 21.2). The genome organization is almost the same (5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR)

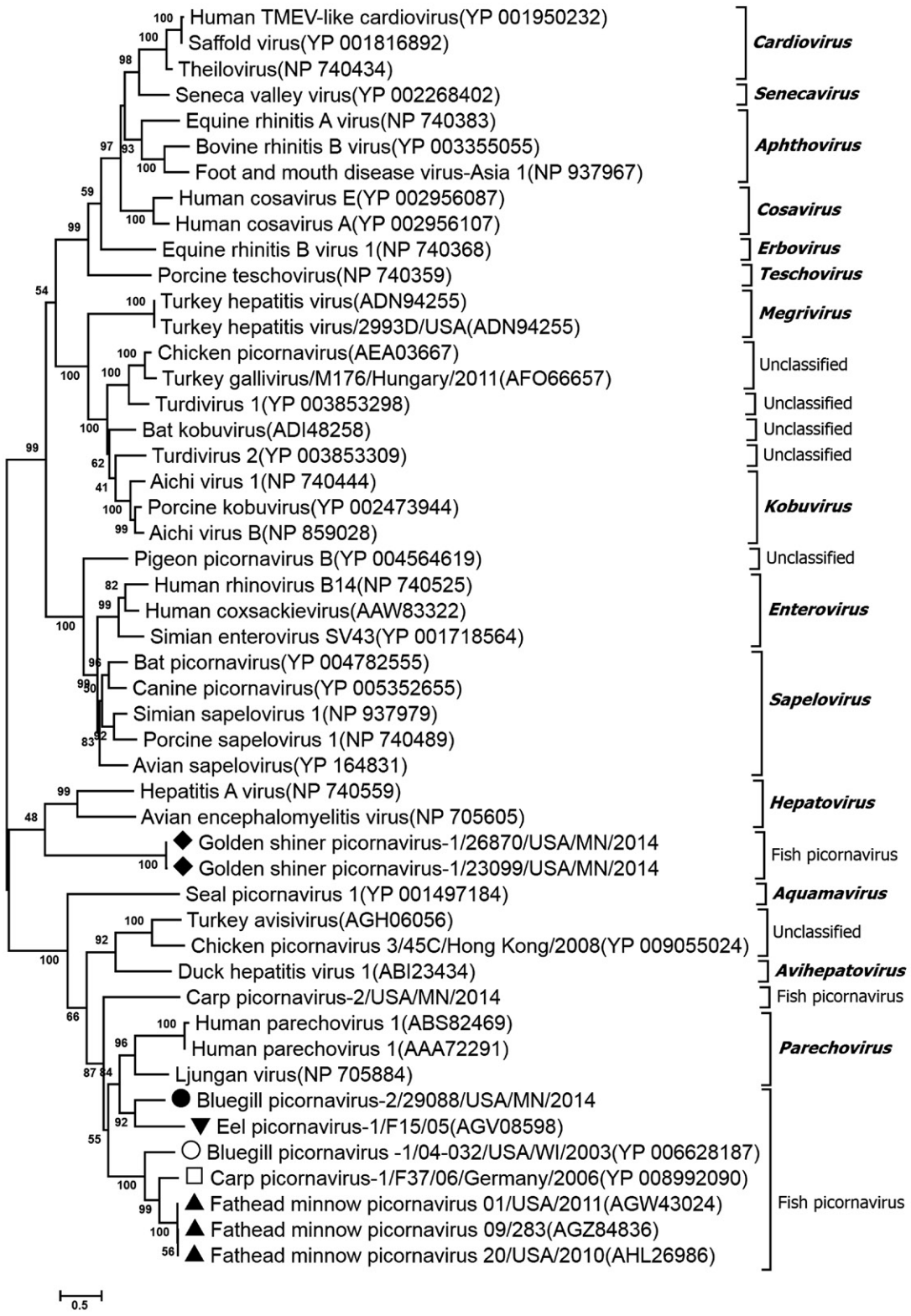

FIGURE 21.3 Phylogenetic analysis based on amino acid sequence of complete picornavirus 3D gene. The evolutionary history was inferred using the Maximum likelihood method with LG+ G+ I model in MEGA6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

TABLE 21.2 Comparison of Complete Genome Sequences of Different Fish Picornaviruses

Virus[a]	Complete Genome (nt)	5′ NTR (nt)	Polyprotein (nt)	3′ NTR (nt)	Genome Organization (5′-3′)	Characteristic Feature	GenBank Accession (Reference)
BGPV-1	8050	712	7008	330	5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR	Poly C tract of at least 21 nts	JX134222 (Barbknecht et al., 2014)
CPV-1	7632	524	6807	301	5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR	Insertion of nine amino acids adjacent to YGDD motif	KF306267 (Lange et al., 2014)
CPV-2[b]	7811	530	6948	297	5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR		Mor et al., unpublished data
EPV-1	7496	488	6777	231	5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR		KC843627 (Fichtner et al., 2013)
FHMPV	7834	557	7933	344	5′NTR-1AB-1C-1D-2A1-2A2-2A3-2B-2C-3A-3B-3C-3D-3′NTR	Predicted 2A3 protein	KC465953 (Phelps et al., 2014)
GSPV-1[b]	7707	510	7035	162	5′NTR-1AB-1C-1D-2A1-2A2-2B-2C-3A-3B-3C-3D-3′NTR		Mor et al., unpublished data

[a]*EPV-1, Eel picornavirus-1; FHMPV, Fathead minnow picornavirus; BGPV-1, Bluegill picornavirus-1; CPV-1, Carp picornavirus-1; CPV-2, Carp picornavirus-2; GSPV-1, Golden shiner picornavirus-1.*
[b]*Nearly complete genome sequence obtained by Illumina MiSeq. 5′ and 3′ NTRs may be incomplete.*

in all fish picornavirus except FHMPV in which there is an additional 2A3 protein. Interestingly, a poly C tract of 12–21 nt was observed in BGPV-1, which has not been reported in other picornaviruses (Table 21.2; Barbknecht et al., 2014).

21.4 EPIDEMIOLOGY

The family *Picornaviridae* is very diverse, having numerous genera and serotypes across a broad host range. Hence, it is not surprising to find more than a single picornavirus among fish species.

21.4.1 Host/Species Affected

The picornavirus fish host range has expanded to include ray-finned fishes (class *Actinopterygii*). Although they are all in the same class (*Actinopterygii*), FHM, bluegill and European eel belong to three different orders, *Cypriniformes*, *Perciformes* and *Anguilliformes*, respectively. In the order *Cypriniformes*, *Cyprinidae* is a large family of freshwater fishes, including the carps, golden shiners and minnows. Within this family, carp, shiners and minnow are infected with more than three different types of picornaviruses, which are suggested to form three or more different genera in the family *Picornaviridae*. In addition, BGPV-1 has been isolated from common carp, channel catfish (*Ictalurus punctatus*), pumpkinseed (*Lepomis gibbosus*), green sunfish (*Lepomis cyanellus*), black crappie (*Pomoxis nigromaculatus*) and largemouth bass (*Micropterus salmoides*), in addition to bluegill, indicating that a single virus has the potential to infect fishes of different families. High divergence in fish picornaviruses suggests that a high diversity of picornavirus is yet to be characterized in fish species.

21.4.2 Prevalence

BGPV-1 is widespread in bluegill populations of Wisconsin and perhaps across the entire range of bluegills as indicated by the studies of Barbknecht et al. (2014) and McCann (2012). Barbknecht et al. (2014) tested 30 bluegills from each of 17 sites in Wisconsin between Aug. and Nov. 2008 and found five sites (29%) to be positive for BGPV-1 by reverse transcription polymerase chain reaction (RT-PCR). These authors suggested that 30-fish sampling may not be enough to reliably detect the virus in waters if the prevalence is relatively low. Hence, sampling greater number of bluegills from waters previously testing positive, and from additional waters, will help to better understand the incidence and prevalence of BGPV-1.

A picornavirus from bluegill and black crappie (*P. nigromaculatus*) was associated with a fish kill in Lake Monona, Madison, Wisconsin. In this case, successive fish kills involving thousands of bluegills and black crappie occurred in winter and spring of 2009. Diseased fish of both species tested positive for BGPV-1 (Lasee, unpublished). Similarly, high mortality of bluegills and black crappie was reported in Minnesota in 2014. A novel picornavirus (BGPV-2) was detected from moribund fish, which was suspected as a causative agent of high mortality (Mor et al., unpublished data).

McCann (2012) reported that 8 of the 15 baitfish cases with unknown viruses tested positive for FHMPV by RT-PCR. Phelps et al. (2014) isolated 20 FHMPV isolates from FHMs in Illinois, Minnesota, Montana and Wisconsin, and from brassy minnows (*Hybognathus hankinsoni*) in Montana. These samples were collected from 2003 to 2011 indicating widespread time-space distribution of FHMPVs.

21.4.3 Time-Space Distribution

The FHMPV was isolated from a broad geographical area and different species over a time period from 2003 to 2011. Interestingly, all 20 isolates were genetically identical (Fig. 21.3). BGPV-1 and BGPV-2 were isolated from mortality events in bluegills and black crappies from two adjoining states of Wisconsin and Minnesota, respectively. However, the two viruses showed less than 40% aa (amino acids) identity with each other based on P1, P2 and P3 regions. Hence, we propose to place BGPV-2 in a novel genus different from the one proposed for BGPV-1 (*Lemavirus* genus). The possible reasons for this divergence could be the presence of yet unknown picornaviruses circulating in fish populations or due to a change in virus over time. Similarly, CPV-1 and CPV-2 isolated from common carp in Germany and the United States, respectively, at different time points also shared less than 40% aa identity based on P1, P2 and P3 regions, indicating that they may also belong to two different genera.

21.4.4 Seasonal Prevalence

It has been known that seasonality influences the prevalence of baitfish viruses including picornaviruses. McCann (2012) reported higher prevalence of FHMPVs in the summer. Similarly, BGPV-2 and GSV-1 were detected during summer (Mor

et al., unpublished data). This could be due to three main reasons: (1) rapidly warming water temperatures impose a greater amount of physiological host stress that may lead to an increase in host susceptibility; (2) population dynamics may also play a role in the high viral prevalence during summer; during the spring spawning season, adult fish are often highly aggregated (Lorenzen et al., 1991) resulting in increased viral transmission within the population; and (3) once fry hatch, they are at an increased risk of viral infection due to a lack of acquired immunity (Bergmann and Fichtner, 2008), leading to a high viral prevalence during late spring and early summer when these fish are most vulnerable. Furthermore, any surviving fish will likely clear the virus by fall or winter, thus acquiring some natural immunity and further limiting the spread of the disease.

21.5 PATHOLOGY AND IMMUNITY

Little information is available on the pathology of picornaviruses in different fish species. Three experimental studies have been conducted to fulfill Koch's postulates in bluegill, carp and eel, respectively. In an experimental study, Barbknecht et al. (2014) injected groups of 20 juvenile bluegills intraperitoneally (i.p.) with PBS (control), 1.5×10^4 p.f.u. (low-dose), or 1.5×10^7 p.f.u. (high-dose) of BGPV-1. Significant pathology was observed in virus-inoculated groups with the most common symptom being inflammation and redness at the base of the fins. There was no difference between low and high viral doses. In a second experimental study, infection was given by immersion of fish in water containing 1.5×10^5 p.f.u. BGPV-1/mL for 1 h, but no fish became infected, indicating that the pathology of BGPV-1 may depend on the route of infection, immune status of the fish or both. In addition, stressors such as other infections, rapid temperature change, low dissolved oxygen, crowding and spawning have been associated with bluegill fish kills in the past and may be important factors in making fish susceptible to infection and disease (Barbknecht et al., 2014; Rottmann et al., 1992).

Fichtner et al. (2013) did an experimental study by infecting groups of 16 glass eels by bathing them in virus-containing water (10^6 $TCID_{50}$/mL of water) for 1 h. The control group of 12 glass eels was treated with the same volume of an analogous cell culture medium without adding virus to the bath. Subsequently, the eels were kept in 400-L aquaria at a water temperature of 20°C for 21 days. A total of 7 of the 16 (43%) eels from infected group died without external signs at the end of the experiment. In the untreated control group, only one eel died as a consequence of an injury. Histological examination of all organs showed decreased numbers of fat vacuoles in hepatocytes of euthanized and dead eels compared to uninfected controls (Fichtner et al., 2013). Also present were increased single-cell necrosis, scattered small necrotic foci and mild infiltrations of lymphocytes and macrophages in the liver parenchyma. In the kidneys, vacuolization and hyaline droplet degeneration of renal tubular epithelium and multiple small necroses in the interstitium were observed. The virus was re-isolated from 10 of 10 infected (euthanized or dead) eels and was identified as a picornavirus by means of indirect immunofluorescence assay and EM. No virus was isolated from the control group.

Lange et al. (2014) conducted an experimental study with CPV-1 in specific pathogen-free (SPF) carp. Groups of five SPF carps (approx. 25 g) were infected by either i.p. injection of 10^6 $TCID_{50}$ CPV-1 F37/06 or by exposure of fish to virus-containing aquarium water (5 L of water containing 10^6 $TCID_{50}$ virus per mL) for 1 h. Subsequently, the carp were kept in 400-L aquaria for 21 days at a water temperature of 20°C. Two control groups of five carp each were treated and kept under the same conditions; the same volume of analogous cell culture medium without virus was administered or added to the aquarium water as a control. Carp that were i.p. infected with CPV-1 showed no clinical signs, but virus was re-isolated at various time points from different tissues such as 2 days postinfection (p.i.) from brain, 10 days p.i. from liver and heart and 21 days p.i. from brain and heart. Virus re-isolation was further confirmed by indirect immunofluorescence using 5C9 (a monoclonal antibody against CPV-1) and Cy3-conjugated goat antimouse IgG.

The results of these experimental studies show that picornaviruses have a wide distribution in different tissues after infection. Additional experimental infections with these viruses in different host species can help to better understand the pathogenicity of the virus for the same or different species of fish, pathogenesis of virus infection, influence of stressors on the susceptibility of fish to disease, age susceptibility and possible routes of infection.

21.6 CONVENTIONAL DIAGNOSTIC METHODS

21.6.1 Gross Pathology

There are no documented pathognomonic lesions for the detection and confirmation of fish picornavirus infection. As mentioned earlier, the picornaviruses have been detected in apparently healthy to dead fish with or without clinical signs or lesions. Some affected fish, such as FHMs and bluegills, can have hemorrhages in the skin and base of fins, erythema and pale and swollen internal organs (Barbknecht et al., 2014; Phelps et al., 2014). The affected eels have increased mucus production, mucous ulcers and reddened skin, but no hemorrhages (Fichtner et al., 2013).

21.6.2 Histopathology

The knowledge on histopathological changes due to infection of fish picornaviruses is poor at this point. Glazebrook et al. (1990) discovered picornalike viruses in degenerative areas of the brain and retina in 15-, 17- and 18-day-old barramundi larvae from a hatchery in North Queensland. Histopathological changes seen in longitudinal or sagittal sections included extensive vacuolation and neuronal degeneration of the mid and hindbrain and vacuolation of the retina, as well as severe hypertrophy of the respiratory epithelium. Vacuolation was also a feature of the intestinal mucosa in the hindgut with hyaline droplet formation. Vacuolation was also reported by Bloch et al. (1991) in infected brain of turbot larva. Vacuolated cells were present in all parts of the brain and in comparatively smaller numbers in medulla.

Fichtner et al. (2013) reported detailed examination of different organs especially liver and kidney after experimental inoculation of virus in glass eels. Liver of infected eels showed decreased numbers of fat vacuoles in hepatocytes, increased single-cell necrosis, scattered small necrotic foci and mild infiltrations of lymphocytes and macrophages in the liver parenchyma as compared to uninfected controls. In the kidneys, vacuolization and hyaline droplet degeneration of renal tubular epithelium and multiple small necrosis in the interstitium were observed.

21.6.3 Immunofluorescence

Indirect immunofluorescence can be used for the detection of fish picornaviruses as has been described for CPV-1 and EPV-1. Lange et al. (2014) described a method for the detection of CPV-1 in infected monolayers of FHM cells. Briefly, infected monolayers of FHM cells (after 24 h of incubation) were washed with isotonic buffer (NaCl 8.28 g/L, $Na_2HPO_4 \times 2H_2O$ 1.186 g/L, KH_2PO_4 0.2 g/L), fixed in acetone and rehydrated in isotonic buffer. After rehydration, 25 mL of 5C9 hybridoma supernatant was added and incubated at room temperature for 1 h. Hybridoma 5C9 was prepared after immunization of female BALB/c mice with concentrated and partially purified CPV-1 suspended in complete Freund's adjuvant. Indocarbocyanin conjugated goat antimouse IgG antibody was used as secondary antibody. The fluorescence intensity was rated from not detectable (–) to strong (+++) after microscopic examination (Fichtner et al., 2013). The cross-reactivity of monoclonal antibody 5C9 was tested on cells infected with other fish viruses including eel picornavirus. This method was also used by Fichtner et al. (2013) for detection of EPV-1 by using rabbit antiserum T51 and goat anti-rabbit IgG–FITC fluorescein isothiocyanate with counterstaining of nuclei with propidium iodide (Fichtner et al., 2013).

21.6.4 Electron Microscopy

Negative contrast EM is a good method for the detection of fish picornaviruses. The aggregation of nonenveloped spherical (30–32-nm) virions can confirm the presence of members of *Picornaviridae* (Fig. 21.2).

21.6.5 Virus Isolation

In order to maximize virus recovery, a variety of tissue samples should be processed, including kidney, spleen and encephalon, and plated onto cell cultures within 48 h of collection (AFS-FHS, 2010). Several cell lines have been used for the isolation of different fish picornaviruses such as eel embryonic kidney (EK-1) cells for EPV-1, bluegill fry-2 (BF-2) cells for BGPV-1 and FHM cells for CPV-1. In addition, four cell lines [epithelioma papulosum cyprini, FHM, CHSE-214, BF-2, or rainbow trout gonad (RTG-2)] have been used for FHMPV isolation at 15°C–26°C. The characteristic cytopathic effects (CPEs) consist of rounding and aggregation of cells with eventual widespread epithelial cell sloughing in FHMPV-infected cells (Phelps et al., 2014). CPEs involve cell elongation and accumulation of large cytoplasmic vesicles in BGPV-infected BF-1 cells (Barbknecht et al., 2014).

21.7 MOLECULAR STUDIES

21.7.1 Reverse Transcription Polymerase Chain Reaction

Barbknecht et al. (2014) developed a highly sensitive and specific RT-PCR assay for detection of BGPV-1. In this gel-based RT-PCR assay, forward and reverse primers were designed to anneal within the 3′ NTR, producing a 180-bp amplicon (Table 21.3). The lower detection limit of this assay was 1.9×10^4 p.f.u. Mor et al. (2015) developed a multiplex RT-PCR assay for the detection of BGPV-1, FHMPV and EPV-1. Three primer sets specific for each virus were designed to amplify conserved RNA-dependent RNA polymerase (RdRp) region (Table 21.3). This assay was found to be very sensitive with

TABLE 21.3 Details of Primers Used for PCR Amplification and Sequencing of Fish Picornaviruses

RT-PCR Assay	Virus[a]	Primers	Product Size (bp)	References
RT-PCR	BGPV-1	F 5′-CTCGATAGTGTATGACTCGGACC-3′	180	Barbknecht et al. (2014)
		R 5′ CATGGGGTTCAACACTCACA-3′		
Multiplex RT-PCR	EPV-1	EPV-1_mul_F 5′CAATGGCACATGGAGAGTTG 3′	558	Mor et al. (2015)
		EPV-1_mul_R 5′ CCTTGTTCGATTTCCTCCAA 3′		
	FHMPV	FHMPV_mul_F 5′ACAATGAGRAGTTTCATGCC 3′	821	
		FHMPV_mul_R 5′AATCACGGTCTCATGAAGTCT 3′		
	BGPV-1	BGPV-1_mul_F 5′GGCACTTGGAAAGACGAGAG 3′	1043	
		BGPV-1_mul_R 5′ ATTGTCAAACGGTCCGAGTC 3′		

[a]*EPV-1, Eel picornavirus-1; FHMPV, Fathead minnow picornavirus; BGPV-1, Bluegill picornavirus-1.*

a detection limit of 81.9 pg/μL of extracted RNA from a pool of FHMPV and BGPV-1 and was able to detect 501 and 224 gene copies/μL of BGPV-1 and FHMPV, respectively. The assay was highly reproducible and did not cross-react with other closely related pathogens.

Although, these assays are very sensitive and specific, there are some limitations. These assays are based on one or few picornavirus sequences of a particular fish species and hence there may be a possibility that some divergent strains (that may evolve over time) may not be amplified by these gel-based assays. For example, recently detected BGPV-2 is divergent from BGPV-1 and was not detected by the aforementioned assays. Hence there is a need to use more advanced techniques for detection of currently unknown divergent fish picornaviruses.

21.7.2 Random Amplification, Cloning and Sequencing

This is one of the best methods available for the detection of unknown pathogens. In this method, extracted RNA is reverse transcribed using random primers followed by cloning of amplified product and sequencing of positive insert clones. Barbknecht et al. (2014) used this technique for amplification and detection of BGPV-1. Briefly, varying amounts of extract RNA were reverse-transcribed in a total volume of 25 μL using 200 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase in standard buffer with 0.01 M DTT, 0.5 mM dNTPs and 0.4 mM reverse primer 3′ RACE A (5′CGATCGCTCGAGAATAGCCCTTTTTTTTTTTTTTTTTTT-3′) for 45 min at 37°C. PCR was performed with 2 μL cDNA, 2.5 U *Taq* polymerase in standard buffer with 0.2 mM dNTPs, 0.4 mM reverse primer and 0.4 mM of either the IAYGDD (5′-ATIGCITAYGGIGAYGA-3′) or ICYGDD (5′-ATITGYTAYGGIGAYGA-3′) forward primer. Amplified DNA smears ranging in size from 300 to 2000 bp were gel purified. Gel extracts and amplicons were cloned into the pCR8/GW/TOPO vector (Invitrogen) and sequenced. Sequence data for clones were manually trimmed of vector and primer sequence and assembled into contigs, which were further matched with previously known closest relatives of picornavirus sequences in GenBank database. A similar approach with some modifications was used by Phelps et al. (2014) for complete genome amplification of FHMPVs. Briefly, cDNA was synthesized using the superscript III RT kit (Invitrogen) and Oligo $(dT)_{20}$ supplied with the kit. The PCR reaction was carried out on amplified cDNA by using universal primer 5′-CCGACTCGAGINNNNNNTGTGG-3′. The amplified products were purified and then cloned with Zero blunt PCR cloning kit (Invitrogen). Cloned plasmids with positive insert were sequenced and sequences obtained were analyzed to match with those available in GenBank. These methods of random amplification, cloning and sequencing are very good and have been used for detection of unknown pathogens. Unfortunately, they are time consuming and may require hit and trial method of using different types of primers.

21.7.3 Next-Generation Sequencing

Sequencing methods have improved greatly since the first version of the Sanger technique (eg, first-generation sequencing) was described (Belák et al., 2013; Smith et al., 1986). This has facilitated the development of very powerful tools for detecting and identifying various pathogens, such as viruses, bacteria and other microbes. The advanced second-generation sequencing is also known as *high-throughput sequencing* or *NGS*. These technologies are becoming more popular and are

being commonly used for detection and complete genome amplification of known and unknown pathogens due to dramatic reduction in DNA sequencing costs. There are three main platforms for NGS, for example, 454 pyrosequencing (Roche), SOLiD (ABI) and Illumina (Illumina/Solexa). Illumina is of two types, HiSeq and MiSeq, of which Miseq is more popular and most commonly used. This technique has been used for detection and complete genome amplification of BGPV-2, CPV-1, EPV-1, golden shiner picornavirus-1 (GSPV-1) and FHMPV (Fichtner et al., 2013; Lange et al., 2014; Mor et al., unpublished data; Phelps et al., 2014).

21.7.3.1 Illumina Sequencing

Phelps et al. (2014) used Illumina Miseq for detection and complete genome amplification of FHMPVs. Briefly, good quality total viral RNA free from ribosomal RNA and DNA was subjected to Illumina Miseq. The library was prepared by using TruSeq RNA v2 followed by sequencing on a 300-PE run on the MiSeq. The obtained sequence reads were analyzed by CLC Genomics Workbench 7.5 (www.clcbio.com). After trimming adapter sequences and sequence quality testing, contigs were prepared by *de novo* assembly. Extracted contigs were analyzed by BLASTx analysis on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORFs were predicted by the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A similar method has been used for complete genome amplification of BGPV-2, CPV-2 and GSPV-1 (Mor et al., unpublished data).

Fichtner et al. (2013) used Illumina/Solexa method for complete genome amplification of EPV-1. Viral RNA was purified from EPV-1-infected EK-1 cells for Illumina sequencing. Sequencing was performed on a GAIIx (Illumina) instrument to create reads with lengths of 76 nt. The obtained Illumina sequences were assembled by *de novo* assembly and sequence reads were mapped to the NCBI sequence database to identify similar picornavirus reads. A similar approach was used by Lange et al. (2014) for complete genome amplification of CPV-1.

21.8 PREVENTION AND CONTROL

Given the broad host and geographic range or fish picornaviruses, these viruses have likely been spread through natural and human pathways for a long period of time and are only recently being described due to advancements in diagnostic technology and awareness. Prevention and control for fish picornaviruses is challenging due to a variety of factors, including inconsistent or lack apparent signs of disease, limited use and availability of sensitive and specific diagnostic assays and inconsistent regulations and oversight of fish health concerns.

Although a confirmed outbreak of fish picornavirus has not been associated with farmed fish, species of aquaculture interest are known to be susceptible including bluegill, black crappie, FHM, carp, eel and others. Strict biosecurity to prevent the introduction of the virus from wild fish and water sources is critical. Such a program should include routine diagnostic investigation of imported brood fish, eggs, or forage fish, decontamination of fomites and the use of a clean water source. No therapeutics or vaccines are available for picornaviruses of fish. In addition, disinfectants have not been evaluated for potential sources such as eggs, fomites and water.

In wild fish populations, there is no feasible control once a population has become infected, therefore for picornaviruses of concern, programs to prevent spread through human routes should considered. All potential routes and conditions for transmission have not been evaluated for picornaviruses and thus a conservative approach to management is justified.

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Chapter 22

Caliciviruses of Fish

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22.1 INTRODUCTION

The family *Caliciviridae* is composed of small, nonenveloped viruses, consisting of a nonsegmented, polyadenylated, positive-sense, single-stranded RNA (+ssRNA) genome of about 7.3–8.5 kb in length, enclosed in an icosahedral capsid of 27–40 nm in diameter. At present there are five genera, the *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* (International Committee on Taxonomy of Viruses, http://ictvonline.org). Two other genera have been proposed recently, the *Recovirus* and the *Valovirus*, and several unclassified caliciviruses have also been presented (Farkas et al., 2010, 2008; L'Homme et al., 2009; Wolf et al., 2012). The viruses infect a wide range of hosts and cause a wide spectrum of disease conditions, from gastroenteritis in humans of all ages and several animals like pigs, calves, cats and dogs, to vesicular lesions and reproductive failure in pigs and sea lions, respiratory infections in cats and cattle, fatal hemorrhagic disease in rabbits and infectious stunting syndrome in chickens (Bank-Wolf et al., 2010; Scipioni et al., 2008; Smith et al., 1998). Still, viruses are also found in species where the hosts are asymptomatic (Farkas et al., 2008; Tse et al., 2012; Wang et al., 2005; Wolf et al., 2012, 2011, 2009).

The vesiviruses include a group of viruses characterized as marine caliciviruses, infecting hosts of several species of pinnipeds and cetaceans (ie, seals, sea lions, walruses, whales and dolphins) (Smith et al., 1973). This group also includes the first detection of calicivirus in fish, as the findings of the San Miguel sea lion virus (SMSV) was presented in 1980 in opaleye perch (*Girella nigricans*), a wild-caught marine fish with a range around the Californian and Mexican coast (Smith et al., 1980). Almost 35 years later, a second calicivirus infecting a marine fish was presented, the Atlantic salmon calicivirus (ASCV). ASCV is found at high prevalence in farmed Atlantic salmon in Norway. Initially, it was incorrectly assumed to be related to the marine vesiviruses. It represents a unique branch-sharing root with the branch of noroviruses infecting humans and the unassigned Tulane virus and St-Valérien-like viruses, infecting rhesus monkey and pig, respectively, with relatively large distance to the marine calicivirus subgroup of vesiviruses (Mikalsen et al., 2014; Fig. 22.1).

Neither SMSV nor ASCV is yet shown to cause any disease of their respective fish hosts, the opaleye perch and Atlantic salmon (Table 22.1). SMSV cause vesicular exanthema of swine and the marine hosts of this virus are mainly described as a virus reservoir (Smith et al., 1973). Thus the marine caliciviruses of the vesivirus genera are considered as reportable viruses due to their potential of producing this exotic disease of swine. ASCV is recently presented and although the infection seems to be asymptomatic, there is still much work to be done to conclude its pathogenic potential.

22.2 SPECIFIC MAIN FISH DISEASES

As mentioned, none of the two caliciviruses infecting fish have been shown to cause disease in their respective hosts. ASCV was originally found after extensive cell culturing of a heart tissue sample from an Atlantic salmon diagnosed with heart and skeletal muscle inflammation (HSMI), but screening for the virus has shown its presence in fish experiencing HSMI, cardiomyopathy syndrome (CMS), pancreas disease (PD), symptoms of malabsorption syndrome of salmon (also associated with floating feces in the fish pens) and in asymptomatic fish (Mikalsen et al., 2014; own observations). ASCV was recently characterized and more work is needed to conclude on a possible link to a disease condition(s), or if ASCV is a by-stander of other fish pathogens that can augment or modulate disease outcome. The published results on SMSV in relation to its fish host, opaleye perch, is scarce and has not been updated since it was originally presented. For the latter virus, presence and replication is shown both in field and experimental challenges, but without any evidence of signs of disease (Smith et al.,

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00022-X
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1980, 1981). In addition, the viral infection is not considered important in relation to aquaculture because opaleye perch is generally considered an important game fish and may incidentally be caught in commercial fishing activities. The fish was used for feeding of marine pinniped mammals and swine, and virus from infected fish has historically been shown to cause serious diseases in these animals [ie, the vesicular exanthema of swine (VES), an epizootic that swept across much of the United States in past decades] (Smith et al., 1998, 1981).

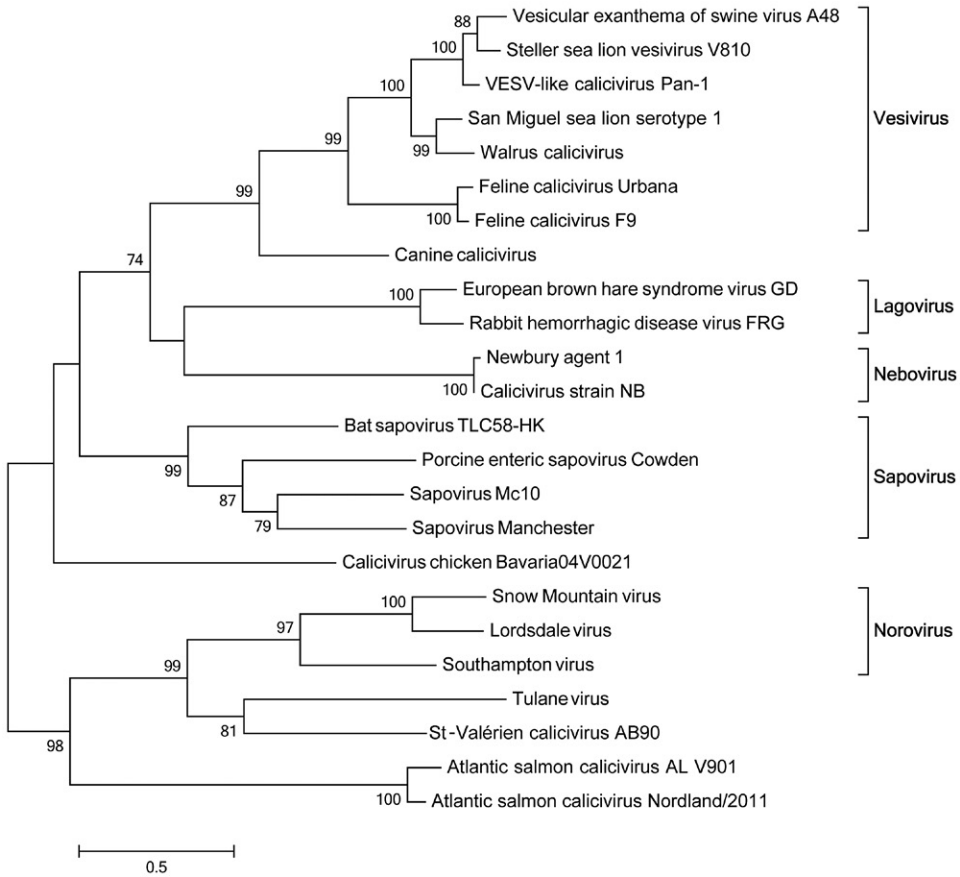

FIGURE 22.1 Evolutionary relationships of ASCV with members of the family *Caliciviridae*. Phylogenetic analysis is based on sequence regions encompassing the capsid or putative capsid of representatives of the five known genera of caliciviruses, proposed new genera and unassigned published viruses. Members of the family *Caliciviridae* included in the tree are as follows: Vesicular exanthema of swine virus (VESV) serotype A48 (NP786889); Steller sea lion vesivirus V810 (ABP88255); VESV-like calicivirus Pan-1 (AAC61759); San Miguel sea lion (SMSV) serotype 1 (AAG13639); Walrus calicivirus (NP786921); Feline calicivirus (FCV) Urbana (NP78331); Feline calicivirus (FCV) F9 (from AAA79327); Canine calicivirus (NP786912); European brown hare syndrome virus (EBHSV) GD (from CAA93445); Rabbit hemorrhagic disease virus (RHDV) FRG (NP740333); Newbury agent 1 (from YP529550); Calicivirus strain NB (AAT35531); Bat sapovirus (Bat-SaV) TLC58-HK (from AFJ39355); Porcine enteric sapovirus (PEC) Cowden (from NP051035); Sapovirus (SaV) Mc10 (YP052971); Sapovirus (SaV) Manchester (CAA60262); Calicivirus chicken Bavaria04V0021 (from ADN88287); Snow Mountain virus (AAN08112); Lordsdale virus (CAA60255); Southampton virus (AAA92984); Tulane virus (ACB38132); St-Valérien calicivirus AB90 (from ACQ44559). Only bootstrap values of 60% and above have been displayed in the output (Mikalsen et al., 2014).

TABLE 22.1 Caliciviruses of Fish

Main Characteristic Group Virus	Main Diseases	Host	References
Atlantic salmon calicivirus (ASCV)	Asymptomatic/unrevealed	Atlantic salmon	Mikalsen et al. (2014)
San Miguel sea lion virus (SMSV)	Asymptomatic	Opaleye perch	Smith et al. (1980, 1981)

22.3 STRUCTURE AND COMPOSITION OF VIRUS

As for all *Caliciviridae* family members, the structure of the SMSV is icosahedral with a T = 3 consisting of 90 dimers of the major capsid protein (Fig. 22.2; Chen et al., 2006, 2004; Prasad et al., 1994; Thouvenin et al., 1997). This is not confirmed for ASCV, but negative staining of electron micrographs of cell-culture-isolated virions shows a size of approximately 42 nm and indications of protrusions and depressions on the viral surface with similarity to other caliciviruses (Fig. 22.3; Mikalsen et al., 2014). In addition, genetic characteristics show similarities in protein expression with the other caliciviruses, which implies that the virus structures are similar.

The genome encapsidated by the viral particle is a nonsegmented+ssRNA with size 7.4 kb and 8.3 kb for the ASCV and SMSV, respectively (Figs. 22.4 and 22.5). The RNA genome is polyadenylated in the 3′ end and all calicivirus are described as having a genome-linked viral protein (VPg) covalently attached to the 5′ end of the RNA (Goodfellow et al., 2005; Herbert et al., 1997). The presence of VPg is not yet confirmed for ASCV (Mikalsen et al., 2014). The genome of all caliciviruses includes two or three open reading frames (ORFs). In ASCV (Mikalsen et al., 2014) and the calicivirus genera lago-, sapo- and nebovirus, the capsid-encoding region of the genome is included in the 3′ end of ORF1, which means it is translated as a part of the polyprotein and then proteolytically processed (Fig. 22.4). All caliciviruses include a small ORF in the 3′ end of the genome, encoding a protein referred to as VP2 (Figs. 22.4 and 22.5). The function of this protein is not fully characterized, but it has been shown to be a structural protein (Glass et al., 2000; Sosnovtsev and Green, 2000). The noroviruses and the vesiviruses, including SMSV, have an ORF1 encoding a polyprotein precursor, which is proteolytically

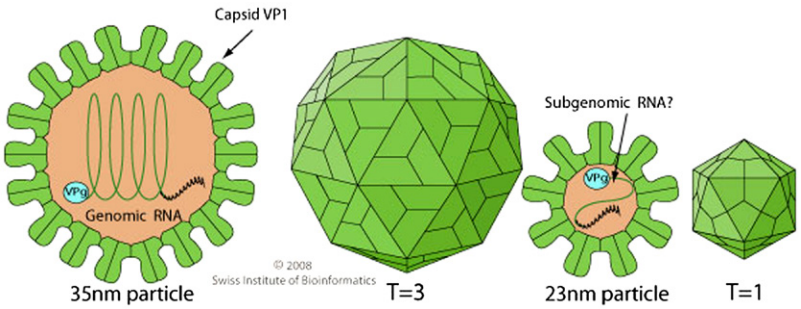

FIGURE 22.2 Calicivirus, with nonenveloped capsid of about 27–40 nm in diameter. There is a T = 3 icosahedral symmetry. The capsid is composed of 180 capsid proteins. *Philippe Le Mercier, ViralZone:* www.expasy.org/viralzone*, SIB Swiss Institute of Bioinformatics.*

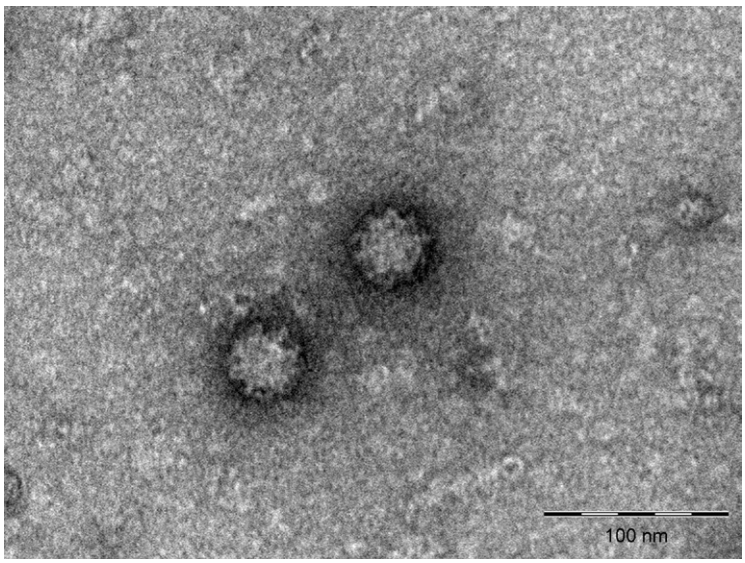

FIGURE 22.3 Negative staining of electron micrographs of ASCV isolated from cell culture showing virus-like particles of approximately 42 nm. Indications of protrusions and depressions on the viral surface. Bar = 100 nm (Mikalsen et al., 2014).

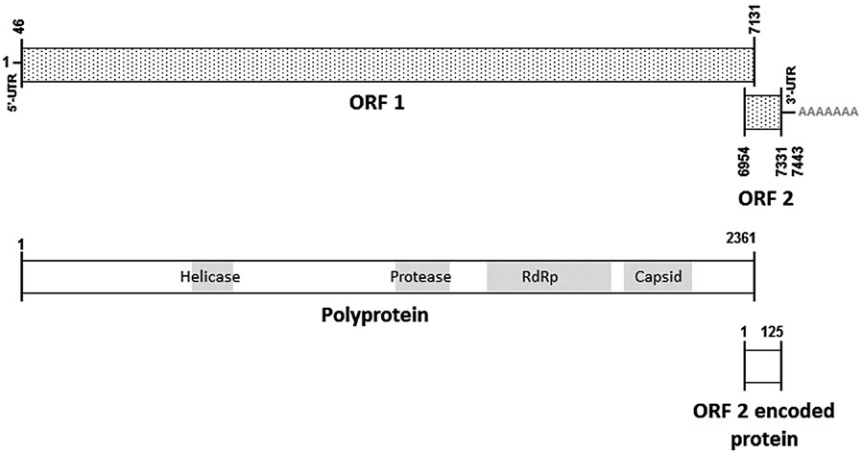

FIGURE 22.4 Schematic presentation of the ASCV genome organization (upper) and predicted encoded proteins (gray shading, lower). The capsid-encoding region is included in the 3′ end of ORF1, similar to the calicivirus genera lago-, sapo- or nebovirus where the capsid is translated as part of the polyprotein also including nonstructural proteins, and proteolytically processed. The RNA genome is polyadenylated in the 3′ end. *Adapted from: Mikalsen, A.B., Nilsen, P., Froystad-Saugen, M., Lindmo, K., Eliassen, T.M., Rode, M., et al., 2014. Characterization of a novel calicivirus causing systemic infection in Atlantic salmon (Salmo salar L.): proposal for a new genus of caliciviridae. PLoS ONE 9, e107132.*

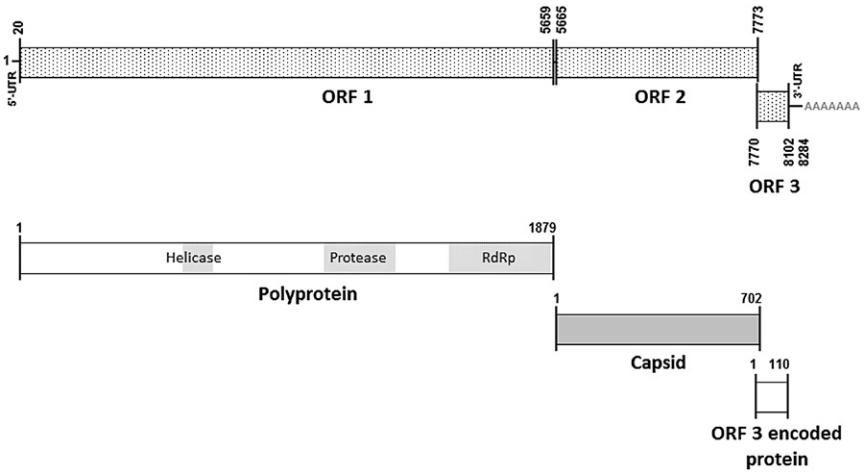

FIGURE 22.5 Schematic presentation of the vesivirus SMSV genome organization (upper) and predicted encoded proteins (gray shading, lower). The virus genomes in vesivirus and norovirus genera include an ORF1 encoding a polyprotein precursor, which is proteolytically processed to result in the nonstructural proteins helicase, genome-linked viral protein (VPg), protease and the RNA-dependent RNA polymerase (RdRp). ORF2 encodes the major capsid protein.

processed to result in the nonstructural proteins helicase, genome-linked VPg, protease and the RNA-dependent RNA polymerase (RdRp) (Fig. 22.5). ORF2 encodes the major capsid protein separately.

22.4 CLASSIFICATION AND VIRUS REPLICATION

The first characterized marine calicivirus was SMSV, which was presented in the early 1970s (Smith et al., 1973), then isolated from California sea lions. A morphological and antigenic relationship was found to be similar to vesicular exanthema of swine virus (VESV) and this isolate was later described genetically and suggested to be classified as a single genotype within the *Caliciviridae* (Neill et al., 1995; Neill and Seal, 1995). The genus was later called *Vesivirus* and also included feline calicivirus (FCV) (Thouvenin et al., 1997). ASCV has recently been discovered and is not yet officially assigned to a virus family. It has been proposed as member of the *Caliciviridae*, based on size and characteristics of virion and similar genetic characteristics, such as presence and size of ORFs, identification of conserved amino acid motifs and protein

domains in encoded nonstructural proteins and a conserved domain for calicivirus coat protein (Mikalsen et al., 2014). By phylogenetic studies based on amino acid sequences of the capsid of two variants of ASCV and representatives of all genera of *Caliciviridae* and other unassigned caliciviruses, ASCV does not cluster with SMSV or other marine caliciviruses, which all constitute a subgroup of the *Vesivirus* genus. Instead, ASCV is a separate branch rooting with the noroviruses and the unassigned Tulane virus from rhesus monkeys and a novel group of swine caliciviruses, St-Valérien-like viruses (Farkas et al., 2010, 2008; L'Homme et al., 2009; Mikalsen et al., 2014; Fig. 22.1). The calicivirus capsid protein carries an amino acid motif, L/VAGNAF, which is suggested to play a structural role and is conserved across all five genogroups of norovirus and also found in Tulane and St-Valérien-like viruses (L'Homme et al., 2009; Oliver et al., 2003). Alignments of the putative capsid of ASCV the motif came out as VGANNF (Mikalsen et al., 2014), thus not fully conserved, reflecting this is a branch separate from the noroviruses and Tulane and St-Valérien-like viruses. ASCV genome organization is also different from noroviruses and Tulane virus. ASCV, like St-Valérien-like viruses, has two ORFs (Fig. 22.4), while noroviruses and Tulane virus are organized in three ORFs (Fig. 22.5). The predicted VP2 of ASCV is 125 amino acids, ORF2 encoded and is significantly shorter than the VP2 of noroviruses and the Tulane virus, containing more than 200 amino acids (L'Homme et al., 2009). There is also a relatively large overlap between ASCV ORF1 and ORF2 of 175 nt, unique among the caliciviruses, for which the overlap is either 20 nt or less or absent. In conclusion, this suggests that ASCV represents a new genus within the *Caliciviridae* genus and the proposed genus name is *Salovirus* (Mikalsen et al., 2014).

SMSV is shown to replicate to a high titer in mammalian cells in culture and supernatants from the cell cultures can infect the opaleye perch and cause a systemic infection with the highest titers seen in the spleen (Smith et al., 1981). Similarly, ASCV replicates in fish cells in culture and virus-positive supernatants cause a systemic infection in Atlantic salmon. The highest viral load found by genetic detection is in kidney and heart at 8 weeks post-challenge (Mikalsen et al., 2014). Details of replication of the two caliciviruses in their respective fish hosts are not described. In general, the replication cycle of calicivirus is poorly understood, since among the five assigned calicivirus genera and the unassigned viruses, only a few viruses can be easily propagated in cell systems and subsequently used in replication studies. Together with newer technologies, like reverse genetics, common characteristics of the replication of caliciviruses have been found (Alhatlani et al., 2015; Oka et al., 2014). The viral capsid attaches to a cellular receptor and subsequently the virion with the RNA genome is internalized by packaging into endocytic vesicles. The viral+ssRNA genome is then uncoated from the virion and endocytic vesicle and exposed to the cytoplasm, where translation begins. VPg covalently linked to the 5′ end of the genomic RNA uses the cellular translation machinery and positions the ribosome at the initiation codon AUG (start codon) without ribosome scanning and then initiates translation (Putics et al., 2010). The polyprotein precursor for the nonstructural proteins is first translated and cotranslationally processed to result in an Nucleoside Triphosphatase (NTPase), VPg, a protease and the RdRp, which assemble into a replication complex. A negative-strand copy of the genome is then synthesized by this complex, which subsequently is used as a template for synthesis of new copies of the genomic+ssRNA and for the caliciviruses that encode the capsid protein in a separate ORF (norovirus, vesivirus and unassigned viruses), a subgenomic RNA encoding the structural proteins capsid and VP2. For these viruses, the subgenomic RNA is then translated into the two structural proteins using VPg to position the ribosome similarly as described for genomic RNA. In the viruses where the capsid is encoded in the C-terminal end of the polyprotein, including the nonstructural proteins (*sapoviruses*, *lagoviruses*, *neboviruses* and unassigned), the coat protein is released from the polyprotein precursor after processing by the viral protease. Assembly of the structural proteins as well as packaging of the genomic RNA follows the process and a mature virion can finally be released from the cell (Abente et al., 2010; Putics et al., 2010). By analogy, these general characteristics should also apply to SMSV and ASCV.

22.5 EPIDEMIOLOGY

ASCV was initially found as two variants, one cell culture isolate and one field strain. Sequence comparison of the full genome of the field strain versus the cell culture isolate, showed only 70.7% nucleotide identity. The discrepancy was spread throughout the whole genome. For ORF1 encoding, the nonstructural proteins and the capsid, the nucleotide identity is 70.9% and 75.7% for ORF2-encoding VP2. Translated to amino acids, the ORF1-encoded polyprotein of the field strain and cell culture isolate has 74.5% amino acid identity (84.0% when similar amino acids are included) and the ORF2 protein has 73.6% (90.4% including similar amino acids) (Mikalsen et al., 2014). Screening using molecular methods specific for the cell culture isolate has not revealed any presence in the Atlantic salmon aquaculture, but the dataset is limited. However, screening using methods specific for the field strain have shown a high prevalence in Norwegian Atlantic salmon farms. Field screening related to fish with heart pathology show ASCV prevalence from 10% to 100% in combinations with presence of Piscine orthoreovirus (PRV)-causing HSMI, Piscine myocarditis virus (PMCV), causing CMS and/or SAV3, causing PD, but ASCV can also be found in 100% of salmon with no heart pathology with previous history of symptoms

of malabsorption and floating feces in the fish pens or in asymptomatic fish (Mikalsen et al., 2014; own observations). Although ASCV is found coincidently with other viral infections and diseases, the present epidemiologic knowledge does not point to any relation to these or other infections or diseases.

Detailed molecular epidemiological studies have not been carried out for ASCV because it was described for the first time fairly recently. A small study of field samples within a limited geographical region showed virus strains with high genetic homogeneity (Mikalsen et al., 2014). Within the two genome regions studied, one in 5′ end of genome with high variability between the cell culture isolate and the field strain presented and one in the capsid-encoding region, the lowest nucleotide identity found was 97% and the lowest amino acid identity was 99% (Mikalsen et al., 2014).

Opaleye perch is not used in aquaculture and only occurs in wild habitats on the west coast of North America from Cape San Lucas, Mexico, to San Francisco, California. Epidemiological data on SMSV infections of opaleye perch does not exist, but SMSV has generally been shown to be widespread among marine and terrestrial mammals, such as sea lions, seals, whales, amphibians, reptiles, swine and primates (Barlough et al., 1998; Smith et al., 1986, 1983a).

22.6 PATHOLOGY AND IMMUNITY

Although marine caliciviruses have been shown to cause serious pathology in marine mammals, like vesicular lesions in the mouth and on flippers of pinnipeds (Schaffer and Soergel, 1973; Smith et al., 1983b), no pathology has been described for ASCV or SMSV infected fish (Mikalsen et al., 2014; Smith et al., 1980, 1981).

Studies on the immune response to infection have not been performed, but for ASCV, a vaccine candidate prepared from formalin-inactivated virus has been tested in laboratory experiments and showed that infection of Atlantic salmon with ASCV can be reduced or eliminated by vaccination. The preliminary experimental vaccination and challenge were done using a cell culture-grown isolate, which is different from the ASCV strains generally found in samples collected from the field. The isolate established a systemic infection in nonvaccinated fish and parallel vaccine-injected fish showed complete protection to infection in three of seven individuals at each of the two samplings performed. The remaining four fish at each sampling were virus negative in all organs tested, with the exception of the kidney, where ASCV was detected at low levels compared with nonvaccinated controls (Mikalsen et al., 2014).

22.7 DIAGNOSTIC METHODS

The diagnostic methods for caliciviruses of fish are based on detection of the virus (or the virus genome) because ASCV and SMSV cause no clinical signs or pathology in infected fish. A real-time polymerase chain reaction (PCR) specific for an ASCV field strain was established using primers against the putative capsid-encoding region. This has been demonstrated to be specific for field isolates from Atlantic salmon farms geographically distributed along the Norwegian coastline. A similar real-time PCR was established specifically for the cell culture-adapted isolate of ASCV, but since the adapted strain is genetically distant to the field strains, this PCR method is not optimal for testing of field samples (Mikalsen et al., 2014). Genetic homogeneity of field strains is high and it thus seems unnecessary to sequence field strains. The presence of strains in the field with high homogeneity to the cell culture isolate is apparently low.

Although there is one isolate of ASCV that can replicate in a cell line, the information on the replication capacity of the field strains is scarce and cell culture is not a reliable diagnostic method. Since no antibodies have yet been produced against ASCV, diagnostic antibody-based techniques like enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFAT) and immunohistochemistry are not available.

Diagnostic methods for use in detecting SMSV in its fish host, Opaleye perch specifically, have not been presented since the original detection of the virus in this host by cell culture and serotyping using a virus neutralization test in the early 1980s (Smith et al., 1980). The virus is widely distributed among and can cause disease in other hosts, and is therefore considered as a reportable virus due to its potential to cause VES, several host nonspecific diagnostic methods are available. More recently, detection of virus genome by real-time reverse transcription PCR and detection of antibodies against the virus by ELISA have been presented for SMSV (McClenahan et al., 2009, 2010).

22.8 PREVENTION AND CONTROL

The need for prevention and control of the infection currently seems limited because none of the caliciviruses infecting fish are associated with clinical signs of disease. ASCV is widely distributed in the Norwegian Atlantic salmon industry, but before any prevention and/or control measures are implemented, the importance of the presence of the virus would need further research. If ASCV infection can be linked to a particular disease entity, vaccination could be a good alternative for

prevention because preliminary studies has shown a good potential of limiting the infection using a formalin-inactivated vaccine (Mikalsen et al., 2014).

The fish host of SMSV, opaleye perch, is not used in aquaculture. It is only present in wild habitats and generally considered an important game fish and may only incidentally be caught in commercial fishing activities. Prevalence data of SMSV in opaleye perch is also nonexistent and taken together, prevention and control measures against SMSV infection of opaleye perch are not relevant.

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Chapter 23

Togaviruses of Fish

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23.1 INTRODUCTION

Togaviridae are enveloped, spherical viral particles with icosahedral virions with diameters of 60–70 nm. Salmonid alphavirus (SAV) belongs to the genus *Alphavirus* and is the only known aquatic member of *Togaviridae*. SAV is the causative agent of pancreas disease (PD) and sleeping disease (SD) in salmonid fish (McLoughlin and Graham, 2007). These two diseases are basically the same, but are named differently for historical reasons.

23.2 PANCREAS DISEASE AND SLEEPING DISEASE

PD was first described in farmed Atlantic salmon in seawater. The name of the disease was based on the changes in exocrine pancreatic tissue, detected by histopathological examination (Munro et al., 1984). Similarly, SD was later described in farmed rainbow trout in fresh water. The name of that disease was based upon the observed clinical signs. The diseased fish were found resting on their sides on the bottom of the tanks, and when disturbed, they swam for some time before returning silently to the bottom (Boucher and Baudin Laurencin, 1994). Further studies of the two diseases indicated that they were closely related (Boucher and Laurencin, 1996).

Clinical signs may also include a sudden drop in appetite a week or two before mortality occurs. Some fish may be observed swimming slowly at the water surface, or the fish may be found resting at the bottom of the tank or the cage. Mortality may vary between fish farms, geographical regions and SAV subtype. Subclinical PD has also been reported (Graham et al., 2006).

After a disease outbreak, a variable proportion of the survivors may develop into long, slender fish with lower weight and less fat tissue than the rest of the population (McLoughlin and Graham, 2007; Taksdal et al., 2014). This may add significantly to the economic losses caused by the disease (Aunsmo et al., 2010). At present, PD and SD are confined to Europe (Table 23.1).

23.3 STRUCTURE AND COMPOSITION OF THE VIRUS

Togaviridae consists of two genera: *Rubivirus* and *Alphavirus*, where the SAV is the only known aquatic member. Alphavirus virions are icosahedral with a diameter of approximately 70 nm and are composed of a single RNA genome of 11–12 kb, surrounded by the capsid protein, which forms a spherical shell. Outside the capsid, repeated units of the E1 and E2 transmembrane glycoproteins transverse a lipid bilayer derived from the host plasma membrane (Kuhn, 2007). Another membrane-associated protein, 6K is also found in the virus particle and is involved in membrane permeabilization, protein processing and virus budding. The 6K protein is necessary for the generation of infectious SAV particles (Guo et al., 2014).

The RNA genome contains a 5′ terminal 7-methylguanosine and a 3′-terminus that is polyadenylated. The coding regions for the nonstructural (nsP1, nsP2, nsP3 and nsP4) and structural (capsid, precursor E2 (p62) and E1) proteins are organized in two open reading frames (ORFs), with the replication region in the 5′ two-thirds and the structural proteins in the 3′ one-third. Nonstructural replication proteins are translated directly from the genome RNA. In contrast, structural proteins are translated from a subgenomic messenger RNA (mRNA). A schematic overview of the RNA genome is presented in Fig. 23.1. The nonstructural proteins of *Alphavirus* are multifunctional. The main activities are methyltransferase and guanyltransferase (nsP1), NTPase, helicase (nsP2) and RNA-dependent RNA polymerase (nsP4) (Kuhn, 2007). The functions of the structural proteins are described in more detail in the next section of this chapter.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00023-1
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TABLE 23.1 Salmonid Alphavirus (SAV) Genetic Subtypes, Diseases and Distribution in Europe, 2015

SAV Subtype	Host and Environment	Country
SAV 1 (PD)[a]	Atlantic salmon (SW) Rainbow trout (FW)	United Kingdom (Ireland, Scotland)
SAV 2 (SD)	Rainbow trout (FW)	France, Germany, Italy, Spain, Switzerland, Poland, United Kingdom (ie, England, Scotland)
SAV 2 Marine (PD)	Atlantic salmon (SW) Rainbow trout (SW)	Norway, United Kingdom (Scotland)
SAV 3 (PD)	Rainbow trout (SW) Atlantic salmon (SW)	Norway
SAV 4 (PD)	Atlantic salmon (SW)	Ireland, United Kingdom (Scotland)
SAV 5 (PD)	Atlantic salmon (SW)	United Kingdom (Scotland)
SAV 6 (PD)	Atlantic salmon (SW)	Ireland

[a]*PD, pancreas disease; SD, sleeping disease; SW, seawater; FW, fresh water.*

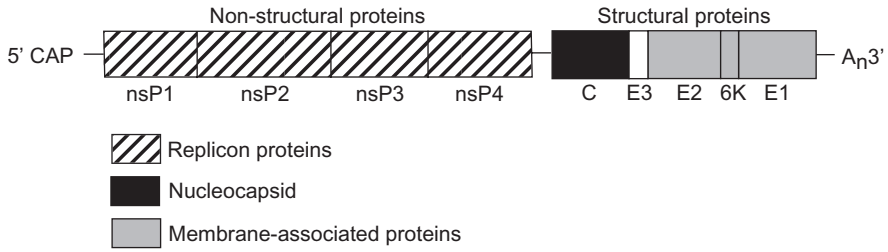

FIGURE 23.1 A schematic diagram of the RNA genome of Salmonid alphavirus (SAV).

Although SAV shares a structure and composition with other alphaviruses, there are some differences. SAV has larger structural and nonstructural proteins, an unglycosylated E3 protein and a shorter nontranscribing 5′-3′ region compared to the mammalian alphaviruses (Weston et al., 2002).

23.4 CLASSIFICATION AND VIRUS REPLICATION

The first isolation of the virus causing PD was published in 1995. Based on virus characteristics like virion morphology, size and nucleic acid composition, the virus most closely resembled members of the family *Togaviridae*, and based upon the disease caused by the virus, the name *salmon pancreas disease virus (SPDV)* was suggested (Nelson et al., 1995). Later studies placed the virus in the *Alphavirus* genus based on the sequence of the 3′ portion of the genome (Weston et al., 1999). Parallel studies of the virus causing SD gave similar results (Villoing et al., 2000). In 2002 the name SPDV was formally accepted and the virus was classified as belonging to the *Alphavirus* genus by the International Committee on Taxonomy of Viruses (Anonymous, ICTV). Collaborative studies concluded that SPDV and sleeping disease virus (SDV) are closely related subtypes of the same virus, with the proposed name *Salmonid alphavirus* (Weston et al., 2002), SPDV is now usually named SAV1, and SDV named SAV2.

Alphaviruses has been classified as either Old World or New World viruses, depending on their distribution. SAV appears to have diverged early on from the Old World–New World lineages and has no close relatives. They cluster into a separate genotype compared to mammalian alphaviruses, with larger structural and nonstructural proteins. Most alphaviruses are transmitted by arthropod vectors, but vector transmission of SAV has not been reported. Although SAV has been detected from sea lice collected from Atlantic salmon during outbreaks of PD (Petterson et al., 2009), no arthropod-dependent transmission has been demonstrated. Several cohabitation experiments have shown virus transmission directly through water (Taksdal et al., 2014; Xu et al., 2012), and with the current state of knowledge, a role for vectors in the transmission of SAV is considered unlikely.

SAV has been differentiated into six subtypes to date, based on molecular differences (Fringuelli et al., 2008). This categorization does not meet the old serology-based Sub-committee on Inter-relationships Among Cataloged Alphaviruses

(SIRACA) criteria to be considered separate subtypes within an alphavirus species because of cross-reactivity between polyclonal antisera raised in Atlantic salmon against all six subtypes and the virus subtypes 1–6 (Graham et al., 2014). However, division in genetic subtypes is a useful tool for molecular epidemiology, and there is a consensus for using this grouping (McLoughlin and Graham, 2007).

The main elements of the life cycle of SAV are considered to be in accordance with other representatives of the *Alphavirus* genus (Weston et al., 2002). Binding and uptake of virus is dependent on the virus glycoproteins E1 and E2, cell surface receptors, a low pH in endosomes and fusion with membrane lipids (Kuhn, 2007). The target organs of SAV in fish comprise of high lipid-metabolizing tissues (Ferguson, 2006), and use of cholesterol-rich membranes in virus entry has also been described for other alphaviruses (Ng et al., 2008). A putative receptor for SAV has not yet been identified. A variety of different receptors have been demonstrated for other alphaviruses, such as the laminin receptor, heparin sulfate and integrin α1β1 (Kuhn, 2007). A naturally occurring substitution from proline to serine in the receptor-binding protein E2 in position 206 in SAV3, has been linked to adaptation to Atlantic salmon. Modeling of the three-dimensional (3D) structure of the uncleaved E3-E2 precursor (p62) shows a localization of this position on the surface of the B-domain of E2, which is associated with receptor attachment in alphaviruses. Recombinant mutants containing serine produce a less persistent infection and faster shedding in experimental studies, although both variants are highly infectious to Atlantic salmon and produce similar viral loads and histopathological changes. In Bluegill fry (BF-2) cell cultures, the recombinant variant containing proline rapidly outcompetes the serine-variant (Karlsen et al., 2015). The virus enters the cell through receptor-mediated endocytosis into coated vesicles using a clathrin-dependent pathway. The nucleocapsid core is then released into the cytoplasm and disassembled, and replication proteins (nsP1-P4) are translated directly from the released genome RNA as a polyprotein. Studies of ultramorphogenesis of SAV1 have demonstrated that virus-like particles can be observed inside membrane-bound vesicles as early as 1 h after contact between virus and cell. After processing, the cleaved proteins form a membrane-dependent replication complex in the cytoplasm (Herath et al., 2012). A subgenomic mRNA is produced later in the infection and translates into a structural polyprotein, which is proteolytically cleaved into mature structural proteins.

The capsid protein is translated first and acts as an autoprotease, and then it associates with the ribosome. Cytoplasmic assembly of capsid and genome RNA into a nucleocapsid appears to be both rapid and efficient. Although the capsid protein is mainly located in the cytoplasm after translation, it contains a nuclear localization signal and can also be detected in the nucleus of some cell types (Karlsen et al., 2010).The remaining envelope proteins (p62, 6K and E1) are translocated into the endoplasmic reticulum (ER) and undergo posttranslational modification. The p62 and E1 glycoproteins form a trimeric spike, which is then processed further by the host enzyme furin, separating E3 from E2 in the *trans*-Golgi network. The spikes are then released in the cytoplasm and finally appear on the cell surface.

Membrane trafficking and surface expression of the structural SAV protein E2 is dependent on low temperature and the presence of E1, and it may be a key factor in restricting SAV infection in vivo to a temperature range of 10°C–15°C (Hikke et al., 2014). The final stage of the alphavirus life cycle is the interaction between capsid protein and glycoproteins to promote virus budding from the cell surface (Kuhn, 2007). Budding of mature enveloped virions can be detected for SAV approximately 24 h postinfection (Herath et al., 2012). During natural infection with SAV, defective viruses containing genome deletions (defective interfering particles) are also generated. Imprecise homologous recombination has been proposed as an explanation for the generation of defective viruses. The role of these defective interfering particles is not yet understood (Petterson et al., 2013).

23.5 EPIDEMIOLOGY

Transmission of SAV occurs horizontally. The virus has been detected in feces and mucus from SAV-infected fish (Graham et al., 2012) and survives in fresh water and seawater (Graham et al., 2007). The spread of PD between neighboring seawater fish farms has frequently been observed.

Horizontal spread through water has been verified by infection experiments where fish injected with SAV have been used as carrier fish that shed SAV into the water. Two to three weeks after injection, SAV is detected in noninjected cohabitant fish (McLoughlin and Graham, 2007; Taksdal et al., 2014). Hydrodynamic modeling indicates that PD likely spreads by passive drift of SAV in the water current (Viljugrein et al., 2009). Risk factors involved in local transmission are shared ownership of different fish farms, close seawater proximity and infection pressure (Aldrin et al., 2010; Kristoffersen et al., 2009).

Transportation of live fish from SAV-infected areas to yet unaffected areas has probably spread the infection to new places (Karlsen et al., 2006). The source or sources of infection in such cases are unknown. However, possible contamination of well boats or introduction of SAV through seawater intake during such transportation has been discussed. Likewise, tracking sources of the spread of SD between freshwater fish farms has been difficult (Schmidt-Posthaus et al., 2014; Scott, 2004). Vertical transmission of SAV is not likely (Kongtorp et al., 2010; McLoughlin and Graham, 2007).

The origin of SAV in aquaculture is thought to be multiple introductions from wild reservoirs. Such introductions have probably then caused self-sustainable epizootics in aquaculture. Based on studies of SAV genomes, it is suggested that all six subtypes known at present developed prior to the first attempts to farm rainbow trout in Europe (Karlsen et al., 2014).

In wild fish, SAV genomes have been identified in the flatfish species common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*) from seawater around the Shetland Islands, Scotland and Ireland. The detections of SAV were both in the vicinity of aquaculture and in regions detached from salmon aquaculture. Subtypes 1, 2 and 5 were identified in the SAV-positive samples (Bruno et al., 2014; McCleary et al., 2014; Snow et al., 2010). One dab-derived SAV, subtype 5, was also grown in cell culture (Bruno et al., 2014).

Studies of the distribution of different subtypes of SAV from Scotland, Ireland and Norway show a clear clustering of subtypes in marine aquaculture. The first SAV subtype to be characterized was SAV1, which was isolated from Atlantic salmon from Ireland in 1995 (Nelson et al., 1995). This subtype dominates in Ireland, as well as in some areas of Scotland. SAV2 has been detected in farmed rainbow trout in fresh water in France, Germany, Italy, Spain, Switzerland, Poland, England and Scotland, and also at some marine sites for Atlantic salmon in Scotland (Graham et al., 2012). In 2008 a variant of SAV2 was reported in sea-reared Atlantic salmon in Scotland (Fringuelli et al., 2008). Retrospective studies have shown that this variant, marine SAV2, has been circulating in Scotland for years and now dominates in seawater fish farms in Shetland and Orkney Islands (Graham et al., 2012). Until 2010, the PD outbreaks in Norway were caused by SAV3, a subtype so far only found in Norwegian aquaculture. From 2010, marine SAV2 has been detected in an increasing number of seawater fish farms in Norway. Presently, the two subtypes constitute two different epidemics of PD, with SAV3 dominating in the south and western Norway and marine SAV2 in northwestern Norway and mid-Norway (Hjortaas et al., 2015). An introduction of SAV2 into the SAV3 area was reported in 2014 (Bornø and Lie Linaker, 2015). The low genetic variation among both SAV2 and SAV3 isolates in Norway, supports a single introductions of both subtypes into Norwegian Aquaculture (Hjortaas et al., 2015; Karlsen et al., 2014, 2006).

23.6 PATHOLOGY AND IMMUNITY

At necropsy of clinically diseased fish, yellow mucoid gut contents is a consistent finding, as is typical for fish that are not eating. In some cases, signs of circulatory disturbance such as scale pocket edema, exophthalmos and ascites can be seen. This is similar to other fish diseases that cause circulatory disturbances. Some fish show pale hearts. Heart rupture, seen as extensive amounts of pericardial blood, may also be observed. In some fish, petechiae in the pyloric region may be detected. None of these changes are, nevertheless, pathognomonic for PD.

By histopathological examination, the disease is characterized by necrosis or loss of exocrine pancreatic tissue, myocarditis and skeletal myositis. These changes develop in a time-dependent way during the progress of the disease. In the early acute phase, severe necrosis of exocrine pancreatic tissue cells shortly precedes necrosis of heart myocytes and the development myocarditis and epicarditis. Some time later in the course of the disease, skeletal muscle responds with degeneration, inflammation and, even later, signs of regeneration of muscle fibers (McLoughlin and Graham, 2007).

The knowledge on immunity against SAV is limited. After experimental infection, enhanced phagocytic activity in head-kidney leukocytes and increased lysozyme and complement levels, as well as the development of neutralizing antibodies against SAV, indicates stimulation of both innate and specific immunity (Desvignes et al., 2002). Experimental studies have also demonstrated that SAV-3 infections induce mRNA transcripts of IFN-α and its stimulated genes at early time, followed by IFN-γ, inflammatory genes and T cell–related genes as the infection progresses. Although SAV3 infection in Atlantic salmon induces a broad range of host immune responses, the onset of innate responses seems too late to limit virus replication (Xu et al., 2012).

Most research on immunity against SAV has focused on the development of neutralizing antibodies against SAV, which develops gradually 2–3 weeks after infection (McLoughlin and Graham, 2007). Sera from infected fish neutralizes SAV in vitro (Houghton and Ellis, 1996; McLoughlin et al., 1998). In vivo, injection of naïve Atlantic salmon with PD convalescent serum protects the fish against experimental infection with PD-infective material (Houghton and Ellis, 1996; Murphy et al., 1995). Similarly, after an experimental challenge of fish with PD- and SD-infective material, reinfection of all fish groups with either PD- or SD-infective material a month later failed to induce the diseases, showing fully acquired cross-protection (Boucher and Laurencin, 1996).

Nevertheless, under large-scale fish farming conditions, SAV infection has been detected after introduction into a fish population until slaughter (Jansen et al., 2010a, b). Also at an individual level, both neutralizing antibodies and SAV were detected in the same individuals (Jansen et al., 2010b). This indicates that the immune reaction against SAV is not fully protective under large-scale fish farming conditions. Moreover, the detection of SAV in experimentally infected fish 6 months after infection suggests that a persistent infection of SAV occurs (Andersen et al., 2007).

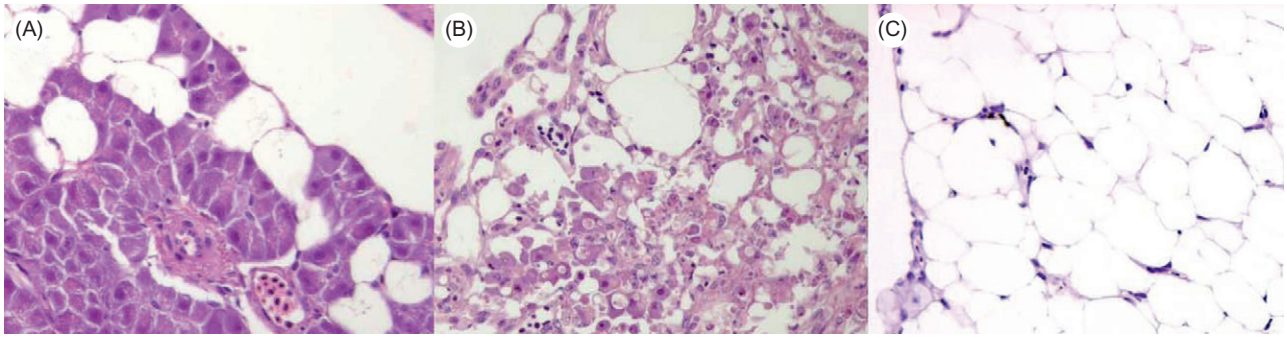

FIGURE 23.2 (A) Light microscopy of normal pancreas of noninfected Atlantic salmon. (B) Acute necrosis of pancreas. (C) Loss of pancreatic tissue in pancreas disease (PD)-diseased Atlantic salmon.

23.7 DIAGNOSTIC METHODS

Diagnostic methods include necropsy and histopathological, virological and serological examinations. The main features of the diagnostic methods are described below. More details are found in Chapters 2.3.5 of the OIE Manual of Diagnostic Tests for Aquatic Animals. The manual is available online (Anonymous, OIE) and is regularly updated.

Characteristic histopathological tissue changes in diseased fish are shown in Fig. 23.2. It must be noted, however, that these changes develop gradually and not simultaneously in all tissues and in all fish in the infected population (McLoughlin and Graham, 2007; Nelson et al., 1995; Taksdal et al., 2014).

SAV can be detected by polymerase chain reaction (PCR) throughout the infection period. The recommended PCR in the OIE manual amplifies a conserved area in the *nsP1* gene (Hodneland and Endresen, 2006). For phylogenetic analysis, the E2 and nsP3 areas are usually targeted (Fringuelli et al., 2008; Graham et al., 2012; Hjortaas et al., 2015). The most suitable organ for virus detection is the heart, but the virus may also be detected from head-kidney, pseudobranch and other organs, and in the viremic stage, also from serum/plasma (Andersen et al., 2007; Graham et al., 2010). SAV can be detected by PCR at slaughtering in fish from sites first diagnosed up to 19 months earlier, although the amount of virus detected and the prevalence seem to decrease (Jansen et al., 2010b). During the early stages of infection, live virus can be isolated from heart, head-kidney and serum/plasma by inoculation onto permissive cell cultures like CHSE-214, BF-2 and TO. The optimal temperature for growth of SAV in cell culture is 15°C (Graham et al., 2008). At later stages of the infection, isolation in cell culture may be difficult (Christie et al., 1998; Graham et al., 2007; Petterson et al., 2013). Specific antibodies in blood samples are detected by using a serum neutralization test (NT) (Graham et al., 2003). During infection with SAV, neutralizing antibodies can be detected as early as 15 days postinfection, with the highest levels from 30 days on (McLoughlin et al., 2006). For screening purposes, the detection of SAV by real-time reverse transcription polymerase chain reaction (RT-PCR) and the detection of specific antibodies in blood samples using the serum NT may be used, as there are no published enzyme-linked immunosorbent assays available for antibody detection.

23.8 PREVENTION AND CONTROL

Farmed SAV-infected fish are the main reservoir of the virus. The most efficient prevention measure is therefore to avoid introduction of live fish from SAV-affected areas into yet unaffected areas. To protect fish health and support safe trade, SAV has been listed by the World Organization for Animal Health (OIE), which makes it possible to prohibit imports of live fish from countries or areas that are not documented as free of SAV.

Transport of live fish may constitute a significant risk for the spread of PD. This is probably the cause of sporadic outbreaks in areas considered free of the disease in Norway. The precise sources of infection in those cases were not identified, however. In general, all possible sources of horizontal transmission of SAV such as aquaculture equipment, well boats, transportation tanks, service boats, personnel and all other contacts between infected and noninfected fish farms, waters and areas should be avoided.

After introduction of SAV into a new area, control and eradication of the infection have been difficult. In a limited number of cases, however, slaughtering of all farmed fish and coordinated fallowing in the area has eliminated the infection. Such areas have contained a single farm or a small group of farms.

The early documentations of immunity against experimental reinfection with SAV led to the idea of using formalin-inactivated SAV (McLoughlin, 1999) or a laboratory-adapted SAV strain (Lopez-Doriga et al., 2001) as a vaccine against

PD. Since 2007, a vaccine has been commercially available. Later, an epidemiological cohort study of the effect of vaccination against PD showed that vaccination reduced the number of PD outbreaks, decreased cumulative mortality and the number of fish that was discarded at slaughter (Bang Jensen et al., 2012). This reduced the economic impact of the disease but did not eliminate SAV or PD outbreaks. Further development of the vaccine technology may give more efficient vaccines in the future, as vaccines against fish pathogenic bacteria usually work well. Breeding of fish strains that are less susceptible to SAV (McLoughlin et al., 2006) probably also will contribute to reducing the impact of the disease.

It is considered that although it is difficult to eradicate SAV in endemic areas, several coordinated regional preventive management measures that are put into place simultaneously may give the best results in reducing the economically negative impact of the disease. Measures to reduce infection pressure such as vaccination, coordinated slaughter and fallowing should be put into place simultaneously in geographically neighboring fish farms. Knowledge of water currents and hydrodynamic modeling may add useful information about reducing the spread of SAV.

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Chapter 24

Hepeviruses of Fish

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24.1 INTRODUCTION

Cutthroat trout virus (CTV) was first identified in adult cutthroat trout (*Oncorhynchus clarkii*) in 1988; however, later surveys found CTV in populations of trout ranging throughout the western United States (Hedrick et al., 1991; Batts et al., 2011). To date, CTV-like viruses have been isolated from seven different species of trout in North America, as well as from Atlantic salmon (*Salmo salar*) in New Brunswick, Canada (Table 24.1; Kibenge et al., 2000, Batts et al., 2011). CTV is a small positive-sense, single-stranded RNA virus, that was determined to be closest to members within the family *Hepeviridae* (ie, human *Hepatitis E virus* (HEV)) based upon genome length, organization and sequence similarity. CTV is avirulent in all fish species experimentally tested via waterborne exposure including: rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) and kokanee salmon (landlocked sockeye salmon, *Oncorhynchus nerka*). However, virus persisted in kidney and spleen tissues of trout juveniles for four weeks post-exposure.

The initial report of this novel virus described a small icosahedral RNA virus detected in four spawning trout populations in northern California (Yun et al., 1988). The virus produced a cytopathic effect different from two other small, round fish viruses detected in smelt (*Osmerus mordax*) and Atlantic salmon, which induced syncytia in cell cultures whereas the trout virus caused only diffuse necrosis. Partial characterizations of these other small RNA viruses of fish have been previously reported (Smith et al., 1980, Moore et al., 1988, Ahne et al., 1990, Hedrick et al., 1990).

In 1991 the first peer-reviewed journal publication described this novel RNA virus. While not associated with disease, the virus appeared to be widespread among trout broodstocks in California (Hedrick et al., 1991). CTV could be isolated using CHSE-214 cells, in which it produced a slow, focal type of cytopathic effect that did not result in destruction of the entire monolayer (Fig. 24.1). This cell-culture system was used to demonstrate that the small, round virus had several characteristics in common with picornaviruses, and that detectable levels of the virus were present at spawning in the ovarian fluids of cutthroat trout, brown trout and brook trout.

In 2008 a replicating agent was isolated in cell cultures at the US Fish and Wildlife Service Fish Health Center in Bozeman, Montana, from rainbow trout ovarian fluid samples originating from Glenwood Springs, Colorado (Batts et al., 2011). This virus-like agent was submitted to the Western Fisheries Research Center in Seattle and the University of California in Davis to assist in identification and a small virus (31 nm icosahedral particles) similar in size and shape to picornaviruses was observed by electron microscopy (Fig. 24.2).

A set of 13 degenerate primers were designed from known picornavirus sequences in order to amplify portions of the virus genome using reverse transcription polymerase chain reaction (RT-PCR) and extracted viral RNA. One primer pair produced a >300 bp amplicon that was suitable for sequencing. Authentic virus sequence was obtained and a pair of primers designed to amplify a 262-nt region for sequencing. These primers were tested on a CTV isolate from Mount Shasta, California, which was obtained from the laboratory of Dr. Ron Hedrick at the University of California Davis. Sequence analysis revealed the two viruses were the same replicating agent, only differing by a few nucleotides. Subsequently, a further set of presumed CTV isolates was provided by Dr. Hedrick and confirmed to be CTV. Following PCR amplification, all of these isolates contained sequences similar to the Glenwood Springs 2008 isolate. The index case of CTV from spawning cutthroat trout obtained at Heenan Lake in 1988 was selected for primer walking using 5′ and 3′ "rapid amplification of cDNA ends" (RACE) and fluorescently labeled sequencing techniques. The full length genome of Heenan Lake 1988 CTV consisted of 7269 nucleotides of positive-sense, single-stranded RNA, beginning with a 5′ untranslated region (UTR), followed by three open reading frames for encoding proteins, a 3′ UTR and ending in a polyA tail (GenBank Accession Number HQ731075). Sequence analysis revealed the CTV genome sequence was most closely aligned with HEV by basic

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00024-3
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TABLE 24.1 Cutthroat Trout Virus Hosts and References

Natural Fish Host	Genus Species	References
Cutthroat trout	*Oncorhynchus clarkii*	Hedrick et al. (1991) Batts et al. (2011)
Rainbow trout	*Oncorhynchus mykiss*	Hedrick et al. (1991) Batts et al. (2011)
Brown trout	*Salmo trutta*	Hedrick et al. (1991) Batts et al. (2011)
Brook trout	*Salvelinus fontinalis*	Hedrick et al. (1991) Batts et al. (2011)
Golden trout	*Oncorhynchus aguabonita*	Batts et al. (2011)
Apache trout	*Oncorhynchus apache*	Batts et al. (2011)
Gila trout	*Oncorhynchus gilae*	Batts et al. (2011)
Atlantic salmon	*Salmo salar*	Kibenge et al. (2000) Batts et al. (2011)

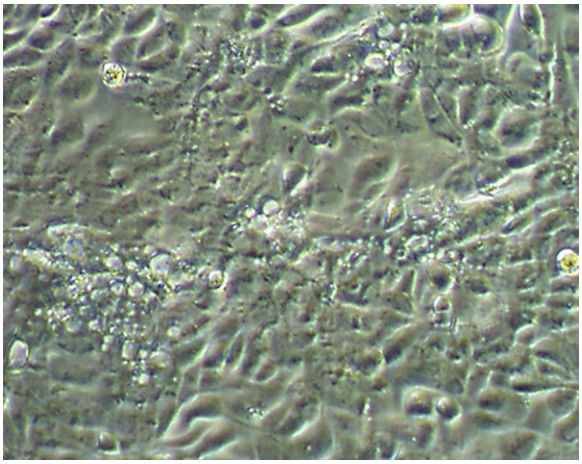

FIGURE 24.1 Cytopathic effect of CTV in a culture of CHSE-214 cells incubated at 15°C. The virus grows relatively slowly, producing a diffuse type of cytopathic effects (CPE) and incomplete destruction of the monolayer. *From: Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Res. 158, 116–123. Reprinted with the permission of Elsevier B.V.*

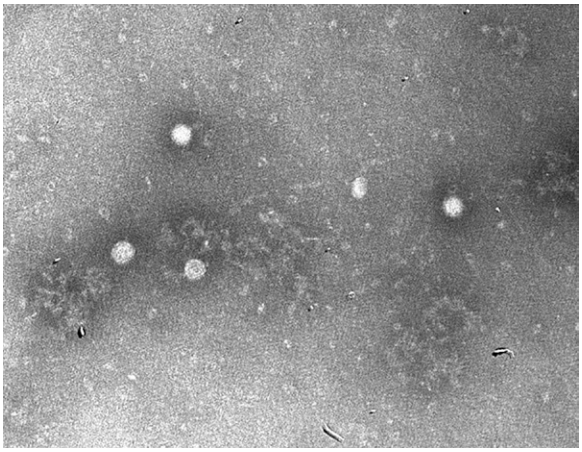

FIGURE 24.2 Electron micrograph of CTV particles within cultured CHSE-214 cells. Negatively stained preparations revealed the small, round virions had a diameter of approximately 31 nm. *From: Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Res. 158, 116–123. Reprinted with the permission of Elsevier B.V.*

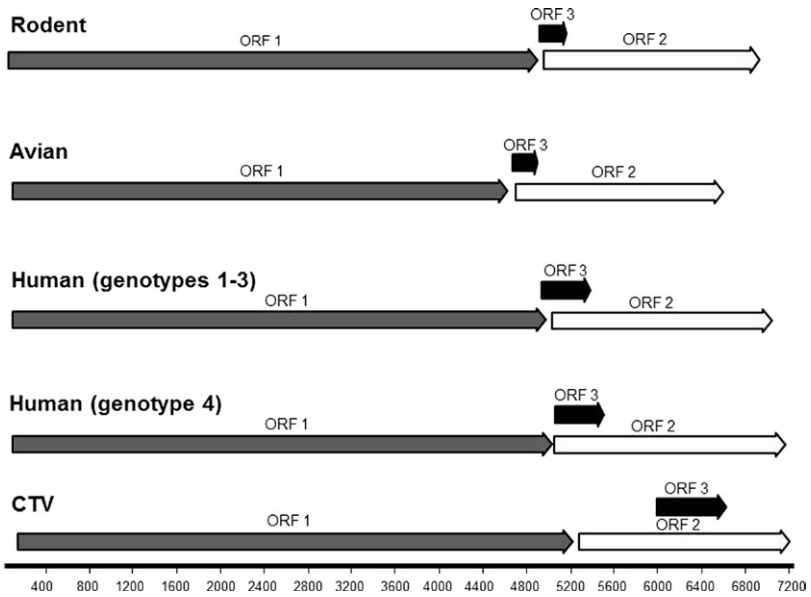

FIGURE 24.3 Genome organization of CTV. The overall genome of 7269 nucleotides of single-stranded RNA organized as three open reading frames, similar to that reported for hepeviruses from human, rodent or avian sources. *From: Batts, W., Yun, S., Hedrick, R., Winton, J., 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Res. 158, 116–123. Reprinted with the permission of Elsevier B.V.*

local alignment search tool (BLAST) searches of the GenBank database (Batts et al., 2011). The genome of CTV was similar to HEV in both size and organization (Fig. 24.3), and was the closest neighbor by sequence alignments and phylogenetic analyses, indicating that CTV was a candidate for inclusion in the family *Hepeviridae*, previously comprised of viruses affecting only mammalian and avian hosts. A new genus, *Piscihepevirus*, is represented by a new species designated *Piscihepevirus A* for the Heenan Lake 1988 cutthroat trout virus (ICTV, 2014). In addition, non-fish viruses of this family are now included as members of a new genus, *Orthohepevirus*.

24.2 CUTTHROAT TROUT VIRUS (NO ASSOCIATED DISEASE OBSERVED)

24.2.1 Structure and Composition of Virus

The nonenveloped CTV particles are reported to have hexagonal or spherical appearance with a mean diameter of 37.5 nm (Hedrick et al., 1991) and 31 nm (Batts et al., 2011). The size of CTV particles is in good agreement with size estimates (32–34 nm) for other members of the *Hepevirus* genus (Purcell and Emerson, 2008). Similar small, hexagonal RNA viruses have been observed as 20–30 nm diameter particles in smelt from New Brunswick, Canada and Germany (Moore et al., 1988; Ahne et al., 1990), while another somewhat larger virus (39.5 nm) was isolated from Atlantic salmon in Washington (Hedrick et al., 1990). All of these viruses share physical properties; however, they produce syncytia in cell cultures, whereas CTV does not.

Early characterization by Hedrick et al. (1991) showed that CTV replicated effectively in the presence of chloroform and the DNA inhibitor 5-bromo-2-deoxyuridine, suggesting the presence of a nonenveloped RNA virus. A 15-min incubation of CTV at 37°C did not reduce virus viability compared with 15°C; however, 15 min incubation at 60°C caused virus reduction of nearly 100,000-fold.

24.2.2 Classification and Virus Replication

CTV is a member of the family *Hepeviridae* based upon genome length, organization and sequence similarity (Batts et al., 2011).

Virus growth for CTV is optimum at 15°C when inoculated onto monolayer cultures of CHSE-214 cells while BF-2 cells have been used for some isolates. While CTV replicates well at 10–20°C, virus replication at 4°C was slow and no evidence of replication occurred at 25°C on CHSE-214 cells (Hedrick et al., 1991). In addition, epithelioma papulosum cyprini (EPC) cells appear to be resistant to infection at 15°C and no replication was evident at 37°C in mammalian Vero cell cultures.

24.2.3 Epidemiology

Although first identified in adult cutthroat trout in 1988, later surveys found CTV in many varied populations of trout ranging throughout the western United States and two Canadian provinces. Overall, CTV has been found in seven different species of trout including: rainbow, cutthroat, brown, brook, golden (*Oncorhynchus aguabonita*), Apache (*Oncorhynchus apache*) and gila trout (*Oncorhynchus gilae*) (Batts et al., 2011). CTV has been isolated from trout in the US states of Washington, Oregon, California, Idaho, Utah, Montana, Colorado, Wyoming, Arizona and New Mexico. In many of these states, only a couple isolates have been found in decades of examinations, while other states have multiple isolations each year. Spawning brown and rainbow trout at one facility in Alberta, Canada, contained an unknown replicating agent subsequently identified as CTV in eight of the years beginning in 2002 (Bev Larson & Emily Port; Alberta Fish and Wildlife, Environment and Sustainable Resource Development; personal communication). Two other recent isolations of CTV were from locations outside the previous geographical range. CTV was detected in fish samples of spawning Wisconsin brown trout in 2012 (Corey Puzach & Susan Marcquenski, LaCrosse Fish Health Center, U.S. Fish and Wildlife Service; personal communication) and spawning Pennsylvania brown trout in 2012 and brook trout in 2014 (Patricia Barbash, Lamar Fish Health Center, U.S. Fish and Wildlife Service; personal communication). Serendipitously, a CTV-like virus was identified in New Brunswick from Atlantic salmon co-infected with the fish orthomyxovirus, *Infectious salmon anemia virus* (Kibenge et al., 2000, Batts et al., 2011); this CTV isolate was initially described as togavirus-like (the next closest group to hepeviruses). Although not technically found in Nevada yet, one CTV-infected fish population in California was only a few miles from the border. Interestingly, this virus has not yet been isolated and identified in Alaska or British Columbia, two large western regions that have numerous trout populations. The overall nucleotide diversity of published CTV sequences is less than 8.4%, although the 63 isolates had an amazing 38 individual sequence types for the short 262 nucleotides examined (Batts et al., 2011).

24.2.4 Pathology and Immunity

Naturally infected fish did not reveal any unusual behavior or show appearance of any external or internal signs of disease. Most of the viral detections occurred from samples of ovarian fluid or tissues of spawning trout during routine egg collections. CTV is avirulent in all fish species tested via waterborne exposure including: rainbow, brown and brook trout, and kokanee, Chinook (*Oncorhynchus tshawytscha*) and coho (*Oncorhynchus kisutch*) salmon. However, virus could be isolated from kidney and spleen tissues of kokanee salmon and rainbow, brown and brook trout juveniles for 3–5 weeks following exposure, but not from Chinook or coho salmon tested after 1–4 weeks. No microscopic pathology was observed for any fish tissue section from naturally or experimentally exposed fish (Hedrick et al., 1991). Interestingly, prior exposures of juvenile rainbow trout to CTV provided significant reduction in mortality compared with unexposed controls following laboratory challenges with the fish rhabdovirus, *Infectious hematopoietic necrosis virus*. The increased resistance could be detected for up to 4 weeks post-CTV exposure and was potentially mediated by induction of an interferon response as reported by Hedrick et al. (1994), who also cited unpublished observations from Europe that fish pre-exposed to CTV were similarly protected when challenged by another rhabdovirus, *Viral hemorrhagic septicemia virus* (VHSV).

24.2.5 Diagnostic Methods

Replication in the CHSE-214 cell line can be used for virus isolation of CTV from samples of tissues and ovarian fluids (Hedrick et al., 1991). Typically, CTV is slow-growing and produces a diffuse type of cytopathic effects (CPE). The virus can be identified using molecular methods, such as RT-PCR for amplification of an appropriate size amplicon band with CTV primers designed to bind at conserved sites of the viral RNA. The suggested PCR test (Batts et al., 2011) includes primers 5′-ACTGTTACACCTCATGTAGC-3′ and 5′-GACTTTACTAGCAGTGTGGAT-3′, located within the conserved helicase domain. Batts et al. (2011) used such PCR primers for 63 CTV isolates and obtained a 262-nt region (position 3178-3439 of GenBank HQ731075) for sequence analysis between these primers. Future PCR amplicons can be sequenced and nucleotides compared for differences from reference strains deposited into the GenBank database (Batts et al., 2011).

24.2.6 Prevention and Control

Efforts to control the spread of CTV in western North America may be too late because many trout populations have already been infected in the western United States and Canada; however, the virus has yet to be reported from trout in other areas of the world. Nevertheless, it seems likely that CTV infections will be detected in trout species from other geographic

locations. The virus appears to have potential for vertical transmission to the next generation because juvenile brown trout and brook trout at two locations with virus-positive broodstocks were found to harbor CTV when examined 1 month after hatching (Hedrick et al., 1991). Control for CTV may be less necessary than for other fish viruses that cause disease because it does not cause any adverse pathological effects on trout. In fact, since CTV has been shown to be effective at reducing IHNV infections on susceptible salmonids for up to 4 weeks post-exposure (Hedrick et al., 1994), a strategy with intact or fragments of CTV may serve to aid in control efforts against IHN disease.

Researchers have begun to use CTV as a surrogate model for HEV infections because CTV has similarities to other hepeviruses. This is especially useful since there is quite high mortality in pregnant women and cell-culture replication of HEV is quite poor. Researchers tested the effect of three sex steroids on in vitro CTV replication, showing inhibition for progesterone and stimulation by testosterone and 17β-estradiol (Debing et al., 2013). An antiviral assay was also developed to screen potential candidate chemicals such as ribavirin and interferons. If suitable CTV or HEV control treatments can be developed, aquatic and mammalian researchers might equally benefit.

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Chapter 25

Nodaviruses of Fish

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25.1 INTRODUCTION

Aquaculture has become the fastest food-producing industry in the world. Due to the high market demand and economic value of marine fish, the aquaculture of it has increased rapidly in recent years. Fish nodaviruses have emerged as the principal pathogens with a high impact on the aquaculture industry. The disease caused by fish nodaviruses is called *viral nervous necrosis* (VNN) disease and is also known as *viral encephalopathy and retinopathy* (*VER*). VNN is a devastating disease among cultured marine fish worldwide and results in huge economic losses to the aquaculture industry. VNN was first recorded in hatchery-reared parrot fish (*Oplegnathus fasciatus*) in Japan (Yoshikoshi and Inoue, 1990) and barramundi larvae (*Lates calcarifer*) in Australia (Glazebrook et al., 1990). The mortality caused by VNN is very high at the larval and juvenile stages (80–100%) and is minor for adult fish. Fish nodaviruses have been isolated from more than 40 species of fish (Table 25.1), including tropical, subtropical and cold-temperate marine fish. A few species of fresh fish are also susceptible to fish nodavirus infection, including guppies (Hegde et al., 2003), catfish (Chi et al., 2003), zebrafish (Lu et al., 2008) and medaka (Furusawa et al., 2006). Therefore, knowledge about the NNV phylogenetic relationship, reassortment and mutation of viral RNA (vRNA), transmission pathways, infection progress, viral life cycle, new diagnosis methods and cytopathogenesis of fish nodaviruses is very important for disease control.

25.2 CLINICAL SIGNS AND HISTOPATHOLOGY OF FISH WITH VNN

The clinical signs of the affected fish include abnormal swimming behaviors (eg, whirling and abrupt darting), dark coloration, loss of appetite and enlargement of swim bladder in some species. The diseased fish are lethargic and usually float near the border of the pond.

The histopathological characteristic of VNN is the vacuolation of the central nervous system, especially the brain and the retina (Grotmol et al., 1997a,b) (Fig. 25.1). Under transmission electron microscopy (TEM), nonenveloped spherical virions with diameters of 20–34 nm were found to pack in paracrystalline arrays in membrane-bound organelles or scatter in the cytoplasm of the affected brain cells (Fig. 25.2); in addition, large vacuoles due to the degeneration of organelles always exist in the heavily infected cells (Chi et al., 1997).

By immunohistochemistry staining (IHC) using specific antibodies, positive signals were also found in lymphocytelike cells, vascular and endocardial endothelium of Atlantic halibut (Grotmol et al., 1997a,b). By in situ hybridization using specific digoxigenin-labeled probe, positive signals were not only limited to the brain and retina, but were also present in the liver and intestine of grouper larvae during acute infection (Chi et al., 2001), indicating that fish nodavirus can spread into many tissues within the heavily affected larvae through viremia. However, vacuolation was only observed in brain, retina and spinal cord, indicating that central nervous tissue is the target for fish nodavirus proliferation, and the abnormal swimming behavior is related to the damaged central nervous system.

25.3 CLASSIFICATION AND PHYLOGENETIC ANALYSIS OF FISH NODAVIRUSES

25.3.1 Viral RNA and Proteins

The genome of members of the genus *Betanodavirus* consists of two single-stranded, positive-sense RNAs, which are capped but not polyadenylated (Mori et al., 1992) (Fig. 25.3). The genome size of betanodavirus is 4.5 kb. RNA1 (3103 nt) encodes RNA-dependent RNA polymerase (RdRp) with a calculated molecular weight of 110.74 kDa. Betanodavirus RdRp contains a mitochondrial localization targeting signal (Guo et al., 2004; Mezeth et al., 2007) and will colocalize with

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00025-5
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TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV)

NNV Genotype	Host Fish	Area	References
RGNNV 25–30°C[a]	*Acanthurus triostegus*	Tahiti[w]	Thiery et al. (2004)
	Aeoliscus strigastus	South Korea[c]	Gomez et al. (2008a)
	Alectis indica	China[w]	Liu et al. (2015)
	Amphiprion sebae	India[c]	Binesh et al. (2013)
	Anguilla anguilla	Taiwan[c]	Chi et al. (2003)
	Apogon exostigma	Tahiti[w]	Thiery et al. (2004)
	Apogon lineatus	South Korea[w]	Gomez et al. (2008b)
	Argyrosomus argentatus	South Korea[w]	Gomez et al. (2008b)
		China[w]	Liu et al. (2015)
	Argyrosomus macrocephalus	China[w]	Liu et al. (2015)
	Argyrosomus regius	France[c]	Thiery et al. (2004)
		Spain[c]	Hodneland et al. (2011)
		Iberian Peninsula[w]	Lopez-Jimena et al. (2010)
	Arotrolepis sulcatus	China[w]	Liu et al. (2015)
	Atractoscion nobilis	United States[c]	Curtis et al. (2001), Ciulli et al. (2005)
	Balistapus spp.	Italy[w]	Panzarin et al. (2012)
	Boops boops	Italy[w]	Ciulli et al. (2007)
	Caelorinchus multispinulosus	South Korea[w]	Gomez et al. (2008b)
	Callionymus lunatus	South Korea[w]	Gomez et al. (2008b)
	Caranx djeddaba	China[w]	Liu et al. (2015)
	Caranx oshimai	China[w]	Liu et al. (2015)
	Chanos chanos	South Korea[c]	Gomez et al. (2008a)
	Chelidonichthys cuculus	Italy[w]	Ciulli et al. (2007)
	Chelidonichthys spinosus	South Korea[w]	Gomez et al. (2008b)
	Chelon labrosus	Italy[c]	Panzarin et al. (2012)
	Cromileptes altivelis	Australia[c]	Moody et al. (2009)
		Indonesia[c]	Mori et al. (2003), Ransangan and Manin (2012), Zafran Koesharyani et al. (2000)
	Cromileptes altivelis	Taiwan[c]	Chi et al. (2003)
	Cynoglossus puncticeps	China[w]	Liu et al. (2015)
	Cynoglossus robustus	South Korea[w]	Gomez et al. (2008b)
	Cynoglossus semilaevis	China[c]	Li et al. (2014)
	Cynoglossus sinicue	China[w]	Liu et al. (2015)
	Dascyllus trimaculatus	South Korea[c]	Gomez et al. (2008a)
	Decapterus maruadsi	Japan[w]	Gomez et al. (2004)
	Dicentrarchus labrax	Bosnia and Herzegovinia[w]	Dalla Valle et al. (2001)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
		Croatia[c]	Toffolo et al. (2007)
		Cyprus[c]	Panzarin et al. (2012)
		France[c]	Delsert et al. (1997), Thiery et al. (2004)
		Greece[c]	Panzarin et al. (2012), Sideris (1997), Skliris et al. (2001)
		Greece[w]	Dalla Valle et al. (2001)
		Israel[c]	Ucko et al. (2004)
		Italy[c]	Bovo et al. (1999), Ciulli et al. (2005), Nishizawa et al. (1997), Panzarin et al. (2012), Skliris et al. (2001), Toffolo et al. (2007), Vendramin et al. (2013)
		Italy[w]	Dalla Valle et al. (2001), Panzarin et al. (2012), Vendramin et al. (2013)
		Malta[c]	Skliris et al. (2001)
		Portugal[c]	Skliris et al. (2001)
		Spain[c]	Olveira et al. (2009), Panzarin et al. (2012), Skliris et al. (2001)
		Tunisia[c]	Cherif et al. (2010), Cherif et al. (2009), Haddad-Boubaker et al. (2013), Thiery et al. (2004)
	Diodon holocanthus	Japan[w]	Gomez et al. (2004)
	Doederleinia berycoides	South Korea[w]	Gomez et al. (2008b)
	Engraulis japonicus	South Korea[c]	Gomez et al. (2008a)
	Epinephelus aeneus	Israel[cw]	Ucko et al. (2004)
		Italy[w]	Toffolo et al. (2007)
	Epinephelus akaara	China[c]	Liu et al. (2012), Liu et al. (2015)
		Japan[c]	Gomez et al. (2004), Iwamoto et al. (1999), Mori et al. (1991), Nishizawa et al. (1995, 1997)
		Japan[w]	Gomez et al. (2009)
	Epinephelus areolatus	China[w]	Liu et al. (2015)
	Epinephelus awoara	Taiwan[c]	Lai et al. (2001)
		China[c]	Liu et al. (2015)
	Epinephelus bleekeri	China[c]	Liu et al. (2015)
	Epinephelus coioides	Australia[c]	Moody et al. (2009)
		China[c]	Liu et al. (2015)
		Taiwan[c]	Chi et al. (2003)
		Thailand[c]	Iwamoto et al. (1999)
	Epinephelus costae	Algeria[w]	Kara et al. (2014)
		Greece[w]	Panzarin et al. (2012)
		Italy[w]	Vendramin et al. (2013)
	Epinephelus episticutus	China[w]	Liu et al. (2015)
	Epinephelus fasciatomaculatus	China[w]	Liu et al. (2015)

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TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
	Epinephelus fuscoguttatus	Indonesia[c]	Ransangan and Manin (2012)
		Malaysia[c]	Ransangan and Manin (2012)
		Taiwan[c]	Chi et al. (2003)
	Epinephelus lanceolatus	Taiwan[c]	Lin et al. (2001)
	Epinephelus malabaricus	China[c]	Liu et al. (2015)
		Taiwan[c]	Lin et al. (2001)
		Thailand[c]	Nishizawa et al. (1997), Skliris et al. (2001)
	Epinephelus marginatus	Algeria[w]	Kara et al. (2014)
		Greece[w]	Panzarin et al. (2012)
		Italy[c]	Ciulli et al. (2005)
		Italy[w]	Panzarin et al. (2012), Vendramin et al. (2013)
	Epinephelus megachir	China[w]	Liu et al. (2015)
	Epinephelus merra	China[w]	Liu et al. (2015)
	Epinephelus moara	China[w]	Liu et al. (2015)
		Japan[c]	Iwamoto et al. (1999), Nakai et al. (1994), Nishizawa et al. (1995, 1997)
	Epinephelus septemfasciatus	Japan[c]	Fukuda et al. (1996), Iwamoto et al. (1999), Iwamoto et al. (2000), Nishizawa et al. (1995, 1997)
		South Korea[c]	Cha et al. (2007)
	Epinephelus spp.	Greece[w]	Panzarin et al. (2012)
		Italy[w]	Panzarin et al. (2012)
	Epinephelus tauvina	China[c]	Liu et al. (2015)
		Singapore[c]	Tan et al. (2001)
	Gastrophysus spadiceus	China[w]	Liu et al. (2015)
	Gobius niger	Italy[w]	Ciulli et al. (2007)
	Gobius spp.	Italy[w]	Panzarin et al. (2012)
	Heterodontus japonicus	Japan[w]	Gomez et al. (2004)
	Inimicus japonicus	Japan[c]	Gomez et al. (2004)
	Lagocephalus lunaris	South Korea[w]	Gomez et al. (2008b)
	Lateolabrax japonicus	China[c]	Jia et al. (2015)
		China[w]	Liu et al. (2015)
		Japan[c]	Iwamoto et al. (1999), Nishizawa et al. (1997)
	Lateolabrax sp.	Japan[c]	Gomez et al. (2004)
	Lates calcarifer	Australia[c]	Moody et al. (2009), Nishizawa et al. (1997)
		India[cw]	Binesh and Jithendran (2013)
		Israel[c]	Ucko et al. (2004)
		Malaysia[c]	Ransangan and Manin (2010); Ransangan and Manin (2012)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
		Singapore[c]	Skliris et al. (2001)
		Taiwan[c]	Chi et al. (2003)
	Latris lineata	Australia[c]	Moody et al. (2009)
	Leiognathus berbis	China[w]	Liu et al. (2015)
	Leiognathus bindus	China[w]	Liu et al. (2015)
	Leiognathus brevirostris	China[w]	Liu et al. (2015)
	Leiognathus dussumieri	China[w]	Liu et al. (2015)
	Leiognathus insidiator	China[w]	Liu et al. (2015)
	Leiognathus nuchalis	South Korea[w]	Gomez et al. (2008b)
	Leiognathus rivulatus	China[w]	Liu et al. (2015)
	Lepisosteus platyrhynchus	Italy[w]	Ciulli et al. (2007)
	Lophius litulon	South Korea[w]	Gomez et al. (2008b)
	Lutaanus erythropterus	Taiwan[c]	Chi et al. (2003)
	Lutjanus argentimaculatu	China[w]	Liu et al. (2015)
	Lutjanus fulviflamma	China[w]	Liu et al. (2015)
	Lutjanus johni	China[w]	Liu et al. (2015)
	Lutjanus russelli	China[w]	Liu et al. (2015)
	Lutjanus sanguineus	China[w]	Liu et al. (2015)
	Macquaria novemaculata	Australia[c]	Moody et al. (2009)
	Merlangius merlangus	Italy[w]	Ciulli et al. (2007), Panzarin et al. (2012)
	Merluccius merluccius	Italy[w]	Ciulli et al. (2007)
	Microcanthus strigatus	Japan[w]	Gomez et al. (2004)
	Micropterus salmoides	Italy[c]	Bovo et al. (2011)
	Monacanthus chinensi	China[w]	Liu et al. (2015)
	Monocentris japonica	South Korea[c]	Gomez et al. (2008a)
	Monocirrhus polyacanthus	South Korea[c]	Gomez et al. (2008a)
	Morone saxatilis × *Morone chrysops*	Italy[c]	Bovo et al. (2011)
	Mugil cephalus	China[w]	Liu et al. (2015)
		Israel[c]	Ucko et al. (2004)
		Italy[w]	Ciulli et al. (2007)
		Japan[w]	Gomez et al. (2004)
		South Korea[c]	Cha et al. (2007)
	Mullus barbatus	Croatia[w]	Panzarin et al. (2012)
		Italy[c]	Toffolo et al. (2007)
		Italy[w]	Ciulli et al. (2007), Panzarin et al. (2012)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
	Mullus surmuletus	Italy[w]	Panzarin et al. (2012)
	Mullus spp.	Italy[w]	Panzarin et al. (2012)
	Muraenesox cinereus	South Korea[w]	Gomez et al. (2008b)
	Navodon tessellates	China[w]	Liu et al. (2015)
	Okamejei kenojei	South Korea[w]	Gomez et al. (2008b)
	Oplegnathus fasciatus	South Korea[c]	Cha et al. (2007)
		Japan[w]	Gomez et al. (2004)
	Oplegnathus punctatus	Japan[c]	Skliris et al. (2001)
	Oreochromis niloticus	Thailand[c]	Keawcharoen et al. (2015)
		Western Europe[c]	Bigarre et al. (2009)
	Otolithes argenteus	China[w]	Liu et al. (2015)
	Oxyeleotris lineolata	Australia[c]	Moody et al. (2009)
	Pagellus spp.	Italy[w]	Panzarin et al. (2012)
	Pagrosomus major	China[c]	Liu et al. (2015)
	Pagrus major	South Korea[w]	Gomez et al. (2008b)
		Japan[c]	Gomez et al. (2004)
	Paralichthys olivaceus	Japan[c]	Gomez et al. (2004), Nishizawa et al. (1995, 1997)
		South Korea[c]	Cha et al. (2007)
	Parasilurus asotus	Taiwan[c]	Chi et al. (2003)
	Pelates quadrilineatus	China[w]	Liu et al. (2015)
	Platax orbicularis	Polynesia[c]	David et al. (2010)
	Plectorhynchus pictus	China[w]	Liu et al. (2015)
	Pleuronectes yokohamae	Japan[c]	Gomez et al. (2004)
		South Korea[w]	Gomez et al. (2008b)
	Plotosus lineatus	Japan[w]	Gomez et al. (2004)
	Poicelia reticulata	Singapore[c]	Hegde et al. (2003)
	Pomacentrus coelestis	Japan[w]	Gomez et al. (2004)
	Priacanthus hamrur	China[w]	Liu et al. (2015)
	Pseudorhombus cinnamomeus	China[w]	Liu et al. (2015)
	Pseudorhombus pentophthalmus	South Korea[w]	Gomez et al. (2008b)
	Pterois lunulata	Japan[w]	Gomez et al. (2004)
	Pygocentrus nattereri	South Korea[c]	Gomez et al. (2008a)
	Rachycentron canadum	Taiwan[c]	Chi et al. (2003)
	Rhabdosargus sarba	China[c]	Liu et al. (2015)
	Rhinomuraena quaesita	South Korea[c]	Gomez et al. (2008a)
	Salaria fluviatilis	Spain[c]	Vendramin et al. (2012)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
	Salmo trutta trutta	Italy[w]	Panzarin et al. (2012)
	Sander lucioperca	Italy[c]	Bovo et al. (2011)
	Sardina pilchardus	Italy[w]	Ciulli et al. (2007), Panzarin et al. (2012)
	Sciaenops ocellatus	China[c]	Liu et al. (2015)
		Israel[c]	Ucko et al. (2004)
	Scomber japonicus	South Korea[w]	Gomez et al. (2008b)
	Sebastes inermis	Japan[c]	Gomez et al. (2004)
	Sebastes oblongus	Japan[c]	Gomez et al. (2004)
	Sebastes pachycephalus	Japan[c]	Gomez et al. (2004)
	Sebastes schlegeli	Japan[w]	Gomez et al. (2004)
	Sebastiscus marmoratus	Japan[w]	Gomez et al. (2004)
	Selaroides leptolepis	China[w]	Liu et al. (2015)
	Selene vomer	South Korea[c]	Gomez et al. (2008a)
	Seriola dumerili	Japan[c]	Nishizawa et al. (1997)
		Japan[w]	Gomez et al. (2004)
	Siganus oramin	China[w]	Liu et al. (2015)
	Sillago japonica	South Korea[w]	Gomez et al. (2008b)
	Solea solea	Italy[c]	Panzarin et al. (2012)
	Sparus aurata	France[c]	Castri et al. (2001), Thiery et al. (2004)
		Italy[c]	Dalla Valle et al. (2000), Toffolo et al. (2007)
		Italy[w]	Panzarin et al. (2012)
		Spain[c]	Panzarin et al. (2012)
		Tunisia[c]	Cherif et al. (2009); Haddad-Boubaker et al. (2013)
	Sparus latus	China[c]	Liu et al. (2015)
	Sparus macrocephalus	China[c]	Liu et al. (2015)
	Stephanolepis cirrhifer	Japan[w]	Gomez et al. (2004)
	Takifugu alboplumbeus	China[w]	Liu et al. (2015)
	Takifugu niphobles	South Korea[w]	Gomez et al. (2008b)
	Takifugu pardalis	Japan[w]	Gomez et al. (2004)
	Takifugu rubripes	Japan[c]	Gomez et al. (2004)
	Takifugu xanthopterus	China[w]	Liu et al. (2015)
	Tarphops oligolepis	China[w]	Liu et al. (2015)
	Tephrinectes sinensis	China[w]	Liu et al. (2015)
	Terapon jarbua	China[w]	Liu et al. (2015)
	Thamnaconus modestus	Japan[w]	Gomez et al. (2004)
	Thunnus orientalis	Japan[c]	Sugaya et al. (2009)
	Trachinotus blochii	Malaysia[c]	Ransangan and Manin (2012)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
	Trachinotus falcatus	Taiwan[c]	Chi et al. (2003)
	Trachurus japonicus	South Korea[w]	Gomez et al. (2008b)
		Japan[w]	Gomez et al. (2004)
	Trachurus trachurus	Italy[w]	Ciulli et al. (2007)
	Trisopterus minutus	Italy[w]	Panzarin et al. (2012)
	Umbrina cirrosa	Italy[c]	Nishizawa et al. (1997), Panzarin et al. (2012), Skliris et al. (2001)
		Italy[w]	Dalla Valle et al. (2001), Panzarin et al. (2012)
	Umbrina russelli	China[w]	Liu et al. (2015)
	Zanclus cornutus	Japan[w]	Gomez et al. (2004)
	Zebrasoma flavesenes	South Korea[c]	Gomez et al. (2008a)
	Zebrias zebra	China[w]	Liu et al. (2015)
	Zeus faber	South Korea[w]	Gomez et al. (2008b)
	Zoarces gilli	South Korea[w]	Gomez et al. (2008b)
SJNNV 20–25°C[a]	*Anguilla anguilla*	Spain[w]	Bandin et al. (2014)
	Argyrosomus regius	Iberian Peninsula[w]	Lopez-Jimena et al. (2010)
	Dicentrarchus labrax	Italy[c]	Cutrin et al. (2007)
		Portugal[c]	Cutrin et al. (2007)
		Spain[c]	Cutrin et al. (2007), Toffolo et al. (2007)
	Diplodus sargus	Spain[c]	Garcia-Rosado et al. (2007)
	Pagrus auriga	Spain[c]	Garcia-Rosado et al. (2007)
	Pagrus major	Japan[c]	Nishizawa et al. (1997)
	Pagrus pagrus	Spain[c]	Garcia-Rosado et al. (2007)
	Pseudocaranx dentex	Japan[c]	Iwamoto et al. (2000), Mori et al. (1992), Nishizawa et al. (1995, 1997), Skliris et al. (2001)
	Sparus aurata	Portugal[c]	Cutrin et al. (2007)
		Spain[c]	Cutrin et al. (2007)
	Solea senegalensis	Portugal[c]	Cutrin et al. (2007)
		Spain[c]	Cutrin et al. (2007), Panzarin et al. (2012), Thiery et al. (2004)
TPNNV 20°C[a]	*Paralichthys olivaceus*	Japan[c]	Nishizawa et al. (1997)
	Takifugu rubripes	Japan[c]	Iwamoto et al. (2000), Nishizawa et al. (1995, 1997)
BFNNV 15–20°C[a]	*Dicentrarchus labrax*	France[c]	Thiery et al. (2004)
	Gadus macrocephalus	China[c]	Mao et al. (2015)
		Japan[c]	Nishizawa et al. (1997)
	Gadus morhua	Canada[c]	Johnson et al. (2002)
		Norway[cw]	Nylund et al. (2008)
		United Kingdom[c]	Starkey et al. (2001)
		United States[w]	Gagne et al. (2004)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
	Hippoglossus hippoglossus	Norway[c]	Aspehaug et al. (1999), Grotmol et al. (2000), Grotmol et al. (1995), Johansen et al. (2004a), Korsnes et al. (2005)
		United Kingdom[c]	Starkey et al. (2000)
	Melanogrammus aeglefinus	Canada[w]	Gagne et al. (2004)
	Paralichthys olivaceus	Japan	Iwamoto et al. (1999), Iwamoto et al. (2000)
	Pseudopleuronectes americanus	Canada[w]	Barke et al. (2002); Gagne et al. (2004)
	Solea vulgaris/Solea solea	United Kingdom[c]	Starkey et al. (2001)
	Verasper moseri	Japan[c]	Nishizawa et al. (1995, 1997)
TNNV 13–20°C[b]	*Scophthalmus maximus*	Norway[c]	Johansen et al. (2004b)
Reassortant strains RG/SJ	*Dicentrarchus labrax*	Croatia[c]	Panzarin et al. (2012)
		Greece[c]	Panzarin et al. (2012)
		Italy[c]	Panzarin et al. (2012)
	Solea senegalensis	Italy[c]	Panzarin et al. (2012)
		Portugal[c]	Olveira et al. (2009)
		Spain[c]	Olveira et al. (2009)
	Solea solea	Italy[c]	Panzarin et al. (2012)
	Sparus aurata	Cyprus[c]	Panzarin et al. (2012)
		Greece[c]	Panzarin et al. (2012)
		Portugal[c]	Olveira et al. (2009), Panzarin et al. (2012)
		Spain[c]	Olveira et al. (2009)
Reassortant strains SJ/RG	*Dicentrarchus labrax*	Croatia[c]	Toffolo et al. (2007)
		Italy[c]	Toffolo et al. (2007)
Undetected genotype	*Acipenser gueldestaedi*	Greece[c]	Athanassopoulou et al. (2004)
	Aeoliscus strigatus	Singapore	Gomez et al. (2006)
	Chanos chanos	Japan	Gomez et al. (2006)
	Dascyllus trimaculatus	Singapore	Gomez et al. (2006)
	Dicentrarchus labrax	United Kingdom[c]	Frerichs et al. (1996)
	Diplodus sargus	Italy[c]	Toffolo et al. (2007)
	Engraulis japonicus	Japan	Gomez et al. (2006)
	Epigonus telescopus	Italy[w]	Giacopello et al. (2013)
	Epinephelus akaara	Taiwan[c]	Chi et al. (1997)
	Epinephelus coioides	Philippines[c]	Maeno et al. (2002)
	Hoplostethus mediterraneus	Italy[w]	Giacopello et al. (2013)
	Hymenocephalus italicus	Italy[w]	Giacopello et al. (2013)
	Lates calcarifer	France[c]	Comps et al. (1994)

(*Continued*)

TABLE 25.1 The Fish Species Affected by Four Genotypes of Nervous Necrosis Virus (NNV) (Continued)

NNV Genotype	Host Fish	Area	References
		Indonesian[c]	Zafran et al. (1998)
		Tahiti[c]	Renault et al. (1991)
	Monocentris japonica	Japan	Gomez et al. (2006)
	Monocirrhus polyacanthus	Amazon	Gomez et al. (2006)
	Mugil cephalus	Italy[c]	Toffolo et al. (2007)
	Notacanthus bonaparte	Italy[w]	Giacopello et al. (2013)
	Oplegnathus fasciatus	Japan[c]	Yoshikoshi and Inoue (1990)
	Plectropomus maculatus	Thailand[c]	Nopadon et al. (2009)
	Pygocentrus nattereri	Amazon River	Gomez et al. (2006)
	Rhinomuraena quaesita	Indonesia	Gomez et al. (2006)
	Sciaenops ocellatus	South Korea[c]	Oh et al. (2002)
	Scopthalmus maximus	Norway	Bloch et al. (1991)
	Selene vomer	United States	Gomez et al. (2006)
	Stephanolepis cirrhifer	Thailand[c]	Pirarat et al. (2009)
	Umbrina cirrosa	France[c]	Comps et al. (1996)
	Zebrasoma flavesenes	Singapore	Gomez et al. (2006)

The superscript letter "c" means cultured fish and "w" means wild fish.
[a]*Best replication temperature.*
[b]*Fish-reared temperature.*

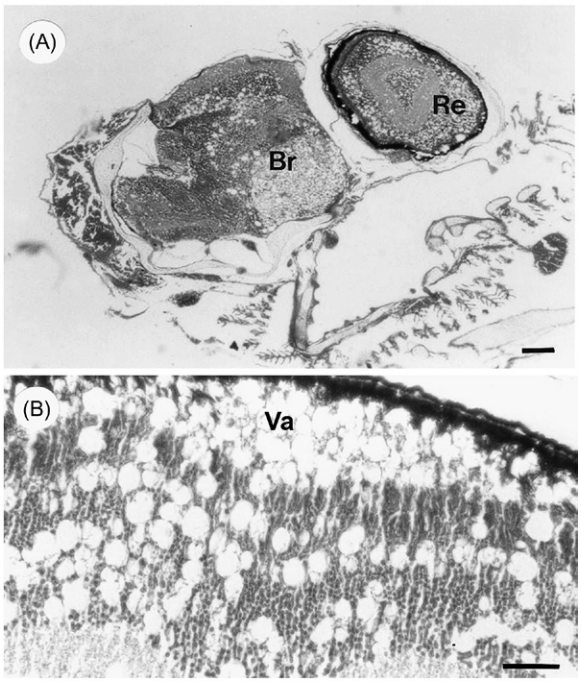

FIGURE 25.1 Light micrography of histological section of (A) the head and (B) the retina in grouper larvae with a betanodavirus infection. Note the severe vacuolation (Va) of the brain and retina. Br, brain; Rt, retina (H & E, bar = 10 μm).

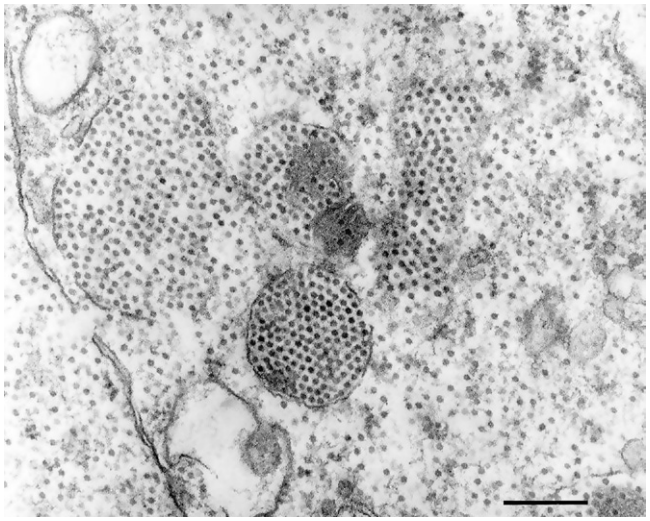

FIGURE 25.2 TEM of betanodavirus particles in the brain of grouper larvae (bar = 400 nm).

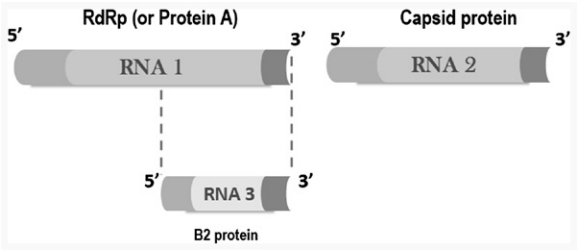

FIGURE 25.3 Schematic genomic organization of fish nodavirus, showing the genomic RNA (RNA 1 and 2) and the subgenomic RNA 3.

mitochondria after NNV infection. Knockdown of the expression of NNV RdRp by specific short hairpin RNA (shRNA) can completely block viral genomic replication (Wu et al., 2008). RNA2 (1433 nt) encodes a capsid protein with a calculated molecular weight of 37.059 kDa (Cherif et al., 2010; Liu et al., 2012; Skliris et al., 2001; Thiery et al., 2006) and contains a highly conserved region and a variable region in its open reading frame (ORF). Capsid protein is the only structural protein of NNV particles and plays a role in inducing apoptosis, followed by secondary necrosis of the cells through a mitochondria-mediated cell death pathway (Wu et al., 2008). The subgenomic RNA3 (371 nt), which is transcribed from the 3′ end of RNA1 and expressed in the virus-infected cells, encodes a nonstructured protein B2, with the function of suppressing host short interfering RNA (siRNA) silencing (Fenner et al., 2006a,b; Iwamoto et al., 2005; Nishizawa et al., 1995; Sommerset and Nerland, 2004) or inducing a necrotic death (Su et al., 2009).

25.3.2 Genotypes and Serotypes

The coat protein gene of a striped jack larvae with VNN disease was first cloned and sequenced, and the sequence similarities was very low (28.6%) between striped jack nervous necrosis virus (SJNNV) and insect nodaviruses, but it is high between SJNNV and other fish nodaviruses (Nishizawa et al., 1995). The insect nodaviruses belong to the *Alphanodavirus* genus of the *Nodaviridae* family, and the fish nodaviruses are classified in the *Betanodavirus* genus in the same family. According to phylogenetic analysis of the partial sequences (427 bases) of the coat protein gene of 25 fish nodavirus isolates collected from Japan, Thailand, Italy and Australia, those betanodaviruses were divided into four groups: SJNNV, tiger puffer nervous necrosis virus (TPNNV), berfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). To date, only these four clusters of fish nodaviruses are accepted as four species of betanodaviruses by the International Committee on Taxonomy of Viruses (ICTV, 2014). The turbot nervous necrosis virus (Johansen et al., 2004a,b) has been proposed as an additional genotype. By cross-neutralization assay using each NNV genotype-specific antiserum, only three serotypes were derived. Although RGNNV and BFNNV are separately isolated from warm- and cold-water fish species, both genotypes share the same serotype (Mori et al., 2003).

25.3.3 Optimal Replication Temperatures of Four Genotypes

The optimal growth temperatures of the four genotypes in the E-11 cell line are 15–20°C for BFNNV, 20°C for TPNNV, 20–25°C for SJNNV and 25–30°C for RGNNV (Iwamoto et al., 2000). BFNNV only affects cold-water fish species in North America, Norway and Japan, and sea bass in France. TPNNV was limited to tiger puffer in Japan (Nishizawa et al., 1997). SJNNV was originally isolated from striped jack in Japan and later in the fish of southern Europe (Thiery et al., 2004). The RGNNV genotype has the broadest host range of warm-water fish species and the widest geographic distribution, including Asia, Australia, the Mediterranean basin and the United States (Munday et al., 2002).

Mass mortalities associated with VNN have been reported during the summer in several species of grouper and sea bass; however, the mortality ceased when the temperature decreased in winter (Fukuda et al., 1996; Le Breton et al., 1997; Mori et al., 1991). The temperature impact of RGNNV replication has been demonstrated in GF-1 cells (Chi et al., 1999). RGNNV can efficiently replicate at 28°C and induce complete cytopathic effects (CPEs) at 7 days postinfection when the viral adsorption temperature was either 20°C or 28°C. No CPEs were observed when RGNNV-infected cells were incubated at 20°C, but viral RNA could still be detected in the cells, indicating that the inhibition of CPEs at 20°C is not related to viral adsorption and is likely due to the temperature impact on viral RdRp activity.

25.3.4 Reassortment and Mutation of Genomic RNAs

Reassortant of betanodavirus with SJNNV RNA1 and RGNNV RNA2 was detected in sea bass caught in Italy and Croatia (Toffolo et al., 2007), and a reassortant betanodavirus with RGNNV RNA1 and SJNNV RNA2 was also found in sea bream and Senegalese sole farmed in Portugal and Spain (Olveira et al., 2009). Betanodaviruses isolates from six countries in southern Europe were analyzed, and 23/120 samples were found to be RGNNV/SJNNV reassortants (Panzarin et al., 2012). Because host specificity of NNV is determined by the capsid protein (Iwamoto et al., 2004), the occurrence of reassortants between SJNNV and RGNNV genotypes will extend the host range for each genotype. It is also surprised that a significantly higher evolutionary rate was found in the viral polymerase (*RdRp*) gene compared to the coat protein gene, suggesting that the evolution of NNV *RdRp* might increase the temperature adaptation of betanodaviruses (Panzarin et al., 2012).

25.4 DIAGNOSIS

Several methods have been developed for the detection of fish nodaviruses. IHC and TEM have been used for histopathological and cytopathological diagnosis, respectively, and dig-labeled probe has been applied to detect tissue distribution of NNV in acutely or persistently infected fish (Chi et al., 1997; Valero et al., 2015).

Direct, indirect and antigen capture enzyme-linked immunosorbent assays (ELISAs) have been established for screening NNV coat proteins in the affected fish (Arimoto et al., 1992; Nishizawa et al., 1995; Shieh and Chi, 2005) or fish antiserum against NNV (Kai et al., 2010; Watanabe et al., 2000). An immunomagnetic reduction assay, using magnetic nanoparticles coated with rabbit anti-NNV antibodies, is developed for monitoring NNV spread, and its sensitivity is about 2×10^1 $TCID_{50}$/mL for NNV (Yang et al., 2012).

The first cell line used for NNV isolation is the SSN-1 cell line (Frerichs et al., 1996), and the cloned E-11 cells derived from SSN-1 cells are highly permissive to the four NNV genotypes (Iwamoto et al., 2000). To date, more and more cell lines have been established for NNV isolation, proliferation and characterization (Chang et al., 2001; Chi et al., 1999; Hegde et al., 2002; Lai et al., 2003; Qin et al., 2006).

Reverse transcription polymerase chain reaction (RT-PCR), as well as nested RT-PCR, are still the major diagnosis methods for NNV in the laboratory (Grotmol et al., 2000; Nishizawa et al., 1994). Real-time quantitative RT-PCR assay (RT-qPCR) is applied for both the detection and quantification of NNV (Panzarin et al., 2010). In addition, a real-time RT-qPCR method has confirmed that the copious content of NNV in grouper fin is similar to that in the brain, so that the fin tissue becomes a new, noninvasive sample source for NNV detection (Kuo et al., 2011). Due to coinfection of RGNNV and SJNNV in the same host fish and reassortants of RGNNV and SJNNV were found in a few species of fish reared in southern Europe, degenerate external primers and two genotype-specific internal primers were designed for simultaneous amplification of NNV products in a single PCR, and the tetra-primer PCR can be an alternative method for virus sequencing within 4 h (Toubanaki et al., 2015a).

Some nucleic acid–based methods are developed for by-pond diagnosis, including the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method (Sung and Lu, 2009); automated microfluidic chip system (Kuo et al., 2012); the CPA-LFD system, which consists of cross-priming isothermal amplification and lateral flow dipstick detection (Su et al., 2015); and a gold nanoparticle-based biosensor for visual detection of the RT-PCR product of NNV nucleic acids

(Toubanaki et al., 2015b). Those methods, being relatively simple, rapid, highly sensitive and specific, are suitable for NNV detection by pond.

25.5 NNV TRANSMISSION AND INFECTION OF FISH

25.5.1 Horizontal Transmission

Clinical signs and mortality can be induced in grouper larvae by immersion challenge with NNV (Kai and Chi, 2008; Mori et al., 1991). Moreover, NNV can spread from diseased to healthy fish by cohabitation when the ratio of the infected to the healthy was 1:100 or more (Arimoto et al., 1993). In advance of cohabituation of the NNV-contaminated feeding prey, like the *Artemia salina* nauplii, is also a route for horizontal transmission (Chi et al., 2003; Skliris and Richards, 1998). NNV-positive wild fishes from sea areas have been detected by RT-PCR, suggesting that wild carrier fish could be the source for VNN outbreak in the offshore aquaculture; on the other hand, wild fish population may be horizontally infected by NNV originating from the diseased fish released from the culture farm into open sea, or via virus-contaminated water or invertebrates (Gomez et al., 2009).

25.5.2 Vertical Transmission

NNV has been detected by RT-PCR in the ovary, eggs and hatched larvae from the infected broodfish, and the outbreak of VNN occurred only in the hatched larvae from NNV-positive spawners, but not from NNV-negative fish (Mushiake et al., 1994), suggesting that NNV can spread vertically from the infected female fish to the offspring. It is also noted that the stressful procedures, such as repeated spawning, may increase the frequency of NNV vertical transmission. Moreover, NNV has been detected in the Sertoli cells and the germ cells in the testis of European sea bass and gilthead sea bream after experimental infection (Valero et al., 2015), indicating that male fish can either vertically transmit NNV.

25.5.3 Infection of Fish and Progression to the Target Organ

In experimentally infected larval striped jack, NNV multiplication was first found in the spinal cord above the swim bladder, then in the brain and finally in the retina. However, virus multiplication was also observed in epidermal cells of skin and intestine of naturally NNV-infected striped jack larvae during acute infection, and it is suggested that NNV particles might enter the host fish through infecting the epithelium of skin and intestines (Nguyen et al., 1996). Because NNV exhibits neurotropism, it is possible that NNV gains access to the central nervous system via peripheral nerves, such as automatic nerves linked to digestive tract or sensory or motor nerves linked to the skin epithelium. In severely infected grouper larvae, NNV could be detected in many organs, such as head kidney, spleen and blood cells, indicating that viremia may cause systemic infection (Chi et al., 2001).

25.6 NNV LIFE CYCLE AND CYTOPATHOGENESIS

25.6.1 Viral Entry Into Cells

NNV-specific monoclonal antibodies with high neutralizing titers were developed (Shieh and Chi, 2005), suggesting that NNV-specific receptors exist on the host cell membrane. By electron microscopic monitoring, NNV was found to penetrate the cells by micropinocytosis and macropinocytosis pathways (Liu et al., 2005). Through virus overlay protein binding assay (VOBPA), grouper heat shock cognate protein 70 (GHSC70) was proved to have a role in the NNV entry step of GF-1 cells, likely functioning as an NNV receptor or coreceptor protein (Chang and Chi, 2015). The binding of naked virions to the cell surface will induce viral penetration into cells through endocytosis, and uncoating will occur between the viral entry and the beginning of viral genome transcription. During this stage, the pH level of endosomes plays a critical role in the uncoating of viruses. Adachi et al. (2007) have demonstrated that the addition of the inhibitors of endosomal acidification will not interfere with the attachment and entry of NNV into host cells, but can inhibit NNV RNA synthesis. In a study of alphanodavirus (namely, Flock house virus (FHV)), the exposure of FHV to low endocytic pH can change the structure of its capsid protein to disturb the endosomal membrane, resulting in the release the viral genome into the cytoplasm (Odegard et al., 2009). Betanodavirus belongs to *Nodaviridae* and might exhibit a similar uncoating mechanism as that of alphanodavirus.

25.6.2 Viral Proliferation

The life cycle of betanodavirus is illustrated in Fig. 25.4. As soon as NNV enters the cytoplasm of cells (<1 h), viral particles quickly uncoat (within 3–4 h) for viral genome release and viral gene expression. Subsequently, NNV RdRp is translated from genomic RNA1 and translocates to mitochondria membrane to synthesize vRNAs, and minor viral proteins are expressed at this stage (Wu et al., 2010a,b). During 1–12 hpi, the B2 protein is expressed and suppresses the host's siRNA response. At the late stage of the replication cycle (24–48 hpi), capsid protein is strongly expressed and assembled, inducing apoptosis and secondary necrosis (Chen et al., 2006, 2007; Su et al., 2009).

25.6.3 Apoptosis and Necrosis

Both B2 and capsid protein are death factors for NNV-infected cells. NNV B2 protein triggers death signals through the upregulation of the proapoptotic gene *Bax* to enhance mitochondria membrane potential (MMP) loss at the middle stage of the viral life cycle (12–24 hpi), and cells were disrupted at the late stage (24–48 hpi). The caspase-independent pathway cell death can be blocked by B2-specific siRNA, the antiapoptotic protein Bcl-xL and the adenine nucleotide translocator-specific inhibitor BKA (Su et al., 2009). NNV capsid protein-induced cell death occurs through postapoptotic necrosis, phosphatidylserine exposure at the early apoptotic stage and loss of MMP for cytochrome *c* releases at the midapoptotic stage. Subsequently, RNA2-activated caspase-8 and 3 trigger postapoptotic necrosis cell death at the late apoptotic stage. The loss of MMP and caspases activation can be blocked by the overexpression of antiapoptotic protein zfBcl-x_L in GF-1 cells (Wu et al., 2008).

Mitochondrial damage and NNV-induced necrotic cell death were assessed in stable cell lines, expressing the antiapoptotic Bcl-2 proteins, zfBcl-xL or zfMcl-1a. Both antiapoptotic Bcl-2 proteins strongly inhibited RGNNV-induced necrotic cell death (Chen et al., 2007), and the overexpression of zfBclxL could dramatically block the cell death induced by capsid protein (Wu et al., 2008) and B2 protein (Su et al., 2009). In addition, RGNNV may preferentially induce caspase-independent death in grouper liver cells (Chen et al., 2006, 2007; Su et al., 2011), but it induces caspase-dependent death in sea bass cells (Guo et al., 2003).

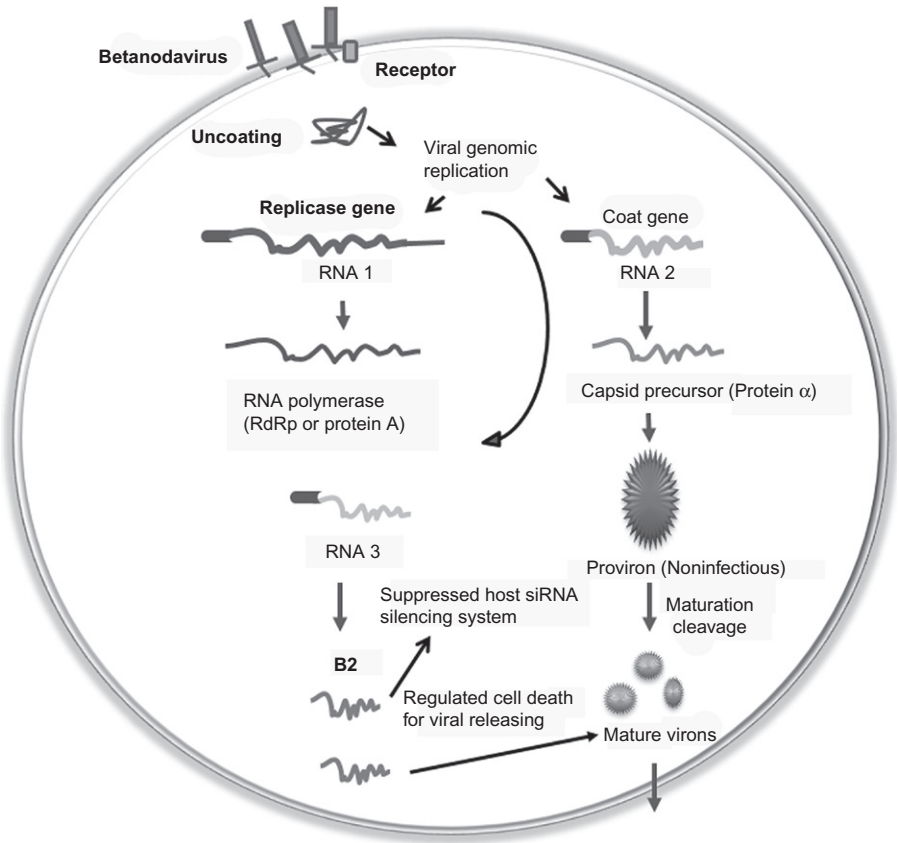

FIGURE 25.4 Schematic illustration showing genomic organization and replication of betanodavirus.

25.6.4 ROS Production

Mitochondria are the main production houses of reactive oxygen species (ROS) during RGNNV infection, and ROS will trigger oxidative stress, which may contribute to the secondary necrotic cell death (Chang et al., 2011; Reshi et al., 2014a,b). Capsid protein induces mitochondria-mediated cell death via caspase-3-dependent pathway, and B2 does the same via a Bax-mediated pathway (Su et al., 2009). Mitochondria complexes I and II of the electron transport chains are the major sites of ROS production (Su et al., 2009; Sugioka et al., 1988). The inhibition of complexes I (retenone) and II (Antimycin) and the oxidation of both complexes, lead to the increase of ROS production (Ott et al., 2007; St-Pierre et al., 2002; Turrens et al., 1985; Turrens and Boveris, 1980). However, ROS is also important for the activation of the body's antioxidant enzymes such as SOD and glutathione peroxidase (Ott et al., 2007). In RGNNV-infected cells, ROS was produced at 24 hpi with a gradual regulation of catalase and Nrf2 transcription factors (Cadenas, 1997; Kaspar et al., 2009; Peterhans, 1997).

25.7 NNV PERSISTENT INFECTION

NNV persistent infection (PI) has been found in many fish species. PI fish without clinical signs of VNN still can release infectious virions into the environment. In Norway, NNV has been detected in Atlantic halibut (*Hippoglossus hippoglossus*) with subclinical signs of VER (or VNN), and large amount of virus particles were found in macrophagelike cells in the central nervous system, including brain and retina (Johansen et al., 2002). The NNV isolated from Atlantic halibut was used to bath-challenge spotted wolffish (*Anarhichas minor*), and half the infected fish died within 8 weeks. At 16 weeks postchallenge, the surviving fish did not show any clinical signs of VER, but NNV could be detected in the eye, brain and abdominal organs, including intestine, liver and spleen, and reisolated by using SSN-1 cells, suggesting that the surviving fish carried infectious NNV and became a horizontal infection source (Johansen et al., 2003). Moreover, after the smolts of Atlantic salmon (*Salmo salar*) were intraperitoneally challenged with tissue homogenate of NNV-infected Atlantic halibut (*Hippoglossus hippoglossus*), NNV was found to transport to the medulla oblongata and replicate in the survivors, resulting in a persistent NNV infection (Korsnes et al., 2005). It is noted that B2 protein, a nonstructure protein of NNV, can be detected only in recently infected Atlantic halibut and Atlantic cod, but not in chronically infected fish, suggesting that acute and PI may be distinguished by detection of B2 (Mezeth et al., 2007).

Interferon response is identified as one of the entities for the maintenance of NNV PI in a barramundi brain (BB) cell line, which was derived from the brain tissue of NNV-persistently infected barramundi *L. calcarifer* (Chi et al., 2005; Wu and Chi, 2006). Infectious NNV could be detected in the supernatant of BB cells, and nearly 1% of BB cells are NNV positive after immunostaining using NNV-specific antibodies. The PI of BB cell line was cured by NNV-specific antibodies treatments for five subcultures, and an NNV-free cBB cell line was obtained. It is found that IFN response was active in NNV-persistently infected BB cells and could be induced in cBB cells by NNV infection. NNV PI was able to reestablish in cBB cells if the multiplicity of infection (MOI) was low ($\leq$1). A two-step induction of IFN response was responsible for the establishment and maintenance of viral PI. The first IFN response occurred in the initially NNV-infected cells, resulting in the release of IFN into culture supernatant. The released IFN was then interacted with the noninfected cells to induce the second IFN response and develop antiviral activity to prevent those cells from lytic infection. Although IFN response was induced in some initially infected cells, those cells lysed finally, and released virions into culture supernatant. It is likely that the cells would be lysed if the entry of NNV was ahead of IFN induction of antiviral activity, and the cells would survive if IFN response started before the entry of viruses. Mx protein is a type I IFN-inducible protein, and the anti-NNV activity of fish Mx proteins has been confirmed in barramundi (Wu and Chi, 2007; Wu et al., 2010b) and grouper (Chen et al., 2008; Lin et al., 2006). Endogenous BMx induced in cBB cells by NNV infection was able to downregulate NNV RNA synthesis through sequestering viral RdRp to perinuclear lysosomes for degradation (Wu et al., 2010b). Moreover, both endogenous grouper and barramundi Mx proteins are revealed to facilitate the clearance of NNV RdRp through autophagy and lysosomes (Wu et al., 2016). Once the balance of NNV replication and IFN response-induced anti-NNV antagonism is achieved, the PI will form. The progression of PI in cBB cells after NNV infection was illustrated in Fig. 25.5.

Temperature is another factor for the development of NNV PI. The *Solea senegalensis* were infected with a reassortant betanodavirus, whose genome consists of RGNNV RNA1 and SJNNV RNA2, and reared at different temperatures. The mortality of fish reared at 22°C was 100%, but it was 8% for the fish reared at 16°C, revealing that 16°C is an unsuitable temperature for replication of the reassortant NNV with RGNNV RdRp. However, viral replication was detected in the surviving fish reared at 16°C, but no clinical signs were observed in those fish. Once the water temperature increased from 16°C to 22°C, the viral load quickly elevated, resulting in the outbreak of mortalities. The rearing temperature is thus suggested to be another factor for NNV PI (Souto et al., 2015).

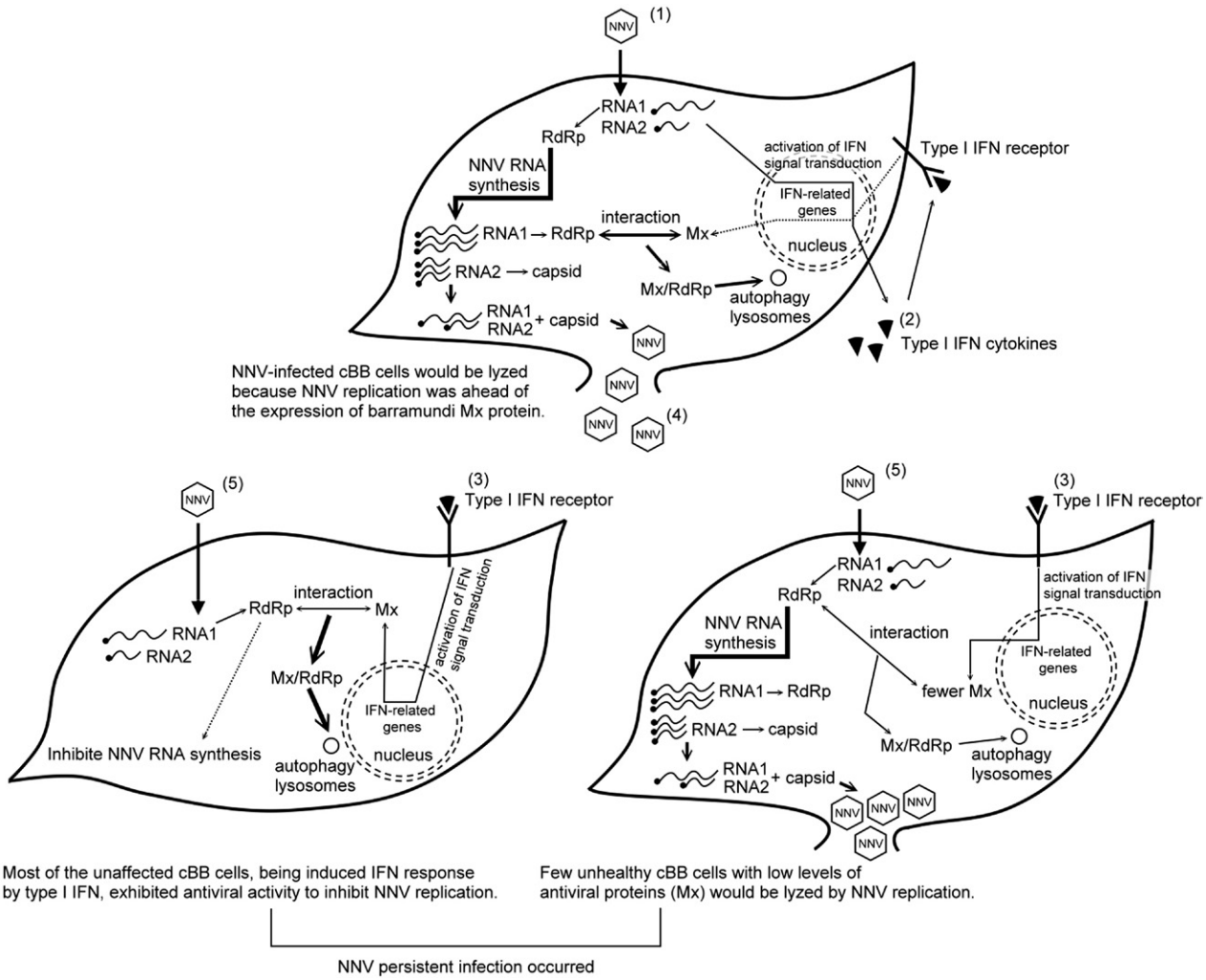

FIGURE 25.5 Schematic summary of NNV PI in cBB cells and the antiviral mechanism of an endogenous Mx protein. (1) After NNV infection, RNA1 is first translated to RdRp to synthesize viral RNA1 and RNA2, which encode RdRp and capsid protein, respectively; vRNA was recognized by pathogen recognition receptors. (2) Next, vRNA turns on the type I *IFN* gene to release type I IFN cytokines, which bind to their receptors to activate the IFN response to produce Mx proteins. Mx proteins would sequester RdRp to the perinuclear area for the degradation of RdRp through autophagy and lysosomes. (3) The type I IFN released from the infected cells induces IFN response in other unaffected cells. (4) The first NNV-infected cells are lysed; the progeny virus released and then (5) infected the unaffected cells that already contain IFN-induced antiviral proteins, including Mx protein. Most of the unaffected cells had antiviral activity against NNV; few unhealthy cells with low levels of antiviral proteins would be affected with NNV. When the dynamics between NNV replication and IFN defense achieve balance, NNV-PI occurs.

25.8 CONTROL STRATEGIES

NNV can be distributed within aquaculture facilities through contaminated equipment and water. Therefore, good husbandry and biosecurity are necessary to prevent NNV infection. It is necessary to disinfect fish transport vehicles, rearing water or live feed. To minimize vertical transmission, the selection of virus-free spawners and reducing the spawning-frequency are important for disease control (Mushiake et al., 1994). By washing fertilized stripe jack eggs and disinfecting seawater with ozone, the occurrence rate of VNN decreased (Arimoto et al., 1996). Each new batch of incoming fish should be maintained in separate ponds from the old batches of fish and avoid sharing equipment among ponds. Monitor of NNV among cultured fish should be done regularly. In the early outbreak of VNN, it is urgent to remove the moribund fish from noninfected fish as soon as possible. When the epidemic situation is out of control, the dead fish and the pond water should be sterilized prior to disposal.

Immunizing the fish with NNV vaccine or any immune compound, combined with appropriate delivery methods designed for different stages of the fish, are good ways to control VNN. To date, NNV vaccines have been developed

internationally and shown to reduce disease incidence and mortalities efficiently after experimental trials. The highest mortality due to VNN always occurs at the larval stage; however, vaccination at the larvae stage is difficult because the immune system of fish is not well developed and the fingerlings are too small to handle for injection immunization. To solve this problem, the protocols of bath immunization using inactivated NNV (Kai and Chi, 2008) and oral immunization using recombinant NNV expressed by *Escherichia coli* or *Vibrio anguillarum* and encapsulated using Artemia (Chen et al., 2011; Lin et al., 2007) were established and showed high protection efficiency with values of relative percentage survival (RPS) of 79–95 for bath vaccine and 69.5 for oral vaccine to protect the larvae against NNV infection. In addition, vaccination of broodstock by intramuscular injection of inactivated NNV 1 month before the breeding season is demonstrated to be very helpful to reduce the risk of vertical transmission of NNV to the offspring (Kai et al., 2010).

The intraperitoneal injection of recombinant vaccine in turbot and Atlantic halibut (Husgag et al., 2001) and viruslike particles vaccine in grouper and sea bass (Liu et al., 2006; Thiery et al., 2006) induce high titer of neutralization antibodies or good protection against NNV infection. Moreover, a field test has revealed that a single administration of the inactivated betanodavirus vaccine could effectively mount a specific immune response and long-term protection against VNN in grouper at the grow-out culture system (Pakingking et al., 2010). By the way, rapid nonspecific and delayed specific protection against VNN can be induced when the groupers were injected with a combination of inactivated betanodavirus and aquabirnavirus (ABV) (Yamashita et al., 2009). In addition to NNV vaccines, several antiviral compounds, including synthesized tilapia hepcidin 1–5 (TH1–5) and cyclic shrimp antilipopolysaccharide factor, have been demonstrated to be potent antiviral agents (Chia et al., 2010; Wang et al., 2010).

Recently, the RNAi technology as a control method against aquatic virus infection has been developed and evaluated (Reshi et al., 2014a,b). NNV RdRp is translated from genomic RNA1 at the very early stage of viral infection, and is necessary for the synthesis of viral RNAs. Wu et al. (2008) have demonstrated that RGNNV capsid protein can cause MMP loss and induce apoptosis. A stable cell line producing RGNNV B2, capsid protein and RdRp was established (Su et al., 2009). By using this cell line, RGNNV B2 protein was found to modulate host siRNA at the early stage of viral replication and trigger the death signals which induced MMP loss and the following cell apoptosis. The apoptotic cell death could be blocked by B2-specific siRNA (Su et al., 2009). Therefore, the siRNA specific to viral RNA1, RNA2 and RNA3, being translated to RdRp, capsid protein and B2 respectively, may have potential for inhibiting NNV replication. A vector-based delivery manor for stable-producing siRNAs specific to RdRp, capsid protein and B2 genes are developed and tested in groupers, and it is found that siRNA specific to NNV RNA1, RNA2 and RNA3 can effectively interrupt the cell death caused by B2 and capsid proteins (Dr. Hong, unpublished data). These findings will open a door for aquaculture industry that RNAi technology might be used as a therapeutic/antiviral agent against fish nodavirus.

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Part IV

DNA Viruses of Crustaceans

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Chapter 26

Nimaviruses of Crustaceans

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26.1 WHITE-SPOT SYNDROME VIRUS

26.1.1 Structure and Composition of Virus

White-spot syndrome virus (WSSV) is a large, double-stranded DNA virus of 250–420 nm in length and 80–120 nm in width (Inouye et al., 1994; Wang et al., 1995; Durand et al., 1997; Kanchanaphum et al., 1998; van Hulten et al., 2001). The genome of WSSV ranges from 292–307 kb, containing 507–684 open reading frames, of which only 181–184 encode its 50 or more functional proteins. Many of these functional proteins have no homology to any other known proteins (van Hulten and Vlak, 2001; Yang et al., 2001). Three isolates of WSSV from China, Thailand and Taiwan have been completely sequenced and submitted to the GenBank database under accession numbers AF332093, AF369029 and AF440570.

Examination of WSSV-infected tissue using transmission electron microscopy (TEM) reveals rod or elliptical-shaped, enveloped, nonoccluded virions within hypertrophic nuclei. The nucleocapsid is composed of rings of stacked subunits (Wang et al., 1995) formed from a capsid originator, and the membrane is produced de novo in the nucleoplasm (Inouye et al., 1994). Many of the virions contain an appendage at one extremity measuring 270–310 nm in length and 30 nm in width. This appendage appears to be extension of the apical envelope (Durand et al., 1996), although its function remains undefined. The nucleocapsid has a superficially segmented appearance with 8-nm diameter subunits arranged in two parallel rows comprising each segment. The nucleoplasm is 180–420 nm in length and 54–85 nm in width and is tightly packed within the virion. The viral envelope is 6–7 nm thick and has a trilaminar unit membrane structure. The distance between the envelope and the nucleocapsid is 2.0–7.5 nm (Durand et al., 1997).

26.1.2 Classification and Virus Replication

WSSV has had several different names since its discovery in 1992 including: rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ) (Inouye et al., 1996), hypodermal and hematopoietic necrosis virus (HHNBV) (Cai et al., 1995), systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya et al., 1995) and white-spot baculovirus (WSBV), among others (Durand et al., 1996; Lo et al., 1996a). In 1995, the virus was assigned to the family *Baculoviridae* and subfamily *Nudibaculovirinae* (Wongteerasupaya et al., 1996), however an analysis of several nucleic acid sequences from the virus proved that it did not belong in this family, and it remain unclassified until 2002 (Lo et al., 1997). During this time, it became apparent that all of the White-spot disease outbreaks were caused by the same virus, thus the family Nimaviridae and genus *Whispovirus* were created in 2002 by the International Committee on Taxonomy of Viruses to properly classify the WSSV (Mayo, 2002a,b). To date, *Whispovirus* is the only genus in this family and WSSV is the type species of the genus (Mayo, 2002a,b).

Shotgun proteomics of the WSSV virion has found that at least 58 structural proteins make up the virion, where at least 33 are located in the envelope, and at least nine in the nucleocapsid (Li et al., 2007a,b). The most abundant proteins in the WSSV envelope are VP28 and VP26, which together make up almost 60% of the envelope (Tang et al., 2007). VP28 is the major envelope protein and there is evidence that it has a role in the infection process by acting as an attachment protein that facilitates WSSV binding and entry into shrimp cells (Yi et al., 2004). The attachment point on VP28 is believed to be at its N and O-linked glycosylation sites on its N-terminus (van Hulten et al., 2000).

Several other shrimp proteins have been found to interact with WSSV envelope proteins including: *Penaeus monodon* Rab7 (PmRab7) binding with VP28 (Sritunyalucksana et al., 2006), VP53A interacting with *P. monodon* chitin-binding

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00026-7
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protein (PmCBP) (Chen et al., 2007a,b, 2009) and β-integrin interacting with VP28 (Li et al., 2007a,b). Little is known about how DNA viruses mediate their entry into cells, but vaccinia virus has been found to use an eight-transmembrane protein complex to facilitate its entry into host cells (Senkevich et al., 2005). PmCBP has been found to interact with 11 WSSV envelope proteins including VP24, VP32, VP39B, VP41A, VP51B, VP53A, VP53B, VP60A, VP110, VP124 and VP337, however antibody-neutralization experiments demonstrated that only antibody attachment to VP53A significantly, although incompletely, blocked WSSV infection (Chen et al., 2009). These findings suggest that WSSV also uses a protein complex to mediate its entry into cells but of those studied, only VP53 interacts with the surface of the host cell (Huang et al., 2012). This complex might also interact with other host proteins, including glucose transporter 1 (Glut1) because Glut1 interacts with both viral VP53A and host PmCBP. This would allow a WSSV envelope protein complex to interact with a host heterodimer consisting of Glut1 and PmCBP (Huang et al., 2012). Transporters have been linked to viral entry of several other viruses including: Glut1 for T-cell lymphotropic viruses (Yu et al., 2011), the exporter of cytoplasmic heme feline leukemia virus subgroup C receptor for feline leukemia virus C (Tailor et al., 1999; Quigley et al., 2004) and gibbon ape leukemia virus and murine amphotropic retrovirus binding to inducible sodium-dependent phosphate symporters (Kavanaugh et al., 1994). Tissue expression of Glut1 in *Litopenaeus vannamei* coincides with the tissue tropism of WSSV (Huang et al., 2012).

Extracellular domains of host cell integrin proteins are able to bind RGD, Arg-Gly-Asp, motifs. A large number of viruses capitalize on this feature by producing envelope proteins that contain an RGD motif to facilitate binding of the virus to the surface of a host cell through these integrins. There are several WSSV envelope proteins that contain an RGD motif including: VP12, VP31, VP36a, VP36b (VP281, VP37), VP90, VP110 and VP187 (Tsai et al., 2006; Xie et al., 2006; Li et al., 2007b). WSSV may use these RGD-motif containing viral proteins to attach to the cell surface and facilitate entry into the cell. Incubation of recombinant VP31, VP33, VO110 and VP187 with crayfish hemocytes found that although all WSSV proteins adhere to the crayfish hemocytes, only VP33, VP110 and VP187 bind through the RGD motif (Li et al., 2006, 2007a,b, 2011). The WSSV VP187 protein has been found to bind to a shrimp β-integrin expressed on the shrimp cell surface and soluble integrin was found to block, although incompletely, WSSV infection in vivo and in vitro (Li et al., 2007a,b). The WSSV envelope proteins VP26, VP31. VP37, VP90 and nucleocapsid protein VP136 all interact with *L. vannamei* β-integrin (LvInt) (Zhang et al., 2014). Although the RGD motif is important for integrin binding, LDV (Leu-Asp-Val) and YGL (Tyr-Gly-Leu) motifs are also important (Zhang et al., 2014). Neutralizing antibodies targeting VP31 and VP37 offer protection to shrimp cells and this could be via the prevention of their interaction with integrins (Li et al., 2005a,b; Wu et al., 2005; Zhang et al., 2014). Binding of an extracellular ligand to a cell surface integrin is well-known to activate intracellular signaling cascades, but more research is required to outline what signaling pathways these might be.

The fact that WSSV interacts with several host cell surface receptors with many different viral proteins provides evidence that there are multiprotein interaction complexes formed by both WSSV and the host cell. These interaction complexes are sites for WSSV recognition, attachment and migration into the cell (Zhou et al., 2009; Chang et al., 2010; Zhang et al., 2014; others). The WSSV protein complexes that are required to mediate binding to the shrimp cell have been named the WSSV "infectome" (Chang et al., 2010). It is clear that at least nine WSSV proteins (WSV010, VP19, VP24, VP26, VP28, VP37, VP38, VP51A and VP51C) make up this "infectome," however bioinformatic predictions suggest that it is VP28, VP37 and VP51A that have the largest number of surface exposed amino acid residues and are the most likely candidates for binding directly to the host cell surface complex in this infectome (Chang et al., 2010).

Although VP12 has an RGD motif, it was found to interact with a *L. vannamei* mitochondrial adenine nucleotide translocase (ANT) that facilitates the exchange of ADP and adenosine triphosphate across the mitochondrial inner membrane. LvANT and VP12 colocalize to the mitochondria, suggesting that VP12 forms a complex with LvANT that is capable of destabilizing the mitochondria through mitochondrial membrane permeabilization, an early signal of cellular apoptosis (Deniaud et al., 2004; Ma et al., 2014). Another protein that complexes with ANT to form the mitochondrial permeability transition pore is the voltage-dependent anion channel (VDAC) protein. Silencing of the expression of VDAC through siRNA has been found to delay the infection of WSSV in several *Marsupenaeus japonicus* organs (Wang et al., 2010). WSSV has been demonstrated to induce apoptosis in infected cells to release replicated virions that spread the WSSV infection to other cells (Sahtout et al., 2001; Wongprasert et al., 2003; Leu et al., 2013). The mechanism of apoptosis induction may be through a VP12-ANT-VDAC complex formed early in the infections process.

Infection by most animal viruses, WSSV included, result in a cellular stress response characterized by the increased expression and localization of heat-shock proteins. Heat-shock cognate protein Hsp70 has a role in the trafficking of receptors and clathrin-coated vesicles and has been implicated in facilitating the entry of several animal viruses into host cells and subsequent virion assembly and disassembly (Yasuko et al., 1998; Cripe et al., 1995; Guerrero et al., 2002). Shrimp Hsc70 is upregulated upon WSSV infection, but it is not translocated to the plasma membrane. Hsc70 binds to VP28 during the virus infection up to 24 h postinfection and although the reason for this interaction is unknown, it is postulated that Hsc70 acts as a chaperone protein to aid the virion assembly (Xu et al., 2009).

VP26 acts as a link between the envelope and the nucleocapsid where its highly hydrophobic N-terminus is anchored in the envelope, and the more hydrophilic C-terminus is bound to the nucleocapsid (Xie and Yang, 2005). Shrimp cellular protein actin and actin-associated proteins can be bound by VP26. This association with cellular structural proteins is a well-known method for viruses to use cytoskeletal machinery to facilitate their transport through the cytoplasm to the target organelle. The target organelle for WSSV is the nucleus, where transcription of the WSSV genome occurs. WSSV VP26 has been found to bind to a protein only found in the hemolymph of WSSV-infected shrimp called the WSSV binding protein (WBP) because it has no homologue in NCBI's GenBank (Youtong et al., 2011). VP26 is known to bind to hemocyte membrane proteins (Liu et al., 2011), but neutralization studies incubating recombinant WBP with WSSV prior to incubation on shrimp hemocyte primary cultures provide significant, although incomplete, protection of the cells to WSSV infection (Youtong et al., 2011). This suggests that WSSV VP26 is binding, at least partially, to the hemocytes through shrimp WBP.

Both VP26 and VP28 form projected trimers in the viral envelope that is similar to the structure of other viral envelope fusion proteins. Viral fusion proteins have an important role in the infective interaction between the viral envelope membrane and cell surface receptors on host cells. All enveloped viruses currently known facilitate entry into a host cell via fusion of their lipid envelope with the host cell plasma membrane. Based on its structural similarity, and that the attachment of VP28 to host cells is known to facilitate WSSV entry into cells through receptor mediated endocytosis (Yi et al., 2004; Kulkarni et al., 2013), it is likely that VP26 and VP28 are involved in mediating WSSV entry into host cells in a similar manner (Xie and Yang, 2005).

The other nucleocapsid proteins of WSSV are much less understood than those located in the envelope. One of the major structural nucleocapsid proteins, in addition to VP26, is VP15. VP15 encodes a putative 90 amino acid protein where almost 25% of the amino acids are serines, and almost 45% of the protein is made up of basic amino acids. The high number of serines and basic amino acids is characteristic of many DNA-binding proteins. VP15 is able to interact with itself, but doesn't bind to other major structural proteins in the virion. However, VP15 has been shown to preferentially bind to supercoiled DNA and likely facilitates the organization and packaging of the WSSV genome into the nucleocapsid (Zhang et al., 2001; Witteveldt et al., 2005; Liu et al., 2010). VP15 has also been shown to interact with *P. monodon* protein PmFKBP46. PmFKBP46 is able to bind DNA and colocalizes to the same subcellular location in the nucleus as VP15 and, therefore, may assist in the packaging of the viral genome into the nucleocapsid during virion assembly (Sangsuriya et al., 2011).

Another WSSV nucleocapsid protein is VP35; a protein that has two potential nuclear localization signals (NLSs). A key requirement for WSSV replication is translocation to the nucleus and cell localization studies have shown that the target for VP35 is the nucleus (Chen et al., 2002). Many large DNA viruses locate a nuclear pore and release a complex of proteins containing NLS sequences tightly associated with the viral genome to facilitate the transport of the viral genetic material into the nucleus (Kasamatsu and Nakanishi, 1998). These NLS sequences on VP35 could be fulfilling this role of WSSV.

26.1.3 Epidemiology

WSSV outbreaks were initially reported in 1992 in *P. japonicus* in Taiwan (Chou et al., 1995) and in Japanese kuruma shrimp (*M. japonicus*) farms (Nakano et al., 1994), and in *P. monodon* and *Fenneropenaeus penicillatus* from Taiwan in 1993 (Chou et al., 1995). It spread from these locations quickly and was found throughout Southeast Asia within 3 years (Inouye et al., 1994; Nakano et al., 1994; Momoyama et al., 1994; Takahashi et al., 1994; Wongteerasupaya et al., 1995; Chen, 1995; Chou et al., 1995; Rosenberry, 1995; Wang et al., 1995; Winarno, 1995). The first cases in North America were found in Texas (Lightner, 1996), followed by several outbreaks in Central and South American countries by 1999 (*Annual animal healthstatus*—http://www.oie.int/hs2/report.asp). WSSV outbreaks have also been documented in Europe in 1995 (Stentiford and Lightner, 2011), the Middle East in 2002 (Tokhmafshan et al., 2004), in wild shrimp caught off the east coast of the United States in 2004 (Chapman et al., 2004) and in Africa in 2010 (OIE, 2011).

WSSV is among the most lethal viruses known to infect Penaeid shrimp, and is a significant impediment to the growth and sustainability of global crustacean aquaculture. It replicates rapidly and can result in 100% mortality in an infected pond in 3–10 days. A study of WSSV virulence examined WSSV isolates from six geographic regions including: China, India, Thailand, Texas, South Carolina and Washington, DC. All six isolates caused 100% mortality in *Penaeus vannamei*, however the Texas isolate caused mortality the most quickly, while the South Carolina isolate was the slowest. When the different isolates were infected into *Farfantepenaeus duorarum*, the Texas isolate caused 60% mortality, while the South Carolina isolate caused only 35% mortality (Wang et al., 1999). Comparisons have also been made between WSSV isolates with genomes of different sizes (292–307 kb) by injecting the isolates into *P. monodon*. The smaller genome isolate, WSSV-Th, caused 100% mortality in 3.5 days, while the common ancestor WSSV isolate, WSSV-Th-96-II, took 14 days to reach complete mortality. When both isolates were passaged together in equal amounts, the WSSV-Th isolate out-competed

the WSSV-Th-96-II isolate within four passages, and was the only WSSV DNA detectable in the fifth passage. This provides significant evidence that WSSV genomes containing a deletion increase fitness, and thereby virulence, by facilitating more rapid replication of the virus (Marks et al., 2005).

26.1.4 Transmission

Horizontal transmission of WSSV is well-known to occur from ingestion of WSSV-infected tissues, injection of cell-free homogenate from infected tissue or exposure of the body surfaces to virus particles (Chang et al., 1996; Durand and Lightner, 2002). Not all methods of transmission are equal because injection of infected tissue is over one magnitude greater at transmitting WSSV than cohabitation (Lotz and Soto, 2002). Vertical transmission of WSSV can occur if viral inclusions are present in the reproductive organs of female *P. monodon* broodstock (Lo et al., 1997). It is thought that males cannot pass WSSV directly on to progeny through the reproductive process because viral particles have only been detected in the connective tissue surrounding the seminiferous tubules, and the muscle and connective tissue surrounding the spermatophores, but not in the germ cells of males. However, WSSV particles have been found in the follicle cells, ovary, oocytes, oogonia and connective cells of female shrimp. No WSSV infection has been detected in mature eggs, which implies that WSSV killed infected eggs before they were able to mature (Lo et al., 1997).

There is a very large list of documented arthropod species that have been found to be capable of being infected by WSSV, or harboring live viral particles (Table 26.1). In 2008, the European Food Safety Authority (EFSA) made a

TABLE 26.1 A List of the Hosts That Are Known to be Capable of Being Infected With, or Carrying, White-Spot Syndrome Virus

	Species	Common Name	Type of Infection	References
Penaeid shrimp	*Metapenaeus affinis*	Jinga shrimp		Mohan (2008)
	M. brevicornis	Yellow prawn		Mohan (2008)
	M. dobsoni	Kadal shrimp	Nat, Exp	Hossain et al. (2001), Ramirez-Douriet et al. (2005)
	M. ensis	Greasyback shrimp	Nat, Exp	Chang et al. (1998), Lo et al. (1996b), Wang et al. (1998a,b)
	M. lysianassa	Bird shrimp	Nat	Hao et al. (1999)
	M. monoceros	Speckled shrimp	Exp	Ramirez-Douriet et al. (2005)
	Penaeus aztecus	Brown shrimp	Exp	Lightner et al. (1998)
	P. duorarum	Pink shrimp	Exp	Lightner et al. (1998)
	P. canaliculatus	Striped prawn	Nat	Mohan (2008)
	P. chinensis	Chinese white shrimp	Nat, Exp	Lu et al. (1997), Yan et al. (2004), Zhan et al. (1998)
	P. indicus	Indian white shrimp	Nat, Exp	Rajan et al. (2000), Rajendran et al. (1999)
	P. japonicus	Kuruma shrimp	Nat, Exp	Lo et al. (1996b), Lu et al. (1997), Wang et al. (1998a,b), Zhan et al. (1998)
	P. merguiensis	Banana shrimp	Nat, Exp	Edgerton (2004), Hossain et al. (2001), Hameed et al. (2001, 2003)
	P. monodon	Giant tiger prawn	Nat, Exp	Chang et al. (1998), Lo et al. (1996b), Rajan et al. (2000), Rajendran et al. (1999), Wang et al. (1998a,b), Zhan et al. (1998)
	P. penicillatus	Redtail shrimp	Nat	Lo et al. (1996b), Wang et al. (1998a)

(*Continued*)

TABLE 26.1 A List of the Hosts That Are Known to be Capable of Being Infected With, or Carrying, White-Spot Syndrome Virus (Continued)

	Species	Common Name	Type of Infection	References
	P. semisulcatus	Green tiger prawn	Nat, Exp	Lo et al. (1996b), Rajendran et al. (1999), Wang et al. (1998a)
	P. setiferus	White shrimp	Exp	Lightner et al. (1998)
	P. schmitti	Southern white shrimp	Exp	Unzueta-Bustamante et al. (2004)
	P. stylirostris	Blue shrimp	Nat, Exp	Lightner et al. (1998), Lu et al. (1997)
	P. vannamei	Whiteleg shrimp	Nat, Exp	Lightner et al. (1998), Lu et al. (1997)
	Parapenaeopsis stylifera	Kiddi shrimp	Nat	Lightner et al. (1998)
	Solenocera crassicornis	Coastal mud shrimp	Nat	Hossain et al. (2001)
	S. indica (S. crassicornis)	Coastal mud shrimp/red prawn	Nat	Hossain et al. (2001)
	Solenocera choprai	Ridgeback shrimp	Nat	Mohan (2008)
	Squilla sp.	Mantis shrimp	Nat, Exp	Hossain et al. (2001), Chakraborty et al. (2002)
	Trachypenaeus curvirostris	Southern rough shrimp	Nat, Exp	Wang et al. (1998a,b)
Prawn	*Alpheus brevicristatus*	Snapping shrimp	Nat	Takahashi et al. (2003)
	A. lobidens	Apping shrimp		Takahashi et al. (2003)
	Callianassa sp.	Mud shrimp	Exp	Lo et al. (1996b)
	Exopalaemon orientalis	Oriental shrimp	Nat, Exp	Chang et al. (1998), Wang et al. (1998a,b)
	Farfantepenaeus aztecus	Northern brown shrimp	Exp	Lightner (1996)
	F. duorarum	Pink shrimp	Exp	Lightner (1996)
	F. penicillatus	Redtail shrimp	Nat	Chou et al. (1995), Lo et al. (1996b), Wang et al. (1998b)
	F. chinensis	Fleshy shrimp	Nat	Flegel (1997)
	Heterocarpus sp.		Nat	Chakraborty et al. (2002)
	Macrobrachium idella		Exp	Rajendran et al. (1999)
	M. lamerrae	Kuncho river prawn	Exp	Chang et al. (1998)
	M. rosenbergii	Giant river prawn	Nat, Exp	Hossain et al. (2001), Lo et al. (1996b), Rajendran et al. (1999), Wang et al. (1998a,b)
	M. japonicus			Wang et al. (1998b)
	Palaemon adspersus	Baltic prawn	Exp	Corbel et al. (2001)
	P. styliferus	Kiddi shrimp	Nat	Hossain et al. (2001), Chakraborty et al. (2002)
Lobster	*Acetes* sp.	Krill	Exp	Supamattaya et al. (1998)
	Homarus americanus	American lobster	Exp	Clark et al. (2013)
	H. gammarus	European lobster	Exp	Bateman et al. (2012)
	Panulirus homarus	Scalloped spiny lobster	Exp	Rajendran et al. (1999)

(*Continued*)

TABLE 26.1 A List of the Hosts That Are Known to be Capable of Being Infected With, or Carrying, White-Spot Syndrome Virus (Continued)

	Species	Common Name	Type of Infection	References
	P. longipes	Longlegged spiny lobster	Nat, Exp	Wang et al. (1998b)
	P. ornatus	Ornate spiny lobster/ tropical rock lobster	Nat, Exp	Rajendran et al. (1999), Wang et al. (1998a)
	P. penicillatus	Pronghorn spiny lobster	Exp	Chang et al. (1998), Wang et al. (1998a)
	P. polyphagus	Mud spiny lobster	Nat, Exp	Rajendran et al. (1999)
	P. versicolor	Painted spiny lobster	Nat, Exp	Chang et al. (1998), Wang et al. (1998a)
	Scyllarus arctus	Small European locust lobster	Exp	Corbel et al. (2001)
Crayfish	*Astacus astacus*	European crayfish	Exp	Jiravanichpaisal et al. (2004)
	A. leptodactylus	Slender-clawed crayfish	Exp	Corbel et al. (2001)
	Cherax destructor albidus	Yabby	Exp	Edgerton (2004)
	C. quadricarinatus	Tropical blue crayfish	Exp	Shi et al. (2000)
	Orconectes limosus	Spinycheek crayfish	Exp	Corbel et al. (2001)
	O. punctimanus	Spothanded crayfish	Nat	Wang et al. (1998b)
	Pacifastacus leniusculus	Signal crayfish	Exp	Jiravanichpaisal et al. (2001)
	Procambarus clarkii	Red swamp crayfish	Exp	Chang et al. (1998), Huang et al. (2001), Wang et al. (1998a)
Crab	*Atergatis integerrimus*	Bashful crab	Exp	Hameed et al. (2003)
	Calappa lophos	Common box crab	Exp	Wang et al. (1998a)
	C. philarigus	Spectacled box crab	Nat, Exp	Lo and Kou (1998), Hameed et al. (2003)
	Callinectes arcuatus	Swimming crab	Nat	Galaviz-Silva et al. (2004)
	C. sapidus	Blue crab	Nat	Chang et al. (2001)
	Cancer pagurus	Edible crab	Exp	Corbel et al. (2001)
	Carcinus maenas	Common shore crab/green crab	Exp	Corbel et al. (2001)
	Charybdis annulata	Banded-leg swimming crab	Nat, Exp	Hossain et al. (2001), Hameed et al. (2003)
	C. cruciata	Crucifix crab	Exp	Hossain et al. (2001)
	C. feriatus	Red flowery crab	Nat, Exp	Lo and Kou (1998), Lo et al. (1996b), Wang et al. (1998a)
	C. granulata	Swimming crab	Exp	Chang et al. (1998), Wang et al. (1998a)
	C. hoplites	Swimming crab	Nat	Chakraborty et al. (2002)
	C. lucifera	Swimming crab	Nat, Exp	Lo et al. (1999), Hameed et al. (2003)
	C. natator	Hairyback crab	Nat, Exp	Lo and Kou (1998), Hameed et al. (2003)
	C. riversandersoni		Nat	Mohan (2008)

(*Continued*)

TABLE 26.1 A List of the Hosts That Are Known to be Capable of Being Infected With, or Carrying, White-Spot Syndrome Virus (Continued)

	Species	Common Name	Type of Infection	References
	Demania splendida		Exp	Hameed et al. (2003)
	Doclea hybrida		Exp	Hameed et al. (2003)
	Gelasimus marionis nitidus	Indian fiddler crab/orange fiddler crab	Nat	Hossain et al. (2001)
	Grapsus albolineatus	Mottled lightfoot crab	Exp	Hameed et al. (2003)
	Halimede ochtodes		Exp	Hameed et al. (2003)
	Helice tridens		Nat	Lo and Kou (1998), Lo et al. (1996b)
	Liagore rubromaculata		Exp	Hameed et al. (2003)
	Liocarcinus depurator	Sandy swimming crab/ blue-leg swimming-crab	Exp	Kanchanaphum et al. (1998), Rajendran et al. (1999)
	L. puber	Velvet swimming crab	Exp	Kanchanaphum et al. (1998), Rajendran et al. (1999)
	Lithodes maja	Norway king crab/northern stone crab	Exp	Hameed et al. (2003)
	Macrophthalmus sulcatus	Ocypodid	Nat	Hossain et al. (2001)
	Matuta miersi (Ashtoret miersii)		Exp	Hameed et al. (2003)
	M. planipes	Flower moon crab	Nat	Lo et al. (1999)
	Menippe rumphii		Exp	Hameed et al. (2003)
	Metopograpsus sp.		Exp	Rajendran et al. (1999)
	Metopograpsus messor	Alamihi/mangrove rock crab	Nat	Hossain et al. (2001)
	Paradorippe granulata		Exp	Hameed et al. (2003)
	Parathelphusa hydrodomous	Rice-field crab	Exp	Hameed et al. (2001)
	P. pulvinata (Barytelphusa pulvinata)		Exp	Hameed et al. (2001)
	Parthenope prensor		Exp	Hameed et al. (2003)
	Phylira syndactyla		Exp	Hameed et al. (2003)
	Podophthalmus vigil	Sentinel crab/long-eyed swimming crab	Exp	Hameed et al. (2003)
	Portunus pelagicus	Sand crab/blue swimming crab/flower crab	Nat, Exp	Lo and Kou (1998), Lo et al. (1996b), Supamattaya et al. (1998)
	P. sanguinolentus	Three spot swimming crab	Nat, Exp	Chang et al. (1998), Lo and Kou (1998), Lo et al. (1996b), Hameed et al. (2003), Wang et al. (1998b)
	Pseudograspsus internedius	Mosaic crab	Nat	Chakraborty et al. (2002)

(*Continued*)

TABLE 26.1 A List of the Hosts That Are Known to be Capable of Being Infected With, or Carrying, White-Spot Syndrome Virus (Continued)

	Species	Common Name	Type of Infection	References
	Scylla serrata	Giant mud crab	Nat, Exp	Kanchanaphum et al. (1998), Lo and Kou (1998), Lo et al. (1996b), Rajendran et al. (1999), Hameed et al. (2003), Supamattaya et al. (1998)
	S. olivacea	Mud crab	Exp	Somboona et al. (2010)
	S. paramamosain	Mud crab	Exp	Somboona et al. (2010)
	S. tranquebarica		Exp	Rajendran et al. (1999)
	Sesarma sp.	Marsh crab	Exp	Kanchanaphum et al. (1998), Rajendran et al. (1999)
	S. oceanica (Labuanium rotundatum)		Nat	Lo et al. (1999)
	Somanniathelpusa sp.	Black rice crab	Nat, Exp	Flegel (1997)
	Thalamita danae	True crab	Exp	Hameed et al. (2003)
	Uca pugilator (Uca (Leptuca) pugilator)	Atlantic sand fiddler	Exp	Kanchanaphum et al. (1998)
Other	*Artemia* sp.		Exp	Hameed et al. (2003)
	A. franciscana		Exp	Li et al. (2003)
	Brachionus urceus	Rotifer	Nat	Yan et al. (2004)
	Crassostrea gigas			Vazquez-Boucard et al. (2010)
	Dragon nymphfly		Nat	Mohan (2008)
	Ephydridae sp.	Shore fly	Nat	Wang et al. (1998b)
	Gerries		Nat	Mohan (2008)
	Marphysa gravelyi	Polychaeta	Nat	Vijayan et al. (2005)
	Notonecta	Backswimmer	Nat	Mohan (2008)
	Nepa		Nat	Mohan (2008)
	Ranatra	Water scorpion	Nat	Mohan (2008)
	Schmackeria dubia	Copepods	Nat	Wang et al. (1998b)
	Sergestoidea Acetes sp.	Krill-like prawns	Nat, Exp	Supamattaya et al. (1998), Edgerton (2004)

Nat, natural infection found in the wild or an aquaculture setting; Exp, infection established in a laboratory.
Species name in brackets indicates a change of the species designation since originally reported in the original scientific manuscript.
Source: This table has been modified from EFSA, 2007. Possible vector species and live stages of susceptible species not transmitting disease as regards certain crustacean diseases. EFSA J. 598, 1–91.

declaration that all decapod crustaceans, more than 20,000 marine, brackish and freshwater species, are susceptible to WSSV infection (EC Directive 2006/88/EC) despite scientific evidence for only approximately 100 arthropod species. In addition, all life stages of decapod crustacean are believed to be susceptible to WSSV infections (Lightner, 1996; Venegas et al., 1999). WSSV is such a devastating disease in crustacean aquaculture that the OIE listed it as a reportable disease in 2000 (OIE, 2014).

Nonarthropod species can facilitate the transfer of WSSV by accumulating live WSSV particles and then releasing them. Polychaetes sampled from live-feed suppliers in shrimp aquaculture areas were examined and found to contain infective

WSSV particles which, when fed to *P. monodon* broodstock, caused WSSV infection (Vijayan et al., 2005). Other noncrustacean carriers of WSSV are bivalves (Vijayan et al., 2005), isopods (sea slaters), rotifers (Yan et al., 2004) and Euphydradae insect larvae. Many of these nondecapod crustacea can act as vectors for WSSV as they accumulate large quantities of live WSSV. For this reason, it is important that WSSV-infected ponds are completely decontaminated; not just the removal of the infected aquaculture species. It is unlikely that sea birds can potentially act as passive vector of WSSV. WSSV was detectable in the faces of seagulls (*Larus atricilla*) fed infective shrimp by polymerase chain reaction (PCR) for up to 72 h, but injection of the PCR positive feces did not result in WSSV infection in Penaeid shrimp (Vanpatten et al., 2004).

26.1.5 Pathology and Immunity

The primary target of infected WSSV is the stomach and gut where WSSV is known to interact with the epithelial cells of the intestinal tract (Arts et al., 2007; Di Leonardo et al., 2005). Once in the stomach and intestinal tract of the host, the viral infectome complex attaches to epithelial tissues to facilitate transcytosis through the epithelial lining and infects underlying connective tissue (Kulkarni et al., 2013). Once into the connective tissue, the virus has been observed in cytoplasmic vacuoles of hemocytes, which is probably due to phagocytosis by the hemocytes (Durand et al., 1997). The open circulatory system of the infected animals, and the circulatory nature of hemocytes, allows WSSV to rapidly travel throughout the animal and infect many different tissues (Di Leonardo et al., 2005). WSSV virions can also be found freely circulating in the hemolymph of heavily infected animals.

Disease outbreaks seem to occur more frequently flowing environmental changes that cause stress to aquaculture animals, such as large changes in temperature or rapid changes in salinity following flooding events. Outbreaks usually occur when water temperatures are between 18°C and 30°C (Song et al., 1996; Vidal et al., 2001). It has previously been shown that WSSV is capable at replicating above 20°C, but at temperature of 16°C and below, it is only capable of attaching to surface of host cells without entering the cell and successfully replicating (Jiravanichpaisal et al., 2006). At the upper temperature range, it has been found that changing the water temperature from 27°C to 33°C significantly decreases mortality in acutely infected individuals, but causes the rapid onset of mortality in chronically infected shrimp (Rahman et al., 2007). Most crustacean aquaculture systems are in outdoor ponds subject to changes in ambient temperatures and environmental conditions, so it is unlikely that water temperatures can be controlled on a large scale. It is possible, however, that short-term changes in temperature (ie, water temperature or storing the animals on ice) may allow farmers the time necessary to conduct an emergency harvest of a WSSV-infected pond and successfully deliver the harvest to a processing facility.

Crustaceans, like all invertebrates, lack an adaptive immune system comparable to the vertebrate system of B and T lymphocytes capable of producing long-term, multiyear protection against pathogenic organisms through humoral antibody production or cytotoxic T lymphocytes. Invertebrates do have very good innate immune systems capable of responding rapidly and effectively to a wide range of pathogens present in their environment. This innate immune system is capable of offering excellent short-term protection however; this response is ultimately ineffective against WSSV.

26.1.6 Diagnostic Methods

Clinical signs of WSSV infection include anorexia, lethargy, loose cuticle, white spots on the cuticle and a reddish discoloration of the whole animal (Fig. 26.1). Preliminary diagnosis of WSSV infection can be made based on the appearance of white calcium deposits on in the subcuticular epithelium, which are approximately 0.5–2.0 mm in diameter (Durand et al., 1997). However, these spots are not present on all hosts, especially nonshrimp hosts, and could also be caused by high alkalinity, stress or appear after bacterial infections in shrimp (Wang et al., 2000a,b,c; Sánchez-Paz, 2010). The combination of white spots and behavioral changes, such as a rapid reduction in food consumption and shrimp localizing at the water surface near the edges of the aquaculture pond, indicate that large-scale mortality will likely begin within a few hours to a few days (OIE, 2014). A marked reduction in the clotting time of crustacean hemolymph usually accompanies heavy WSSV infection; however this symptom also occurs with many other viral and bacterial diseases of crustaceans.

Histopathological characterization of WSSV infection involves the presence of hypertrophic nuclei containing inclusion bodies that are eosinophilic to progressively more basophilic, using hematoxylin and eosin stain (Fig. 26.2). Nuclei at the early stage of infection have marginated chromatin with a ring of unstained nucleoplasm separating the chromatin from a reddish inclusion typical of Cowdry A-type inclusions. Highly infected nuclei contain mature virions assembled in ordered arrays or randomly scattered throughout the nucleus, while forming virions are centrally located (Durand et al., 1997). WSSV infects tissues of ectodermal and mesodermal origins, including epithelial and connective tissues,

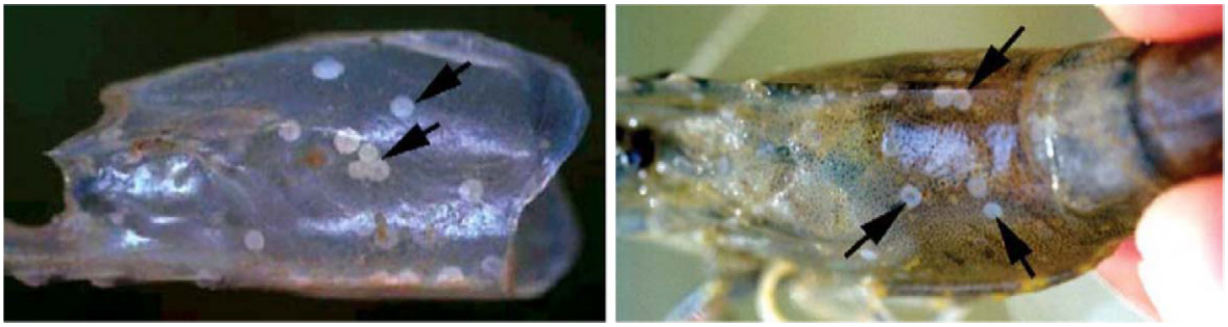

FIGURE 26.1 Gross clinical signs of WSSV infection in penaeid shrimp. White inclusions caused by calcium deposits within the cuticle (*arrows*) are easily visible. *Image taken from Flegel, T.M., 2006. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 256, 1–33.*

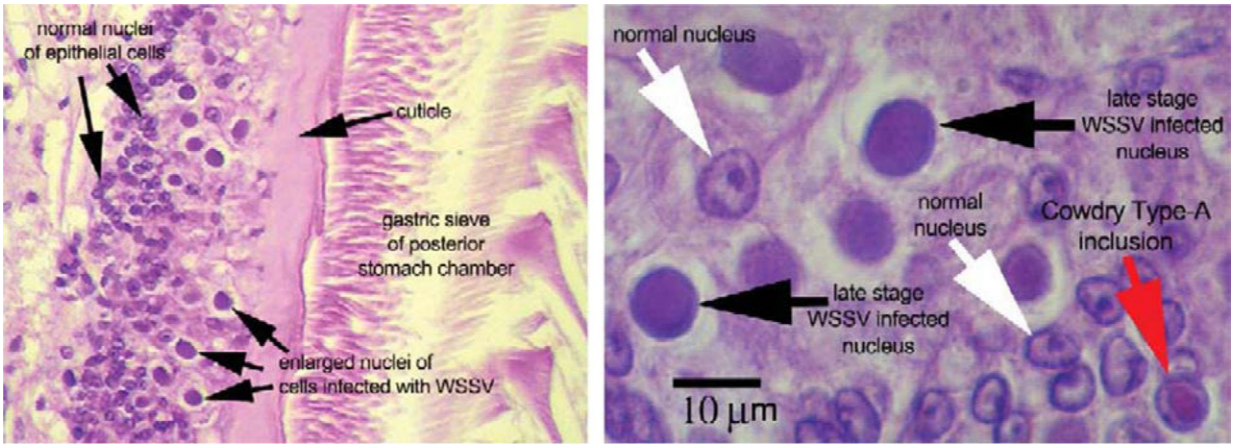

FIGURE 26.2 Histopathological examination of shrimp infected with WSSV and stained with hematoxylin and eosin stain. (Left) Low magnification micrograph showing characteristic inclusions of WSSV in the subcuticular epithelium of the gut. (Right) High magnification including a nucleus with a Cowdry A-type inclusion in the lower right corner. *Image taken from Flegel, T.M., 2006. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture 256, 1–33.*

hematopoietic nodules, gills, epidermis, stomach, nerves and striated muscles (Wongteerasupaya et al., 1995; Durand et al., 1996). Confirmation of WSSV infection can be made using TEM of infected tissues where rod or elliptical-shaped virions of 270–420 nm in length and 80–120 nm in width are visible in hypertrophic nuclei.

Bioassays are one method to prove freedom from WSSV infection. Specific pathogen-free (SPF) shrimp are injected with cell-free homogenate generated from the pleopods of the crustacean in question. If all other tests are negative, and all of the injected SPF shrimp are still alive after 3 days, then it can be assumed the bioassay results are also negative (Nunan et al., 1998). Cell culture is not recommended as a routine method of WSSV detection because there is no crustacean cell line available, and there is a high risk of bacterial contamination. Primary cell culture is also not recommended because there is no standard medium that is recommended and it is difficult to initiate and maintain WSSV in culture. Bioassay is the only method recommended for virus propagation.

Proper sampling procedures and sample selection must occur for WSSV detection using molecular diagnostic methods. The best tissues to sample in shrimp are those of ectodermal and mesodermal origin, with particular interest in the cuticular epithelium or subcuticular connective tissue, or antennal glands and the hematopoietic organ. Some sampling protocols require pleopods, hemolymph, gill, stomach or abdominal muscle for submission (Lo et al., 1997). Screening for WSSV via PCR usually requires a piece of gill, pleopod or hemolymph sample to be submitted. A tissue of endodermal origin, such as hepatopancreas and midgut, are not suitable; nor is the compound eye that contains a PCR inhibitor and makes it inappropriate for PCR-based diagnosis (Lo et al., 1997).

There are several antibody-based methods available for the detection of WSSV and these rely on immunodot, western-blot, immunohistochemistry, indirect fluorescent antibody test or enzyme-linked immunosorbent assay (Huang and Yu,

1995; Poulos et al., 2001; Sithigorngul et al., 2006; Yoganandhan et al., 2004). These antibody tests are rapid and fast, but only have the sensitivity of a one-step PCR test. There are also some experimental and commercial lateral flow kits available that are based on either isothermal amplification or antibodies. These tests are also rapid and have the benefit of increased sensitivity over the aforementioned antibody tests (Sithigorngul et al., 2006; Chen et al., 2007a,b; Srisala et al., 2008; Jaroenrama et al., 2009).

There are multiple PCR-based detection assays that have been used in research and are currently commercially available. The OIE recommended gold standard is a two-step PCR protocol described by Lo et al. (1996a,b). The test is relatively time consuming, but is very sensitive. However, this test has been found to give false-positive results in some nonshrimp crustaceans, while another study also listed the low annealing temperature of the Lo et al. (1996a, 1996) procedure as conducive to occasional false-positives (Chang et al., 2002; Claydon et al., 2004). More modern fluorescent-based tests have been developed that have the same or slightly better sensitivity, and are much faster. Numerous quantitative PCR tests have been described for a variety of crustacean hosts (Durand and Lightner, 2002; East et al., 2004; Jang et al., 2009; Meng et al., 2009, 2010; Fouzi et al., 2010; Zhu and Quan, 2012; Chou et al., 2011; Clark et al., 2013; Mendoza-Cano and Sánchez-Paz, 2013). However any newly developed PCR-based test should be thoroughly validated against the Lo et al. (1996a,b) test.

The OIE recommends the following diagnostic methods for confirmatory diagnosis of WSSV infection: TEM, in situ hybridization with DNA probes, PCR-based amplification and DNA sequencing (OIE, 2014). Detection by DNA probes or PCR amplification methods alone can be problematic because they only indicate detection of the viral genome and do not confirm susceptibility to WSSV. This type of detection doesn't indicate intracellular infection and may simply be viral particles or genetic material within the gut contents or contaminating the surface of the animal. Infection should be confirmed by traditional light and TEM, immunohistochemistry or presence of clinical signs.

26.1.7 Prevention and Control

There is currently no treatment for WSSV in any of its known hosts. The most effective treatment measures so far have been rigorous husbandry and sanitation practices on crustacean aquaculture farms. These practices have been reasonably effective at minimizing outbreaks and preventing their spread once they have occurred. Some of the recommended husbandry practices are to avoid stocking in seasons where the water temperature is between 20°C and 30°C, use biosecure water and culture systems and use SPF, or PCR-negative, seed animals to stock grow-out ponds. WSSV can be inactivated at 50°C for 120 min, or less than 1 min at 60°C (Nakano et al., 1998).

Several natural treatments have been used in shrimp aquaculture where the pretreatment of shrimp with a herbal extract is hoped to significantly improve the host's immune performance on a short-term basis. This could either be through prevention of infection or significantly slowing the progress of infection once established in the host. Many research trials have used water or methanol extractions of traditional herbal plants with some success at prolonging the life of WSSV-infected crustaceans; although none have been shown to be completely effective in preventing WSSV-induced mortality throughout the normal grow-out period (Citarasu et al., 2006; Rameshthangam and Ramasamy, 2007; Balasubramanian et al., 2008; Yogeeswaran et al., 2012). Although some of these treatments have shown limited efficacy, much more scientific validation and extensive reporting of metadata is required before they can be implemented on a large scale.

Another anti-WSSV approach has used WSSV-gene-specific, double-stranded RNA to induce RNA silencing (RNAi) in vivo. Several studies have been conducted and have been shown to inhibit the replication of WSSV in vivo (Robalino et al., 2005; Westenberg et al., 2005; Wu et al., 2007; Xu et al., 2007; Zhu and Zhang, 2011). The efficacy of these treatments remains to be examined in large-scale controlled trials and the traditional method of delivery for these applications as injection is prohibitively expensive and time-consuming. New studies have emerged that use bacteria to express VP28dsRNA which, once inactivated, are fed to shrimp. This approach has been found to reduce the WSSV-induced mortality in *P. monodon* (Sarathi et al., 2008; Ning et al., 2009). The ability to administer an RNAi method through feed would be much more feasible from a production efficiency prospective, but requires further examination and large randomized trials before widespread adoption is recommended.

A more recent approach has been the production of recombinant WSSV envelope proteins and adding them to shrimp diets. Most current approaches use WSSV VP28 because this protein has been shown to bind to the surface of a host cell through the infectome, and facilitate entry into cells, including the epithelial cells lining the shrimp intestinal tract (Chang et al., 2010; Kulkarni et al., 2013). The in vivo studies using this approach have shown some promise (Namikoshi et al., 2004; Witteveldt et al., 2004a,b; Jha et al., 2006; Mustaq et al., 2011) although none are able to provide 100% protection against WSSV. The ability to make these recombinant WSSV proteins quickly and easily in bacteria, and their ability to be administered through shrimp feed, holds promise for future products using multiple recombinant proteins simultaneously.

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Chapter 27

Nudiviruses of Crustaceans

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27.1 SOME NOTES ON THIS CHAPTER

The family *Nudiviridae* is a virus family that was recently approved by the International Committee on Taxonomy of Viruses (ICTV), containing "non-occluded baculoviruses" that had previously been placed in the family *Baculoviridae* because of their large dsDNA genome, replication in the nucleus and hypertrophy of infected cells (Jehle et al., 2013). According to Wang and Jehle (2009), the *Penaeus monodon* baculovirus (variously known as *P. monodon* singly enveloped nuclear polyhedrosis virus (PmSNPV) or Monodon baculovirus (MBV)), the causative agent of spherical baculovirosis in the shrimp *P. monodon*, is also more closely related to nudiviruses than to baculoviruses; these researchers have proposed the name *P. monodon* nudivirus (PmNV) for it. During the writing of this book, it became clear to the editors that it would not be possible to find contributors to this chapter in a timely manner because the international experts who used to work on these viruses are all retired, and there are not many "pure shrimp virologists" out there, as there are no cell lines to grow shrimp viruses. So, most of the work on shrimp viral diseases is a combination of pathology and virology, and the "shrimp pathologists" believe that they are not qualified to write shrimp virology chapters. In order to help foster understanding of PmNV and its capacity to cause disease, readers are referred to the review article by Yongjie Wang and Johannes A. Jehle, "Nudiviruses and Other Large, Double-Stranded Circular DNA Viruses of Invertebrates: New Insights to an Old Topic," from *Journal of Invertebrate Pathology* (2009), which describes the comparative virology of members in the *Nudiviridae* family.

27.2 GENUS *GAMMANUDIVIRUS*

Recently, Yang et al. (2014) used next-generation sequencing and traditional polymerase chain reaction methods to obtain the complete *Pm*NV genome sequence of 119,638 bp encoding 115 putative open reading frames (ORFs). Phylogenetic tree analysis showed that several *Pm*NV genes and sequences clustered with the nonoccluded nudiviruses (NVs), not with the baculoviruses and the *Pm*NV polyhedrin, which is a functionally important protein and the major component of the viral occlusion bodies (OBs) and is sensitive to acid conditions. However, unlike the baculoviral polyhedrin, it is not susceptible to alkali treatment. Yang et al. (2014) concluded that *Pm*NV is not a baculovirus—it should be assigned to the *Nudiviridae* family with the other NVs, and in a new proposed genus *Gammanudivirus*, distinct from the terrestrial NVs. Thus, whereas members of *Baculoviridae* infect only insects, NVs have a considerably broader host range of arthropods, including crustaceans (shrimp).

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Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00027-9
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Chapter 28

Parvoviruses of Crustaceans

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28.1 SOME NOTES ON THIS CHAPTER

The family *Parvoviridae* is subdivided into two subfamilies. Subfamily *Parvovirinae* contains viruses of vertebrates, and subfamily *Densovirinae* contains viruses of insects and shrimp. The shrimp viruses were previously known as the hepatopancreatic parvovirus (HPV) of prawns and shrimp and infectious hypodermal and hematopoietic necrosis virus (IHHNV) of prawns and shrimp. They are now placed in two newly approved genera by the International Committee on Taxonomy of Viruses: *Hepandensovirus*, type species *Decapod hepandensovirus 1* with seven viruses (*Penaeus monodon* hepandensovirus 1–4; *Penaeus chinensis hepandensovirus*; *Penaeus merguiensis hepandensovirus*; *Fenneropenaeus chinensis hepandensovirus*); and *Penstyldensovirus*, type species *Decapod penstyldensovirus 1* with four viruses (*Penaeus stylirostris penstyldensovirus 1 and 2; Penaeus monodon penstyldensovirus 1 and 2*) (Cotmore et al., 2013).

Hepandensoviruses are widespread and highly pathogenic in nature, causing hepatopancreatic disease, and can constitute an economic threat in cultured shrimp populations on rare occasions when larvae from wild-caught shrimp are introduced. Penstyldensoviruses were first identified as pathogens responsible for an economically significant and virulent disease in farmed shrimp IHHNV, are widespread in nature, but are no longer a major economic problem because tolerant shrimp populations have been developed (Cotmore et al., 2013). During the writing of this book, it became clear to the editors that it would not be possible to find contributors to this chapter in a timely manner because the international experts who used to work on these viruses are all retired, and there are not many "pure shrimp virologists" out there as there are no cell lines to grow shrimp viruses. So, most of the work on shrimp viral diseases is a combination of pathology and virology, and the "shrimp pathologists" believe that they are not qualified to write shrimp virology chapters. In order to help foster understanding of the historic importance of penstyldensoviruses (previously IHHNVs) and the capacity of hepandensoviruses (previously HPVs) to cause disease, readers are referred to the review article by Timothy W. Flegel, "Historic emergence, impact and current status of shrimp pathogens in Asia," from *Journal of Invertebrate Pathology* (2012), which describes the status of major shrimp viruses in Asia. Additional information on IHHNV relating to shrimp farming in the Americas can be found in the review article by Don V. Lightner, R. M. Redman, Carlos R. Pantoja, Kathy F. J. Tang, Brenda L. Noble, Paul Schofield, L. L. Mohney, Linda M. Nunan and S. A. Navarro, "Historic emergence, impact and current status of shrimp pathogens in the Americas," from *Journal of Invertebrate Pathology* (2012).

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Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00028-0
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Chapter 29

Circoviruses of Crustaceans

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29.1 INTRODUCTION

Circular, Replication initiator protein (Rep) - encoding single-stranded DNA (CRESS-DNA) viruses are among the smallest viruses known to infect metazoans (about 20 nm). Due to their limited detection in invertebrate hosts, the genomic, morphological and ecological characteristics of crustacean-associated circoviruses remain largely undefined. However, circoviruses identified in aquatic crustaceans exhibit features congruent with vertebrate (porcine, avian)- and insect (dragonfly, damselfly)-associated circoviruses (Dunlap et al., 2013; Hewson et al., 2013a,b; Pham et al., 2014; Ng et al., 2013, Rosario et al., 2015).

Genome architecture is diverse within *Circoviridae*. Nevertheless, all circoviral genomes contain two open reading frames (ORFs) and a nonanucleotide (9-nt) origin of replication encoded in a covalently closed putative stem loop structure. Monopartite genomes are typically ~1.7–3.0 kb (Rosario et al., 2015). The Rep ORF encodes a replication initiator protein (vRep), which targets the origin of replication (Ori) located within the stem loop. Viral propagation occurs via rolling circle replication. Cap ORFs are less conserved than Rep and are responsible for encoding structural protein subunits used to construct a nonenveloped, icosahedral capsid. Unlike other eukaryotic circoviruses, crustacean circoviruses may contain both ambisense and unisense ORFs. Furthermore, one virus detected in *Daphnia* species collected in two lakes in upstate New York represents an unusual putative DNA-RNA virus hybrid, similar to the Boiling Spring Lake RNA-DNA hybrid virus (BSL-RDHV; Hewson et al., 2013b). Thus, there is much to discover about the structure, genetics, transmission and pathology of circoviruses in aquatic crustaceans.

29.2 SPECIFIC MAIN CRUSTACEAN DISEASES

As of 2014, no crustacean-associated circoviruses have been definitively linked to disease. Indeed, no circoviruses have been isolated from invertebrate hosts, restricting our knowledge of their pathology, epidemiology and relationship with host immunity. Transmission electron microscopy or quantification of viral prevalence/load indicates that circoviruses actively infect crustacean tissues and are not simply transient viruses acquired from the environment. Our best understanding of circovirus pathogenicity is derived from two case studies involving *Diporeia* spp., a freshwater benthic amphipod, and *Daphnia* spp., a freshwater cladoceran (Table 29.1).

Annual monitoring programs have been conducted in the Laurentian Great Lakes since 1997 and depict progressive and severe (up to 90%) declines in *Diporeia* spp (Barbiero et al., 2010). Metaviromic analysis has established that Type V circoviruses represent a significant constituent of viral reads (>30%) associated with *Diporeia* spp. collected in Lake Michigan, where *Diporeia* spp. decline is pronounced (Hewson et al., 2013a). Comparably, viral prevalence and load was lower in lakes where *Diporeia* populations were stable (Lake Superior, Owasco Lake). Although no direct causative link has implicated circoviruses in *Diporeia* decline, there is a correlation between viral prevalence and host population stability. In addition, detection of the *Daphnia mendotae*–associated hybrid virus (DMClaHV) has been coincident with population fluctuations in cladoceran hosts. A time series analysis over the summer months in Oneida Lake (upstate New York) reveals a correlation between host population dynamics (abundance, birth/death rates), viral prevalence and viral load. Viral prevalence and load correlated with a slightly delayed host population decline, suggesting that DMClaHV may play a role in *Daphnia* spp. population dynamics (Hewson et al., 2013b). In sum, our knowledge of crustacean-associated circovirus pathogenicity is in its infancy.

CRESS-DNA viruses, including circoviruses, also have been observed in field surveys of pelagic microcrustaceans (Eaglesham and Hewson, 2013, Rosario et al., 2015) and intertidal crustaceans (Rosario et al., 2015), suggesting their ubquity within crustacean hosts. Circovirus-like seuqences have also been discovered in environmental reserviors, such as rice paddy

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00029-2
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TABLE 29.1 Characteristics of Crustacean-Associated Circoviruses

Host	Collection Site	Name	Genome Size (nt)	Putative Rep (aa)	Putative Cap (aa)	Nonanucleotide Motif	Type[a]	ORF Direction	Genbank Accession No.	Reference
Acartia tonsa	Tampa Bay	*Acartia tonsa* copepod circo-like virus (AtCopCV)	1670	376	159	AAGTATTAC	–	Unisense	PRJNA182699	Dunlap et al. (2013)
Labidocera aestiva	Tampa Bay	*Labidocera aestiva* copepod circo-like virus (LaCopCV)	1764	255	227	TATTATTAC	–	Unisense	PRJNA182700	Dunlap et al. (2013)
Diporeia spp.	Lake Michigan	LM29173	1926	260	347	TAGTATTAC	V	Unisense	PRJNA182269	Hewson et al. (2013a)
		LM19063	1944	268	323	TAGTATTAC	V	Unisense		
		LM5751	1430	267	147	TAGTATTAC	VI	Unisense		
		LM3487	2491	402	277	TAGTATTAC	I	Ambisense		
		LM21832	1819	217	318	TAGTATTAC	IV	Ambisense		
		LM3635	1779	314	188	TAGTATTAC	VI	Unisense		
		LM3602	1812	217	319	TAGTATTAC	IV	Ambisense		
		LM28925	2158	296	306	TAGTATTAC	I	Ambisense		
	Owasco Lake	OL7115	3615	563	310	TAGTATTAC	V	Unisense		
		OL36582	2595	237	384	TAGTATTAC	V	Unisense		
		OL7433	1906	291	316	TAGTATTAC	V	Unisense		
Daphnia mendotae & Daphnia retrocurva	Oneida Lake and Cayuga Lake	*Daphnia mendotae*-associated (Cladocera) hybrid virus (DMClaHV)	1173–3648	164–226	131–529	TAGTATTAC	–	Ambisense	PRJNA182249	Hewson et al. (2013b)
Penaeus monodon	Vietnam	*Penaeus monodon* Circovirus (PmCV-1)	1777	266	255	TAATATTAC	–	–	KF481961	Pham et al. (2014)
Farfantepenaeus duorarum (hepatopancreas)	Gulf of Mexico	Shrimp hepatopancreas-associated circular DNA virus (ShrimpCDV)	1956	–	–	AGGTATTAC	IV	Ambisense	KC441518	Ng et al. (2013)

[a]Rosario et al. (2012).

soils (Kim et al., 2008) and virioplankton of an Antarctic lake (Lopez-Bueno et al., 2009). The hosts of these environmental CRESS-DNA viruses are unknown. However, their observation adds to the rapidly expanding known diversity of CRESS-DNA viruses in nature (Rosario et al., 2009; Delwart and Li, 2012). Recent observations of circoviruses in viral metagenomes prepared from echinoderms (Hewson et al., 2014) also illustrates that there is wide diversity of circoviruses in natural reservoirs. The pathology, epidemiology and ecological functions of these viruses remain unknown.

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Part V

RNA Viruses of Crustaceans

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Chapter 30

Totiviruses of Crustaceans

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30.1 INTRODUCTION

Totiviruses largely infect yeast, fungi and protozoa (Ghabrial, 2008), and also infect other species including, crustaceans (Edgerton et al., 1994; Lightner et al., 2004), several insects (Isawa et al., 2011; Koyama et al., 2015; Wu et al., 2010; Yang et al., 2012; Zhai et al., 2010) and fin fish (Lovoll et al., 2010). These viruses are characterized by an isometric virion, approximately 40 nm in diameter that contains a nonsegmented, double-stranded (ds) RNA genome coding for a capsid protein (CP) and an RNA-dependent RNA polymerase (RdRp) (Ghabrial, 2008). There are two totiviruses reported to infect crustaceans: a Cherax Giardiavirus-like virus (CGV) in freshwater crayfish (*Cherax quadricarinatus*) and an infectious myonecrosis virus (IMNV) in the whiteleg shrimp, also called the Pacific white shrimp (*Litopenaeus vannamei*) (Pérez Farfante and Kensley, 1977).

CGV, the first totivirus reported in crustaceans, has been associated with mortalities in farmed freshwater crayfish in North Queensland, Australia (Edgerton et al., 1994). The virus caused high morbidity, with a maximum prevalence of 52.3%, and a high mortality of up to 85% in an affected juvenile crayfish. Although CGV is considered as one of the major viruses infecting freshwater crayfish in North Queensland, knowledge of the sequence and genomic organization of this virus is poor. CGV has not been sequenced or recognized/classified by the International Committee on Taxonomy of Viruses (ICTV). A conclusive taxonomic classification will only be possible when the full genome is sequenced, otherwise it is highly tentative. CGV, which is described as a small, nonenveloped virion that is 5 nm in diameter and contains dsRNA, has never been reported elsewhere outside North Queensland after 2002. Domestication of the Cherax species has removed some of the viruses, including CGV. The virus has never been isolated or sufficiently characterized to confirm that it belongs to the *Totiviridae* family.

The most important totivirus of crustaceans is IMNV, the causative agent of infectious myonecrosis (IMN) in the Pacific white shrimp in Brazil and Indonesia (Lightner et al., 2004; Naim et al., 2014; Poulos et al., 2006; Senapin et al., 2007). IMNV causes mortality from 40% to 70% in cultured *L. vannamei*, and it also increases the food conversion ratio of affected shrimp populations from 1.5 to 4.4, causing huge economic losses to farmers (Andrade et al., 2008). Although *L. vannamei* is the main host, based on experimental studies, IMNV may also infect the giant tiger shrimp (*Penaeus monodon*), the Pacific blue shrimp (*Litopenaeus stylirostris*) (Tang et al., 2005) and the southern brown shrimp (*Farfantepenaeus subtilis*) (Table 30.1) (Coelho-Melo et al., 2014). *Litopenaeus vannamei* is the biggest cultured species of penaeid shrimp, with more than 3 million metric tons being produced in 2012 (FAO, 2012). Unlike CGV, which is restricted to North Queensland, Australia, IMNV is widespread across Brazil and Indonesia. Due to the risk of spread to other countries, IMN has been listed as a notifiable shrimp disease by the World Organization for Animal Health (*Office International des Epizooties*/OIE) (OIE, 2012).

30.2 INFECTIOUS MYONECROSIS VIRUS

IMN is a viral disease of penaeid shrimp caused by IMNV.

30.2.1 Classification

Although IMNV shares key similarities with members of the family *Totiviridae*, it is currently not classified by ICTV (King et al., 2012). The family *Totiviridae* encompasses viruses characterized by an isometric virion, approximately 40 nm in diameter that contains a nonsegmented dsRNA genome coding for a CP and an RdRp (Ghabrial, 2008). There are five genera in the family *Totiviridae*: *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus* and *Victorivirus*. *Totiviridae*

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00030-9
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TABLE 30.1 Characteristics of Totiviruses of Crustaceans

Taxonomy and Virus Name	Disease	Host Species	Genome Size (bp)	GenBank Accession No.	Reference
Family *Totiviridae* (tentative)					
Genus (unassigned)					
Infectious myonecrosis virus, IMNV	IMN	*Litopenaeus vannamei* *L. stylirostris* *Penaeus monodon* *Farfantepenaeus subtilis*	8226–8230	AY570982.3 KJ636782.2	Naim et al. (2015), Poulos et al. (2006), Tang et al. (2005), Coelho et al. (2009)
Cherax Giardiavirus-like virus, CGV	ND[a]	*Cherax quadricarinatus*	ND[a]	ND[a]	Edgerton et al. (1994)

Litopenaeus vannamei is the only natural host to date. L. stylirostris, P. monodon and F. subtilis are susceptible to IMNV based on experimental infection.
[a]ND, not determined.

is a virus family that is not yet assigned to an order, and it has yet to be determined whether IMNV is a new member of the *Giardiavirus* genus, whether it represents a novel genus in the family *Totiviridae*, or whether it warrants a new family of nonsegmented dsRNA viruses that infect invertebrate, or more specifically, the crustacean hosts (together with CGV when the genome sequence becomes available).

Phylogenetic analysis inferred from the RdRp gene suggests IMNV to be most closely related to *Giardia lamblia virus* (GLV), the prototype virus of the genus *Giardiavirus* (Poulos et al., 2006). Interestingly, both IMNV and GLV, unlike other members of the family *Totiviridae*, exist as an infectious virion. In addition, Nibert (2007) reported some features of the open reading frame 2 (ORF2) coding strategy of IMNV that are similar to that of GLV, as well as several other members of the family *Totiviridae*. These features include a 199-nt overlap between the end of ORF1 and the start of ORF2, and a shifty or slippery heptamer motif and pseudoknot in the region of the ORF1-ORF2 overlap. However, the IMNV capsid is organized more like that of *Saccharomyces cerevisiae* virus L-A (ScV-L-A), the prototype virus of the genus *Totivirus*, as well as those of other members of the family (Tang et al., 2008).

Despite sharing major similarities with members of the family *Totiviridae*, IMNV also features different characteristics from that group. These differences include: a larger genome with coding capacity beyond CP and RdRp, a virion with fiber-like protrusions on its surface, the capacity for extracellular transmission and the ability to cause disease in a host (Poulos et al., 2006; Tang et al., 2008). All other members of the *Totiviridae* family (except GLV) lack the capacity for extracellular transmission. Instead, their natural life cycles rely on vertical transmission at cell division or horizontal transmission by hyphal anastomosis. Within the family *Totiviridae*, only IMNV and possibly CGV (Edgerton et al., 1994) were reported to infect a host other than a yeast, fungus or protozoan (Ghabrial, 2008) and to cause a host disease. On the basis of these observations, IMNV and CGV may belong to a new dsRNA virus family with members that infect and cause disease in particular invertebrates and, more specifically, crustaceans.

30.2.2 Structure and Composition of Virus

IMNV is a nonenveloped icosahedral virus, approximately 45 nm (450 Å) in diameter with surface projections of 80 Å and a buoyant density of 1.366 g/mL in cesium chloride (Poulos et al., 2006; Tang et al., 2008). The virion is defined by the characteristic architecture of a single-layered isometric capsid of the 901-aa major capsid protein (MCP) with a predicted mass of 99 kDa (Tang et al., 2008). The icosahedral capsid contains 120-subunit T = 1, which is composed of 60 copies each of two chemically identical subunits, MCP-A and -B, and arranged in a T = 2 fashion (Fig. 30.1A). The A subunits form pentameric structures around each of the 12 fivefold (5 f) axes, and the B subunits form trimeric structures around each of the 20 3 f axes. Moreover, five B subunits lay in staggered arrangement around each A pentamer so that together they form a compact decamer centered at each 5 f axis. Thus, the outer surface of the IMNV capsid consists of 12 pentagons demarcated by wide canyons. Each pentagon has a small, central knob attributed to the fiber foot, which serves as the base of the protrusion. The fiber-like projections extend 80 Å beyond the capsid at the 5 f axes. The protrusions comprise three morphologic domains: an outer knob, a middle stalk and an inner, capsid-anchored foot (Fig. 30.1B).

The interior of the capsid consists of five to six concentric rings (Tang et al., 2008), characteristics of the packaged genome in many dsRNA viruses. The average spacing between the rings is 26 Å, similar to that in orthoreovirus (Reinisch

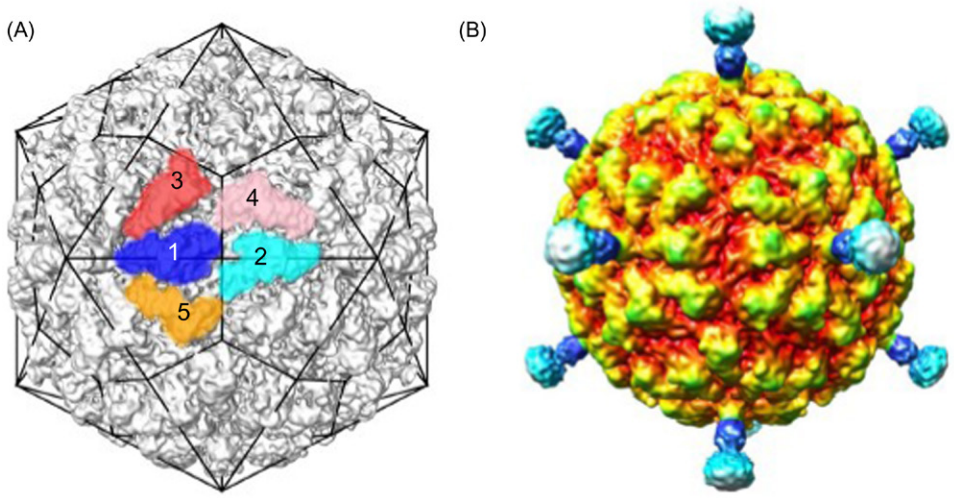

FIGURE 30.1 The ultrastructure of infectious myonecrosis virus (IMNV). (A). Surface view of IMNV virion in black and white, with overlay of T = 1 model and selected MCP-A subunits (1, 2) and B subunits (3, 4, 5) (Tang et al., 2008). (B). Fiber-like protrusions at 5f axes of IMNV virion. The 3D map was calculated at 10Å resolution (Tang et al., 2008). *Reprinted with permission from PNAS. Copyright (2008) National Academy of Sciences, USA.*

et al., 2000), but less than that in ScV-L-A (30Å) (Tang et al., 2008). The presence of the MCP-RdRp fusion proteins projecting into the interior space of the capsid may contribute to this pattern by anchoring at least one end of the dsRNA and by directing its path at the other point in the winding scheme.

Unlike other members of the *Totiviridae*, no pores are observed in the IMNV capsid. This raised the question as to how viral mRNAs are exported and/or nucleic acid elements are imported? As the only potential pores through the IMNV capsid are at the 5f axes, and those are covered by the protrusions, there are at least two possible explanations. One is that the protrusions are lost during or after the cell entry and that the pores are thereby uncovered for release of the transcripts and import of the nucleotides. Another possibility is that the 5f axis, near which the MCP-RdRp protein is incorporated, is not competent to anchor a protrusion so that this particular pore is open for export and import purposes (Tang et al., 2008).

30.2.3 Genome Structure and Expression Strategies

The IMNV genome is a single nonsegmented molecule of dsRNA, thought to be 7560bp in length (Poulos et al., 2006) and 7561bp (Naim et al., 2014; Senapin et al., 2007). These putatively full-length sequences were determined by Sanger sequencing. However, recent GenBank entries (KF836757.1, KJ556923.1) or revised sequences (AY570982.3, KJ636782.2 and KJ636783.2) indicate that the genome of IMNV is >600bp longer than otherwise reported. The additional sequences of 665bp (KF836757.1, KJ556923.1, AY570982.3 and KJ636782.2) or 669bp (KJ636783.2) were determined either by next-generation sequencing or by rapid amplification of cDNA ends (Dantas et al., 2015; Loy et al., 2015; Naim et al., 2014). The additional sequences were located in the 5′ and 3′ untranslated regions (UTRs), adding 646bp and 23bp to the original UTRs, respectively.

Previously, the IMNV genome was thought to contain two nonoverlapping ORFs (Poulos et al., 2006); however, recent works (Dantas et al., 2015; Loy et al., 2015; Naim et al., 2014, 2015; Nibert, 2007) showed that the genome comprises two partially overlapping ORFs located in different reading frames of the genomic plus strand: ORF1 in frame 1 (nt 473-5599) and ORF2 in frame 3 (nt 5398-8136). ORF1 and ORF2 overlap by 199 nt not including the ORF1 stop codon (positions 5398–5596), and ORF2 is in the -1 frame relative to ORF1, leaving a 5′ UTR and a 3′ UTR of 472 and 94 nt, respectively.

Based on the 7561-bp sequence, Poulos et al. (2006) predicted that ORF1 encodes a polyprotein of 1605 aa with predicted mass of 179kDa. The polyprotein is predicted to be cleaved co- or posttranslationally into four distinct polypeptides, designated pep1, 2, 3 and 4 (Nibert, 2007; Tang et al., 2008). Pep1 (93 aa, 10kDa) is predicted to contain a dsRNA-binding motif (dsRBM) and to play a role in either viral replication or modulation of the host's immune response. Pep2 (284 aa, 32kDa) and pep3 (327 aa, 38kDa) have unknown functions, but are speculated to form part of the protrusions. This speculation is recently supported by a finding that the most variable region (hypervariable region) of IMNV genome is located in the first half of ORF1, which probably encodes the capsid protrusions (Dantas et al., 2015). Pep4 (901 aa, 99kDa) has been

identified as the MCP, which appears to be cleaved from a larger ORF1 precursor between Asn1350 and Ile1351 (Nibert, 2007; Poulos et al., 2006; Tang et al., 2008). The processing mechanism between pep1, pep2 and pep3 are predicted to be via two conserved nanopeptides identical to 2A-like motifs: GDVESNPGP and GDVEENPGP at aa 86–94 and 370–378, respectively (Nibert, 2007). The cleavage mechanism between pep3 and pep4 is unknown, but is highly conserved because all (nine) available IMNV sequences encompass this region (Naim et al., 2014; Poulos et al., 2006). Interestingly, the ORF1 translation polyprotein (1605 aa) was not detected in SDS-polyacrylamide gel (Poulos et al., 2006). Taking advantage of the recently amended full-length genome (8230 bp), Naim et al. (2015), as well as Dantas et al. (2015) and Loy et al. (2015) proposed an alternative hypothesis: the 5′ UTR is 472-637-nt long and the ORF1 translation product is 1653-aa long with pep1 extended from 93 aa (Nibert, 2007; Poulos et al., 2006) to 141 aa. Furthermore, Dantas et al. (2015) identified two stem loops and two pseudoknots in the 5′ end, as well as a putative stem loop and a slippery motif located at 3′ end, which are likely involved in the start and termination of translation.

ORF2 covers 2739 nt and encodes a 912-aa product, designated pep5, which shares great similarity with the RdRp from members of the *Totiviridae* family (Nibert, 2007; Poulos et al., 2006). Furthermore, eight motifs characteristic of totivirus RdRp are also found in the RdRp of IMNV. Motifs 1 and 5 are least conserved; motifs 2, 4 and 8 have moderate identity (>40%), and motifs 6 and 7 have 100% identity to the consensus sequence. Motifs 6 (GDD) is universal to RNA polymerase (Jablonski et al., 1991). Poulos et al. (2006) reported that ORF1 and ORF2 are nonoverlapping, but Nibert (2007) predicted that ORF 1 and ORF2 overlap by 199 nt and ORF2 is in the -1 frame relative to ORF1. The latter creates the possibility that ORF2 could be translated as a fusion with ORF1 to produce a *Gag-Pol* protein fusion, similar to those described for other totivirus (Dinman et al., 1991). There are two features of the mRNA that determine the -1 ribosomal frameshift: a shifty or slippery heptamer motif followed by an RNA pseudoknot shortly downstream of the heptamer to slow ribosomal movement at this point (Wicker, 2001). For a *Gag* to be translated as a fusion with *Pol* ORF, a -1 ribosomal frameshifting should occur in the overlapping region. In IMNV, a slippery site of GGGUUUU is found at nt 5449–5455 followed by an H-type pseudoknot at 5464–5508, just downstream of the shifty heptamer. Since this -1 ribosomal frameshifting occurs within the overlapping region of ORF1 and ORF2, and the N terminus of the MCP region at Ile705, the size of MCP-RdRp protein fusion is predicted to be 1734 aa with a molecular mass of 196 kDa (Fig. 30.2).

30.2.4 Biology and Epidemiology

Litopenaeus vannamei has become the major farmed shrimp in the world following the establishment of specific pathogen-free (SPF) shrimp lines in the late 1980s by the USDA Marine Shrimp Farming Program. These SPF lines have subsequently been used by many farmers across the world, including Asia and Latin America. Therefore, though native to the eastern Pacific between Mexico and Peru, *L. vannamei* is now farmed across much of the tropical and subtropical countries, including Brazil and Indonesia, the two countries affected by IMN disease. *Litopenaeus vannamei* was first legally introduced on Brazilian farms in 1983, and Indonesian farms in 2000–01 (Briggs et al., 2004), and has since been cultured in Brazilian and Indonesian provinces.

Even though IMN disease has adversely affected the production of cultured *L. vannamei* in Brazil and Indonesia, the economic impacts and the importance/size of shrimp industry in the two countries are different. Indonesia is one of the main shrimp producers in the world, and in 2014 produced >400,000 metric tons of shrimp, mostly for export with a value of more than $2.8 billion, ~65% of which was from cultured *L. vannamei* (Ministry of Marine Affairs and Fisheries, 2015).

When IMN was first noted in Brazil in 2002, the marine shrimp industry was relatively new to the country, and shrimp disease diagnosis was almost nonexistent. IMN was the first disease that caused significant economic loss for the shrimp industry in Brazil. The Brazilian shrimp farmers had never faced an epidemic situation before (Andrade, 2009). In contrast, when the first IMN outbreak was noted in East Java, Indonesia, in 2005–06, marine shrimp farming had been around quite a while, mostly for *P. monodon*, from 1980 to 2000.

During *P. monodon* era, farmers had experienced different shrimp diseases caused by monodon baculovirus (MBV, occluded baculovirus, double-strand DNA) in 1986, yellowhead virus (YHV, *Roniviridae*, minus-strand RNA) in 1992–94, white spot syndrome virus (WSSV, *Nimaviridae*, double-strand DNA) in 1995–2000 and infectious hypodermal and hematopoietic necrosis (IHHNV, *Parvoviridae*, single-strand DNA). Taura syndrome virus (TSV, *Dicistroviridae*, plus-strand RNA) emerged in Indonesia in 2002, after the introduction of *L. vannamei*. By the time IMN appeared in Indonesia, in contrast to Brazil, farmers had suffered and survived different disease outbreaks. One interesting fact, all diseases were first noted in East Java, partly because the region is one of the centers for shrimp production in Indonesia, and the location of the main hatcheries that provide postlarvae (PL) for the whole country, and for other unknown reasons.

The history and epidemiology of IMN in Brazil were summarized by Andrade (2009) and Nunes et al. (2004). A new infectious disease was noted in August 2002 in the coastal zone of the Brazilian Northeast, in the Piauí state. Farmers

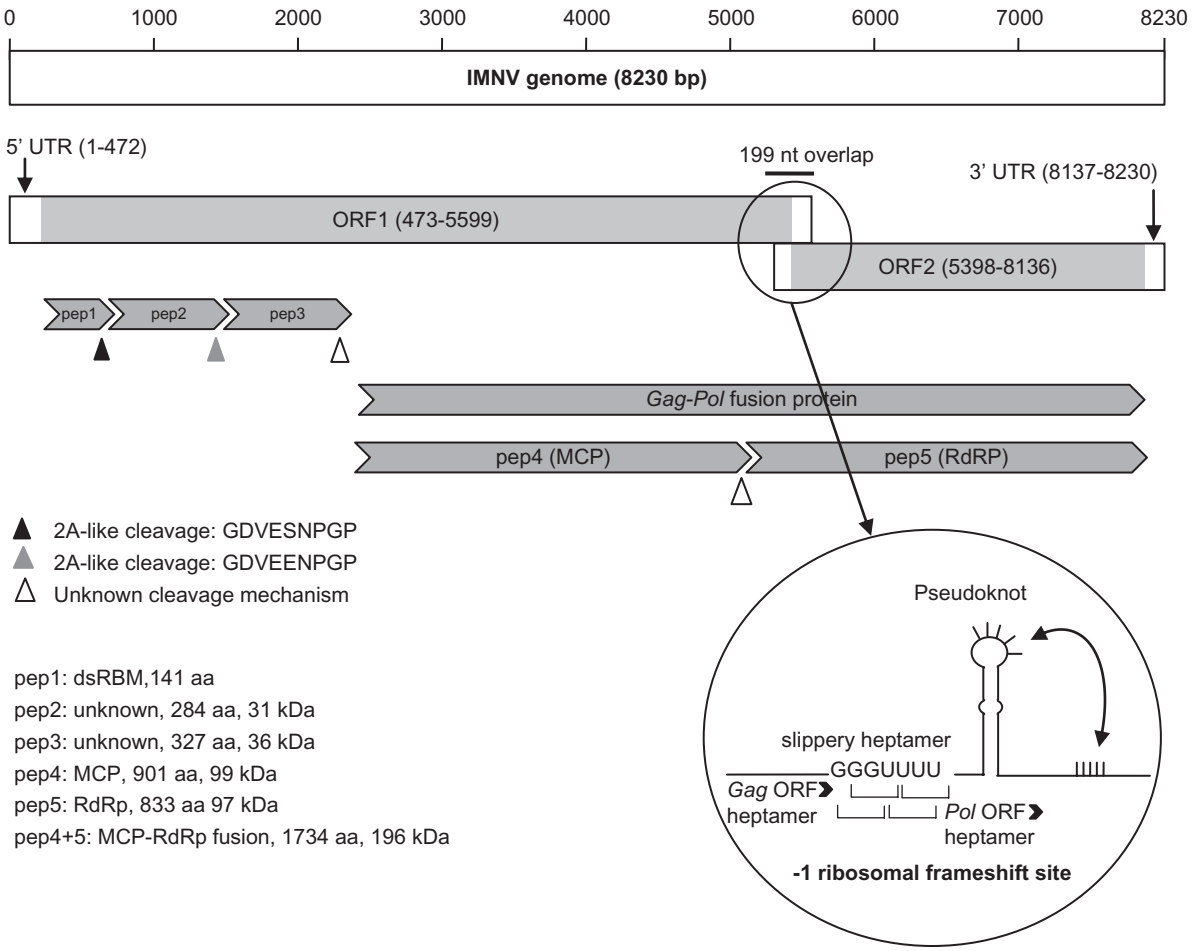

FIGURE 30.2 Genome organization and expression strategies. Upper diagram illustrates the nonsegmented 8230-bp genome of IMNV. Scale indicates nucleotide position within the genome. Middle panel shows IMNV coding schemes: 5′ UTR, ORF1, ORF2, 199 nt overlap between ORF1 and ORF2, and 3′ UTR. Lower panel depicts predicted protein products, after cleavages and -1 ribosomal frameshifting. ORF1 encodes a polyprotein of 1653 aa, which is subsequently processed into pep1, pep2, pep3 and pep4. ORF2 encodes pep5 (RdRp), which is translated as a fusion with ORF1 to produce a Gag-Pol fusion protein. Cleavage mechanisms and predicted sizes as well as putative functions of IMNV proteins are indicated. Insert describes -1 ribosomal frameshift site featuring a slippery heptamer and pseudoknot. dsRBM, double-stranded RNA binding motif; MCP, major capsid protein; RdRp, RNA-dependent RNA polymerase.

named the disease "cotton shrimp disease" because of the whitish muscle appearance, which resembles cotton. Based on histopathology findings, the OIE Reference Laboratory for shrimp diseases at the University of Arizona confirmed that the whitish appearance was not caused by any known disease agents at that time. Later, after several confirmations and following Koch's Postulates, the disease was named IMN (Lightner et al., 2004). After significant outbreaks from November 2002 to March 2003 across Piauí, Ceará and Maranhão states, it was noted that the outbreaks were mainly during the first semester (January–June). By 2004, the disease had spread in seven more states, including Rio Grande do Norte state, Paraíba state and finally Pernambuco state. Ten states have been affected to date, all restricted in the Brazilian Northeast (da Silva et al., 2010; Lightner et al., 2012; Mello et al., 2011).

Andrade (2009) observed the environmental factors associated with IMN outbreaks, which are similar to the outbreaks in Indonesia: decreased dissolved oxygen levels, rapid temperature and salinity variation in the rainy season and high levels of organic materials. IMN was most severe during full and new moon phases, when molting activity was the highest. Moreover, outbreaks in high-density culture suggested that, directly or indirectly, culture densities had contributed to the outbreaks.

Until 2005, only Brazil reported IMN cases. Based on geographical position, the northeast of Brazil where IMNV outbreaks occurred is at the same latitude just south of the Equator as the Indonesian island, the second country hit by IMNV (Naim et al., 2014). There are no shrimp-producing countries other than Brazil and Indonesia that lie at that latitude.

Whether or not IMNV replication and pathogenicity are restricted by environmental factors unique to this latitude needs further supporting evidence.

In May 2006, a mass mortality of cultured *L. vannamei* was reported in Situbondo, East Java. Situbondo is a district well known as a center of penaeid shrimp hatcheries that provides PL in many areas in Indonesia. The affected shrimp showed clinical signs of whitish necrotic muscle and reddened tail fan, similar to those reported for IMN disease in Brazil (Lightner et al., 2004). The outbreak also resulted in elevated mortality that was initially associated with a chronic course of persistent low-level mortality. The results of histopathological examination and PCR test of diseased shrimp confirmed that the outbreak was associated with IMNV infection (Senapin et al., 2007; Taukhid and Nur'aini, 2009). Although IMN has been rumored to occur elsewhere in Asia, this has been disputed (Senapin et al., 2011; Yan et al., 2014). TSV and IMNV outbreaks have taught Indonesian farmers that doing penaeid shrimp culture means dealing with high-risk business because Indonesian farmers had high expectations of *L. vannamei*. Regardless of the species, in many places in the world, if not all, diseases have continued to be the most limiting factor in aquaculture.

Mass cultivation of *L. vannamei* in Indonesia started in 2001. Prior to that, *P. monodon* was the most dominant species. The collapse of the industry in 1995–96 because of WSSV outbreaks, followed by stagnant production in the following years, led to the introduction of *L. vannamei*. The shrimp was illegally introduced to Indonesia by individual farmers in 1999. Following the need to rebuild shrimp farming in Indonesia, the government officially introduced *L. vannamei* from Taiwan and Hawaii (Taukhid and Nur'aini, 2009) for research purposes in 2000 and for aquaculture in 2001. The growth performance of the *L. vannamei* and its relative resistance to WSSV, despite its higher protein requirement feed, shifted many Indonesian farmers to this species instead of *P. monodon* and *Penaeus merguiensis*. This increased shrimp aquaculture production, contributed 37.11% to the national production in 1999, and 41.2% in 2003. Since then, *L. vannamei* aquaculture has gradually dominated the Indonesian shrimp aquaculture, and by 2014, it contributed to about 65% of Indonesian shrimp production (Ministry of Marine Affairs and Fisheries, 2015). The nonnative shrimp can produce high yield and was thought to be resistant to WSSV, and would ensure more stable production compared with *P. monodon*.

Unfortunately, what has happened is unexpected. The introduction of *L. vannamei* led to the emergence of associated diseases, for example TSV in 2002 (University of Arizona Aquaculture Pathology Lab clinical case 02-318) and IMNV in 2005–06 (Senapin et al., 2007; Taukhid and Nur'aini, 2009). Early databases for diseases associated with *P. monodon* from 1980 to 2000, mainly based on clinical reports because PCR or other detection methods were not available in the country at that time. In general, it took 2–3 years for a new disease to impact production significantly following their first appearance in the country. IMNV has affected shrimp production in the country. Total production losses were estimated at $150–200 million from 2009 to 2011 during the peak of the IMNV outbreaks.

According to farmers, IMN outbreaks occurred mainly in shrimp farms without dedicated reservoir ponds. At the beginning, in 2006–07, IMN outbreaks occurred after 70–90 days of culture. In 2008–09, IMNV infected shrimp of 40–60 days of culture. After that, IMN outbreaks have occurred as early as 30 days of culture. It can be concluded that the disease and mortality usually, but not always, occurred in shrimp at 30–90 days of culture.

Since the first outbreak of IMN in 2006 in East Java, by 2014, the disease has spread to other provinces in Indonesia where *L. vannamei* is being cultured, as far as Aceh in the west border and the north tip of Indonesia, to South Sulawesi in the eastern part of Indonesia. East Java and Lampung are the two centers of *L. vannamei* culture that continue to be the two places mostly affected by the IMNV outbreaks (Naim et al., 2014).

Taukhid and Nur'aini (2009) documented the first IMNV cases in Indonesia from 2006 to 2007 in detail. The epidemiology study focused on high-risk areas of East Java and the nearby provinces of Bali and West Nusa Tenggara. In July–August 2006, outbreaks of a disease with similar clinical signs were reported from shrimp farms near the site where the first IMN outbreak occurred. Since then, IMN outbreaks have spread to shrimp ponds in neighboring areas, suggesting that the disease transmission is via natural water movement or seabird involvement. Although it is unclear how the disease spread to different islands, it is suspected that the virus spreads from East Java to neighboring provinces (Bali, West Nusa Tenggara, Central Java, West Java and South Kalimantan) through the movement of infected PL. The spreading toward other provinces in Sumatra Island, however, is not very clear. Molecular study suggests that genetic diversity exists between IMNV strains in East Java and Lampung (Sumatera), as well as between Brazil and Indonesia (Dantas et al., 2015; Naim et al., 2014).

Prior to the first half of 2014, IMNV molecular epidemiology studies were almost nonexistent because of the limited data available in the GenBank. Four complete or nearly complete sequences had been deposited in 2014. By May 2015, there were only six complete or nearly complete genome sequences of IMNV strains that had been deposited in the GenBank database, two strains from Brazil and four strains from Indonesia. Five of them have a span of 8226–8230 bp (GenBank accession numbers AY570982.3, KJ636782.2, KJ636783.2, KF836757.1, KJ556923.1) and one strain has 7561 bp (EF061744.1). Recent publication by Dantas et al. (2015) used two complete strains from Brazil as their data. One of the coauthors of that study (D. Lanza) has generously shared the sequences to reconstruct a phylogenetic tree. One of the two sequences has been deposited in the GenBank very recently (KR815474.1).

Due to the limited data prior to 2014, Senapin et al. (2007) and others (Lightner et al., 2012; Taukhid and Nur'aini, 2009) have suggested that IMNV most likely arrived in Indonesia from Brazil through unregulated transfer of *L. vannamei* broodstocks or PL use in aquaculture. The conclusion is based on the "whole genome" comparison between 7560 bp in Brazilian strain (2003 isolate) and 7561 bp in Indonesian strain (2006 isolate). Subsequent analysis of complete genome sequences revealed that the 2006 Indonesian IMNV shares 99.6% nucleotide identity with 2003 Brazilian IMNV (Senapin et al., 2007). The high nucleotide identity of the genome sequences of the IMNV from Brazil and Indonesia suggests that the Indonesian IMNV may have originated from Brazil.

However, recent publications on genetic diversities by Dantas et al. (2015) and Naim et al. (2014), and subsequent GenBank submissions suggest that Brazilian and Indonesian strains do not intermix, each geographic group is monophyletic. Both publications used different approaches. Dantas et al. (2015) reconstructed rooted trees and Naim et al. (2014) used unrooted trees for analysis. The rooted tree was reconstructed based on 7561 bp, the region covered by eight sequences. Here, as a comparison and to enhance the phylogenetic tree, we reconstructed the unrooted trees (Fig. 30.3) based on the maximum length for each strain (one strain covers 7561 bp, six strains have 8226 bp and another strain has 8230 bp).

The phylogenetic trees shown in Fig. 30.3A and B are consistent with the previous suggestion by Naim et al. (2014), and together with the phylogenetic tree presented in Dantas et al. (2015) confirm the monophyletic branch of each geographic group, even though the latter used a rooted tree.

Furthermore, Fig. 30.3A and B includes two branches of Indonesian strains and two branches of Brazilian strains, which represent diverging lineages of IMNV in Indonesia and Brazil. In Brazil, the 2009 and 2013 strains derived from the same ancestor, while the 2003 strain and the other strain with no information on collection time/year, and fell in the same branch. For Indonesia, the East Java strain and the Lampung strain seemed to have separated because of geographical distance. However, any ongoing exchange of *L. vannamei* broodstocks or PL between different regions in Indonesia, as well as within Brazil, might be expected to obscure or complicate such patterns over longer periods. Strains representing distinct branches of IMNV within Brazil and Indonesia as suggested in Fig. 30.3A and B might nevertheless continue to cocirculate across different regions, unless descendants of one branch came to dominate for particular reasons (Naim et al., 2014).

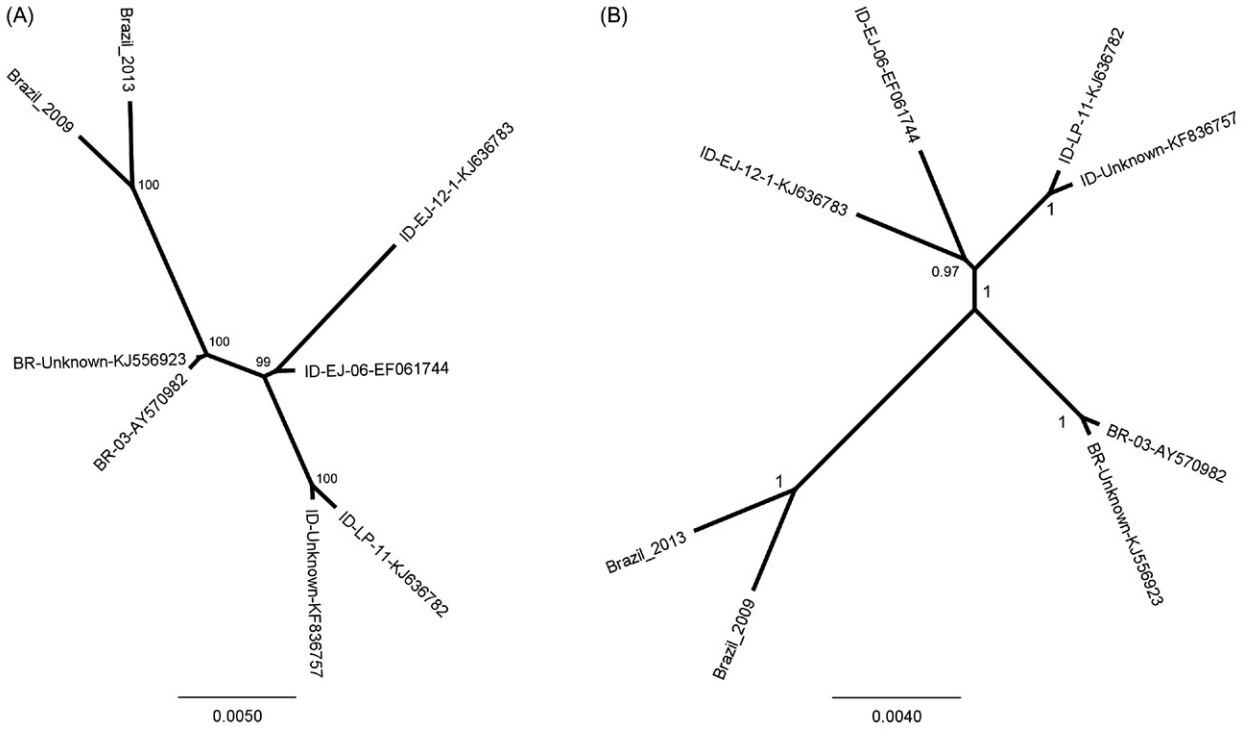

FIGURE 30.3 Phylogenetic comparisons of IMNV whole-genome sequences according to Bayesian inference phylogenetic analysis. The trees were reconstructed using (A) MrBayes 3.2 phylogenetic analysis (Ronquist and Huelsenbeck, 2003) and (B) BEAST 1.8.2 (Drummond et al., 2012). Both trees are unrooted; branches are labeled with the aLRT support values (%). Scale bars indicate nt substitutions per aligned position. IMNV nt sequences were aligned with Kalign2 and then analyzed with jModelTest 2.1 to identify the best nt-substitution model for phylogenetic comparisons. The trees are congruent and displayed as radial phylograms.

To further investigate these findings, phylogenetic comparisons using full length and partial sequences of IMNV were performed. Similar to Figs. 30.3A and B, Figs. 30.4A and B display the phylogenetic trees reconstructed using MrBayes 3.2 and BEAST 1.8.2. One consistent feature in both trees is the clear separation between Indonesian and Brazilian strains. Unfortunately, the phylogenetic trees in Fig. 30.4 are incongruent, and therefore the diversity pattern particularly in Indonesia remains unclear. Based on Fig. 30.4B, the pattern is more consistent with Fig. 30.3A and B, with two immediate branches representing the 2009–13 strains, and another one for the 2003 strain. Also consistent with Fig. 30.3, the different lineages in Brazil in Fig. 30.4B seem to be grouped based on time/location. The two immediate branches represent the 2003, 2004 and 2007 strains in one group, and the 2009, 2011 and 2013 strains in another group.

The pattern for Brazil seems simpler compared with the pattern for Indonesia because IMN confines in the northeast part, on the same land. Andrade (2009) describes that IMN started in the Piauí state in 2002 (represented by 2003 strain), and then had travelled to the north, followed by the south and reached Pernambuco and Rio Grande do Norte in 2004 (the region is represented by the 2009 and 2013 strains). This suggests that the natural spread via water is dominant. The mode of transfer in Indonesia is more complex because Indonesia consists of many islands and shrimp culture spreads across the country. Both natural movement and human involvement (by bringing infected broodstock or PL to different places) occurred.

The earlier appearance of IMN in Brazil, at least as reported to date (Lightner et al., 2004; Nunes et al., 2004; Senapin et al., 2007), is certainly consistent with the earlier suggestion that the Indonesian IMNV originated from Brazil. Moreover, because *L. vannamei* has been legally cultured in Indonesia since only 2000–01, transfer from Indonesia to Brazil at around this same time seemed less likely. Another logical possibility, however, is that the related ancestors of the Brazilian and Indonesian clades may have been separately introduced to the two countries from a third, still unknown, geographical source (Naim et al., 2014).

A wild source of IMNV, outside cultured shrimp, has not been formally reported to date. Furthermore, since IMNV in the lab can infect at least two other shrimp species, *L. stylirostris* and *P. monodon* (Tang et al., 2005) and *F. subtilis* (Coelho et al., 2009), the possible role of such other species, including other decapods (eg, crabs, lobsters or crayfish), the origin and spread of IMNV deserve consideration (Naim et al., 2014). The availability of IMNV-related sequences obtained from other host species, for example, would allow investigations related to virus–host coevolution (Göker et al., 2011) that may shed light on the specific origin of IMNV in Brazilian and Indonesian *L. vannamei*.

To attempt to root the IMNV tree by more empirical means, additional phylogenetic comparisons were performed using outgroup sequences (capsid) from five recently discovered dipteran-arthropod (insect) viruses that are related to IMNV (Isawa et al., 2011; Wu et al., 2010; Yang et al., 2012; Zhai et al., 2010). A Bayesian-inference tree using MrBayes 3.2 was reconstructed. The resulting tree (Fig. 30.5) revealed that with the addition of insect-virus as an outgroup, the two distinct clades between Brazil and Indonesia was consistent with Figs. 30.3 and 30.4. Fig. 30.5 is shown as a cladogram to better elucidate the tree topology.

Phylogenetic comparisons were also performed using capsid sequences from representatives of the five genera in the family *Totiviridae*: *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus* and *Victorivirus*. The resulting trees using various models (not shown) suggest that IMNV is separated from arthropod viruses found in insects (DmTV, AsTV, ORV, TjTV). The viruses in insects fell in the same branch, which is likely a new genus within family *Totiviridae*, or a new family of dsRNA viruses that infect arthropods, together with IMNV and CGV (when the sequence becomes available). An attempt is being done to sequence CGV from a Davidson's fixed embedded in parafilm wax sample (unpublished).

Phylogenetic comparisons shown in Figs. 30.3–30.5 provide new insights into the possible source and ongoing diversification of IMNV in Indonesia and between Indonesia and Brazil. Additional sequencing of IMNV strains in coming years should be expected to clarify the trajectory of IMNV evolution and the propensity of IMNV for spread to other parts of the world (Naim et al., 2014).

30.2.5 Diagnostic Methods

Routine diagnostic methods and confirmatory tests for IMNV are done based on clinical signs, morphological (microscopic histology), in situ hybridization, nested RT-PCR and quantitative RT-PCR (qRT-PCR).

30.2.5.1 Clinical Signs

IMN usually appears acutely on a given farm, with elevated mortalities accompanied by gross signs among postlarval, juvenile and subadult shrimp, but then progresses more chronically, with persistent mortalities accumulating to an overall total of 40–70% (Lightner et al., 2004, 2012).

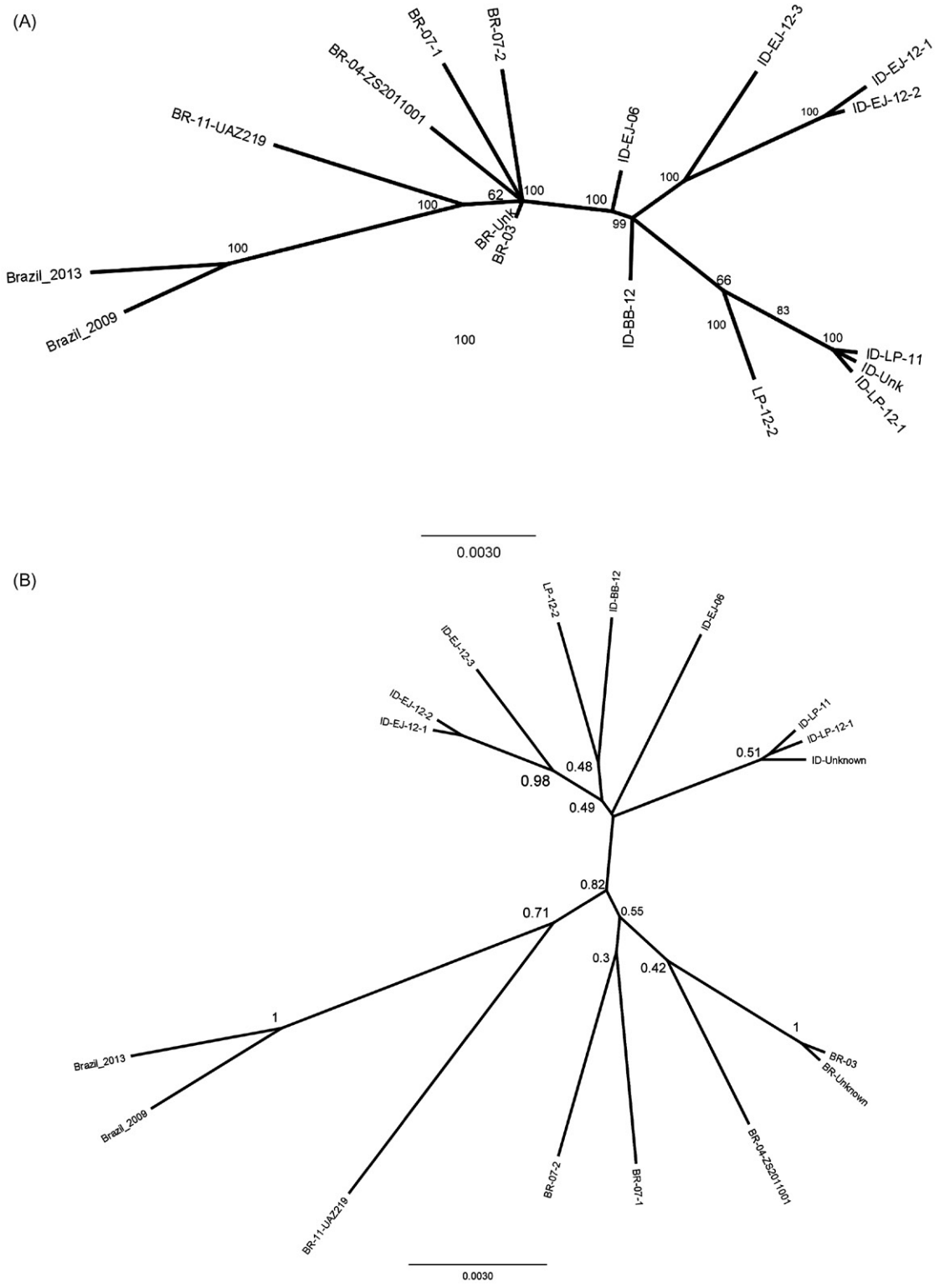

FIGURE 30.4 Phylogenetic comparisons of IMNV (whole genome and partial) sequences according to Bayesian inference phylogenetic analysis. The trees were reconstructed using (A) MrBayes 3.2 phylogenetic analysis and (B) BEAST 1.8.2. Both trees are unrooted, branches are labeled with the aLRT support values (%). Scale bars indicate nt substitutions per aligned position. IMNV nt sequences were aligned with Kalign2 and then analyzed with jModelTest 2.1 to identify the best nt-substitution model for phylogenetic comparisons. The trees are displayed as radial phylograms.

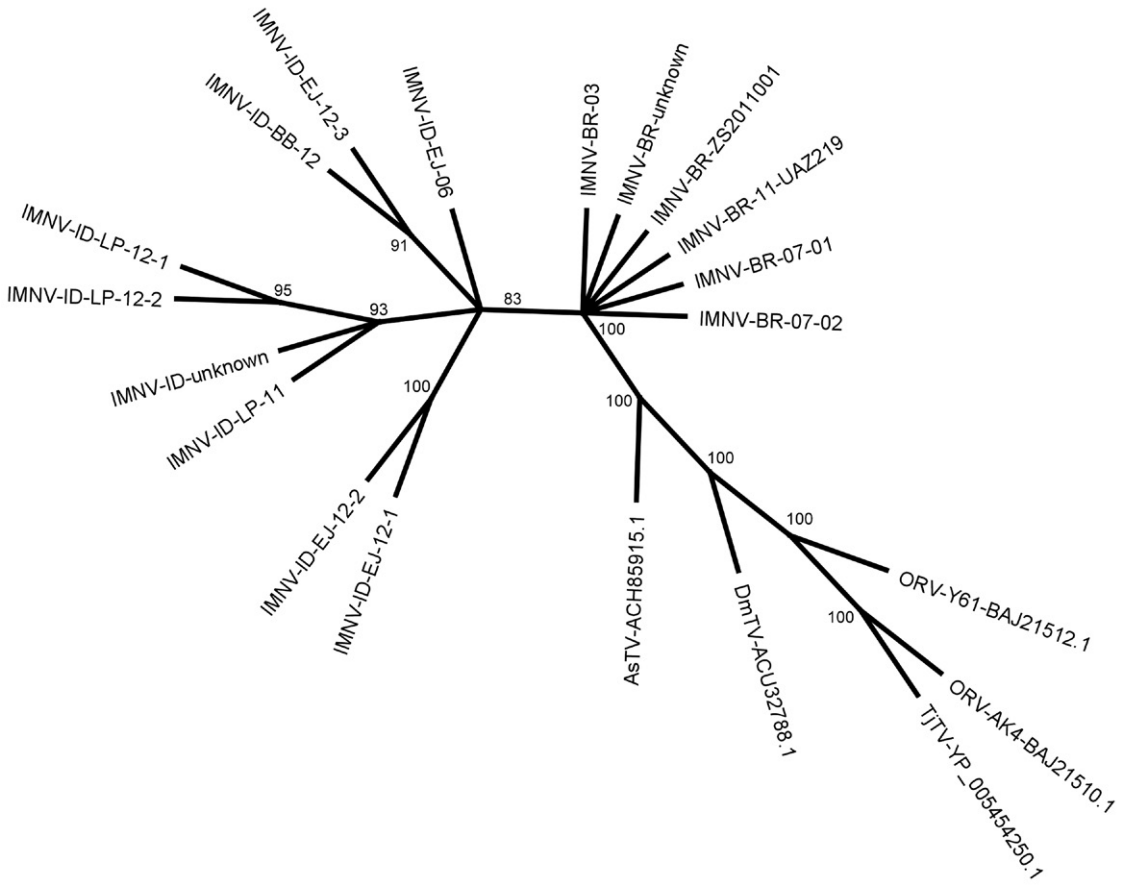

FIGURE 30.5 IMNV and dipteran-virus outgroup aa (capsid) sequences were aligned with Kalign2 and then analyzed with ProtTest 3.3 to identify the best aa-substitution model for phylogenetic comparisons. A Bayesian phylogenetic analysis was next performed using MrBayes 3.2. The tree is displayed as a radial cladogram to allow branch topology in the IMNV clade to be evident.

The gross signs include characteristics of striated muscle necrosis (whitened areas), particularly in the distal abdominal segment and the tail fan (both regions rich in skeletal muscle) forming whitish, opaque lesion (Tang et al., 2005). White discoloration of muscle resulted in muscle color similar to cooked shrimp. Typically, IMN progressed slowly, with large mortality accounts among younger shrimp (juvenile and subadult); however, this disease tends to progress chronically, with persistent low mortality during growth season. At harvest times, mortalities in ponds accumulating up to 70% of the reared shrimp (Lightner et al., 2012). Outbreak of IMN associated with sudden high mortalities after stressful events, such as a sudden change in water salinity or temperature, feeding or capture by cast-feeding. Severely affected shrimp become moribund and mortalities can be instantaneously high following stressful events and continue for several days. Shrimp infected by IMN show behavioral change, such as lethargy and reduced feed consumption. The feed conversion ratio of the infected pond rose from 1.5 to 4.4 and survival rate declined significantly as low as 21% (Andrade, 2009).

30.2.5.2 Histopathology and Pathogenesis

Histopathologically, the lesions are evident on the infected tissues particularly in striated muscle (mostly on skeletal muscle), connective tissue, hemocytes and lymphoid organ. Striated muscle appears to lose the normal striations (Poulos et al., 2006). The lesion is characterized by muscle necrosis (myonecrosis) with infiltration and accumulation of blood cells (hemocytes) resulting in coagulation and edema during the acute phase of IMN. During the chronic or the recovery phase, the lesion progresses into liquefactive necrosis with hemocytic infiltration. Subsequently, hemocytes and inflamed muscle fibers are replaced by a loose matrix of fibrocytes and connective tissue fibers, interspersed by hemocytes and regenerating muscle fiber. In cytoplasm (mostly located on the perinuclear) of hemocytes and connective tissue, pale to dark basophilic inclusion bodies are present (Lightner et al., 2004), consistent with the viral etiology of this disease (Fig. 30.6).

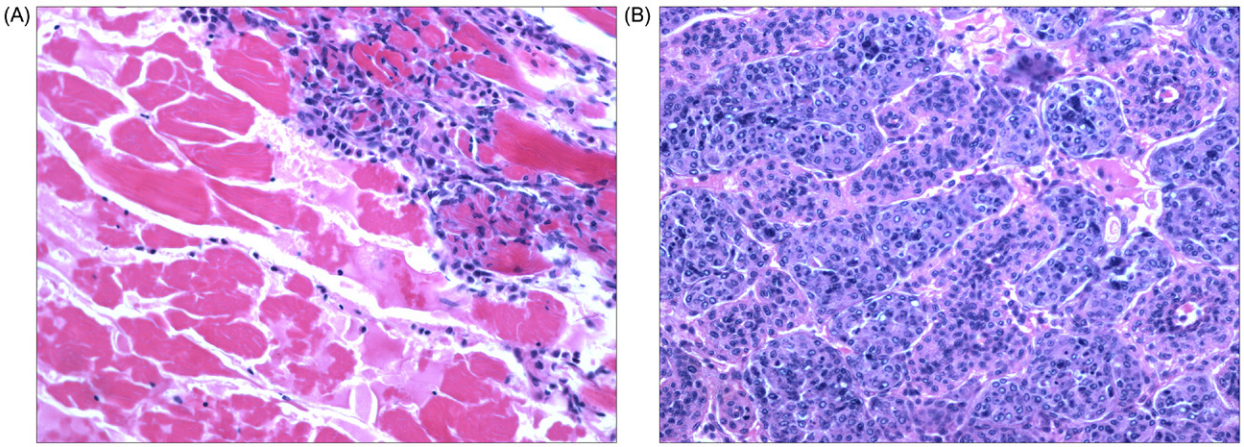

FIGURE 30.6 Photomicrograph illustrating histopathological changes due to IMNV. (A) Acute coagulative necrosis in the muscle fibers, the progression of myonecrosis giving affected muscle fiber a hyaline appearance, nuclear pyknosis, edema and hemocytic infiltration. (B) Lymphoid organ spheroids (LOS), with two normal arterioles are present in the field in the right for comparison.

Significant enlargement (hypertrophy) of lymphoid organ (LO) is found. This is due to accumulation of LO spheroid (LOS) and this is consistent both in acute and chronic-phase of IMN infection. Ectopic lymphoid organ spheroid (ELOS) also often appears on the site near the main body of LO. Common location of ELOS includes the hemocoel and loosed connective tissue, particularly in the heart lumen, hemocoel adjacent of antennal gland tubules (Andrade, 2009).

Mesoderm-derived tissue is the target of IMNV infections. Distinct tissue becomes the target of IMNV during different phase of infection. The main target of acute phase of IMNV infection is the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, hemocytes and the lymphoid organ tubule parenchymal cells. Whereas, at the later (chronic phase), LO is the principal organ where IMN resides. Although IMNV is systemically spread, the virus does not replicate in enteric tissues (gut, caeca and hepatopancreas). Thus the enteric tissue is an inappropriate organ for IMN diagnosis (OIE, 2012).

30.2.5.3 Molecular Detections

Molecular detections for IMNV include at least three different methods: a nested reverse transcription polymerase/RT-PCR (Poulos et al., 2006), the in situ hybridization (Tang et al., 2005) and also a qRT-PCR (Andrade et al., 2007). The nested RT-PCR uses two PCR primer sets that produce a 328-bp amplicon in the first step, and a 139 bp amplicon in the second step. The first step PCR can detect 100 IMNV copies and the second step PCR can detect 10 IMNV copies, the same detection limit as the qRT-PCR with 10 IMNV RNA copies per microliter total RNA. With a detection limit as low as 10 copies, the nested RT-PCR and the qRT-PCR provide the highest sensitivity compared with antibody-based antigen detection methods/monoclonal antibodies to the CP (Kunanopparat et al., 2011).

Results of in situ hybridization using probe specific toward IMNV showed that the probe reacted in the cytoplasm of infected cells (skeletal muscle, lymphoid organ, hindgut, phagocytic cell within hepatopancreas and heart), indicating that the cytoplasm is the most probable cellular compartment where virus replicates, and not in the nucleus (Tang et al., 2005).

30.2.6 Host Range

Natural infections of IMNV have been found only in *L. vannamei* thus far. Experimentally, *L. vannamei*, *L. stylirostris*, *P. monodon* and *F. subtilis* are susceptible to IMN infection (Coelho et al., 2009; Tang et al., 2005). However, *L. vannamei* seems to be the most susceptible species based on the gross signs and mortality rate. *L. vannamei* show initial death 13 days after infection while no mortality was recorded for the two other shrimp tested (Tang et al., 2005). Interestingly, IMNV appears not particularly virulent compared with other known shrimp viruses such as WSSV, YHV and TSV. This might be due to the fact that skeletal muscle (main target of IMNV infection) is not as vital as other virus-targeted organ and the fact that muscle can be repaired at an early stage of infection (Tang et al., 2005).

Two Brazilian shrimp species present with *L. vannamei* on the cultured pond, *F. brasiliensis* and *L. schimitti*, have been suspected as potential hosts of IMN (OIE, 2012). Infectivity of IMN to other crustacean species such as crabs, lobsters,

crayfish and copepods is not known and needs further investigation (Naim et al., 2014). IMNV is positively detected on bivalve mollusks and polychaetes in infected pond, although it is not known whether it is the true host because the probability that it had ingested and accumulated the virus from contaminated pond water cannot be ruled out (Andrade, 2009).

30.2.7 Mode of Spread

IMNV is transmitted both by vertical and horizontal transmission (OIE, 2012). It has been demonstrated that IMNV can be transmitted among shrimps by cannibalism. It is also likely that the virus transmission occurs through water. IMNV is also suspected to be distributed through vectors (seabirds). Similar with other nonenveloped viruses, like TSV, IMNV survives and remains infectious in gut and feces of the seabirds that feed on the infected shrimp. Thus, it can spread within and among the farms through feces or regurgitated shrimp-infected virus on carcasses (Naim et al., 2014; Vanpatten et al., 2004).

IMN remains stable even after frozen storage and several cycles of freezing and thawing. Vertical transmission from broodstock to progeny is also proposed. Surviving shrimps from the IMNV outbreak are known to carry the virus and pass the virus to their progeny through vertical transmission. However, it is still unclear whether it involves trans-ovarium transmission or is a result of contamination of spawning eggs (OIE, 2012).

30.2.8 Prevention and Control

Prevention and control of IMNV and other viral diseases of shrimp relies on the application of biosecurity principles, good management practices and eradication of the virus from affected populations because there are no effective vaccines or therapeutics for IMNV. Biosecurity measures are defined as cumulative steps to protect the introduction of targeted pathogens, and subsequently to contain the spread of the disease if it occurs (Hegngi et al., 2003). In case of IMNV, the main components of biosecurity procedures include isolation and disinfection (Lightner, 2005). Infected shrimp, contaminated water and equipment are the main sources of infection, and therefore protection of a shrimp farm from IMNV must consider these risk factors. The most effective way of preventing the introduction of IMNV into a farm is by avoiding the introduction of new shrimp. If introduction of new broodstock or PL is unavoidable, the shrimp must be IMNV-free. Although there are several diagnostic tools available for IMNV, qRT-PCR is the method of choice for prescreening of broodstock, adults, juveniles and PLs (Andrade et al., 2007). However, because virus that loads in eggs and larvae may be below the detection limit of qRT-PCR, the best available diagnostic tool, certification for IMNV disease-free for these life stages is problematic. For this reason, it is not advisable to transfer spawned eggs and larvae from one area to another, particularly from IMNV areas. Water and equipment must be sterilized before use. IMNV has been detected in zooplankton, crabs and oysters (Lima et al., 2013), and therefore, the water should also be filtered to avoid the introduction of carriers of wild shrimp or other vectors. Shrimp-eating birds have also been thought to be vectors of IMNV, and therefore, applying nets over the ponds is recommended. The virus may spread from farm to farm via contaminated equipment; therefore, sharing of equipment between ponds or farms is discouraged. All equipment including trucks that have been used in other ponds or farms should be cleaned and disinfected.

Once an IMNV outbreak has occurred on a shrimp farm, containment and eradication programs should be implemented to prevent the spread of the disease. These include culling all susceptible shrimp in affected populations, disinfection of used water before discharging it, sun-drying of emptied ponds for at least 2 weeks and disinfection of ponds and all equipment. Once the virus has been eradicated, it is important to prevent reintroduction by applying strict biosecurity measures. IMNV-infected shrimp of all life stages should not be transferred into areas known to be free of the disease. Biosecurity principles can be effective in preventing IMNV outbreaks in isolated or clustered farms, but may not be applicable for shrimp farms which are connected or which share the same water source with nearby ponds or farms. In these open culture systems, good management practices may be applied to reduce the impact of IMNV.

Some good management practices have been successfully applied to reduce the impact of IMN disease in affected shrimp farms. The fallowing and restocking of affected farms with IMNV-free stocks have been proven to be the most effective practices for the prevention and control of other viral diseases (Lee and O'Bryen, 2003; Lightner et al., 2009; Wyban et al., 1992) and should also be applicable for the control and prevention of IMN disease. For example, stocking of culture ponds with WSSV-free PLs significantly increased the rate of successful harvest over those with WSSV-positive PLs (Withyachumnarnkul, 1999). Similar good aquaculture practice should also be applicable to IMNV.

Indonesian and Brazilian shrimp farmers have learned to control and reduce the impact of IMNV in ponds/farms through general measures including proper pond preparation, maintaining water quality and determining the maximum carrying capacity of the pond. Specific measures include: removing soil from the pond bottom and using bioremediation products to

reduce the organic load in the pond; preventing fluctuation in water quality parameters by minimizing water exchange and providing enough aeration; and, reducing stocking density from high (200) to low (below 100) PLs/m^2. If reducing stocking density is not possible for financial reasons, some farmers use partial harvest to maintain carrying capacity of the pond. For example, if a pond was stocked with 150 PLs/m^2, then partial harvests done four times when the average body weights of shrimp were about 10, 15, 20 and 25 grams (Sunarto, 2011). Good management practices may increase the survival rates from 35 to more than 70% in some farms in affected regions (Andrade, 2009).

Development of specific pathogen-free (SPF) shrimp stock requires rigorous testing and screening of broodstock, as well as of the resulting progeny, using robust diagnostic tools, for example PCR, for targeted virus (Lightner et al., 2009; Wyban et al., 1992). Development of IMNV SPF shrimp is warranted because a qRT-PCR assay for IMNV is currently available. There are anecdotal reports that some selected lines of *L. vannamei* provide a better survival rate following challenge with IMNV (OIE, 2012; White-Noble et al., 2010). It has also been reported that a genetic breeding program for *L. vannamei* increased the survival rate from 3.2% in F1 to 55.3% in F7 generation of a selected family following IMNV challenges (Lima et al., 2013). This should provide a genetic basis for the development of IMNV SPR shrimp lines. Stocking of a pond/farm with SPR and/or SPF shrimp to IMNV under biosecure conditions is the most highly recommended GMP for prevention and control of IMN disease.

Despite the lack of published reports on the route of transmission, it has been speculated that, apart from horizontal transmission, IMNV is also transmitted vertically via the trans-ovarian route. Disinfection of spawned eggs and larvae is, therefore, recommended. It has been reported that cultured *L. vannamei* could be dually infected with IMNV and *Macrobrachium rosenbergii* nodavirus (MrNV) (Senapin et al., 2013). Whether this coinfection results in higher mortality of adult cultured shrimp than infection with IMNV alone is presently unknown. However, as MrNV causes significant mortality in juvenile *Penaeus vannamei* (Senapin et al., 2012) and PLs of *P. monodon* and *Penaeus indicus* (Ravi et al., 2009), it is recommended that *L. vannamei* and *M. rosenbergii* should not be reared together or in close proximity because of the risk of spill-over of MrNV from *M. rosenbergii* to *P. vannamei*.

RNA interference (RNAi) has been considered as an emerging solution for the prevention and control of viral diseases in shrimp (Hirono et al., 2011). RNAi is a natural posttranscriptional control process that prevents specific gene expression. RNAi triggers, such as dsRNA which causes the production of small interfering RNA (siRNA), can be provided exogenously to suppress the gene of interest in a sequence-specific manner. A low dose (0.02 μg) of an 81- or 153-bp fragment of dsRNA corresponding to a gene encoding pep1 in the IMNV genome provided 100% protection to *L. vannamei* following IMNV challenge (Loy et al., 2012). Furthermore, dsRNA may also be used as a therapeutic antiviral treatment against IMNV infection. Administration of a single dose (0.5 μg) of the 81 bp dsRNA 48 h postinfection (hpi) provided 50% protection to *L. vannamei* against IMN disease. However, this degree of protection was not observed when dsRNA was administered 72 hpi, suggesting the amount of virus at 72 hpi exceed the capacity of the RNAi antiviral effect (Loy et al., 2013).

In summary, the most effective way of protecting a shrimp farm from IMNV incursion is by blocking the introduction of IMNV into the farm through the application of strict biosecurity measures. Once an IMNV outbreak has occurred in a farm, containment and eradication programs should be implemented to prevent the spread of the disease. Some good management practices have also been successfully applied to reduce the impact of IMN outbreaks in affected shrimp farms.

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Chapter 31

Togaviruses of Crustaceans

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31.1 INTRODUCTION

Members of the family *Togaviridae* are enveloped icosahedral particles of about 70 nm in diameter, with a single-stranded RNA genome of positive polarity, 9.7–11.8 kb in size. Virus multiplies in the cytoplasm and buds from the plasma membrane or from internal membranes (endoplasmic reticulum and Golgi complex). The family has two genera, which contain important viral pathogens: genus *Alphavirus*, consisting of mammalian Group A arboviruses such as *Eastern equine encephalitis virus*, *Venezuelan equine encephalitis virus*, *Chikungunya virus* and the fish *Salmon pancreas disease virus* (Salmonid alphavirus); and genus *Rubivirus*, consisting of the teratogenic human pathogen *Rubella* virus (Powers et al., 2012). Alphaviruses bud from the plasma membrane, whereas *Rubella* virus buds from internal membranes (Powers et al., 2012).

31.2 LYMPHOID ORGAN VACUOLIZATION VIRUS

To date, there is only one published report of partial characterization of a crustacean togavirus called *lymphoid organ vacuolization virus (LOVV)* (Bonami et al., 1992). The virus was observed in the lesions of the lymphoid organ (ie, Oka organ) from a population of cultured *Penaeus vannamei* in Mexico in 1989. Using electron microscopy, Bonami et al. (1992) observed 30-nm-diameter icosahedral nucleocapsids and enveloped 50–60-nm-diameter mature virions closely associated with membranes of the endoplasmic reticulum or Golgi complex. Both nonenveloped (30–31-nm diameter) and enveloped particles (52–54-nm diameter) were also observed after isopycnic centrifugation in CsCl gradients, with buoyant densities of 1.320 and 1.230 g/mL, respectively. In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the nonenveloped particles contained at least two polypeptides of 37 and 38 kDa, whereas the enveloped particles showed an additional two polypeptides of 60 and 70 kDa. Thus, based on this ultrastructural characterization and site of virion maturation, LOVV was considered to be a member of the *Togaviridae* family (Bonami et al., 1992). However, the virus-infected material available was not sufficient to allow the genome of the virus to be characterized; therefore, LOVV is not formally approved by the International Committee on Taxonomy of Viruses as a species within the virus family *Togaviridae*.

31.3 CRUSTACEAN PATHOLOGY

The population of cultured *Penaeus vannamei* that was the source of the LOVV showed no signs of disease or other abnormalities during the 21 months of culture (Bonami et al., 1992). Upon histological examination, the principal lesions were found in lymphoid organs and were characterized as multifocal degenerative lesions, but with no significant inflammatory response within or adjacent to the lesions. The lymphoid organ cells containing virus particles had a highly vacuolated cytoplasm and intracytoplasmic Feulgen negative inclusion bodies (Bonami et al., 1992). Similar histopathological changes of lymphoid organs of many penaeid shrimps (*Penaeus monodon*, *Penaeus penicillatus*, *Penaeus esculentus*, *Penaeus vannamei*, *Penaeus stylirostris* and *Penaeus chinensis*) have been recognized in Asia and Australia by different authors (reviewed by Bonami et al., 1992), suggesting that the virus infection might be common, but with relatively little known importance to shrimp aquaculture (Johnson, 1995) and in wild penaeid shrimp populations.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00031-0
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Chapter 32

Nidoviruses of Fish and Crustaceans

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32.1 INTRODUCTION

The order *Nidovirales* comprises four families of viruses containing long RNA genomes (de Groot et al., 2012b; Gorbalenya, 2008; Gorbalenya et al., 2006), the *Coronaviridae* (de Groot et al., 2012a), *Arteriviridae* (Siddell and Snijder, 2008; Faaberg et al., 2012), *Roniviridae* (Mayo, 2002; Cowley et al., 2012) and *Mesoniviridae* (Nga et al., 2011; Zirkel et al., 2011; Lauber et al., 2012). Viruses in the *Coronaviridae* are segregated into two subfamilies. The *Coronavirinae* comprises four genera (*Alpha-*, *Beta-*, *Gamma-* and *Deltacoronavirus*) of viruses infecting diverse mammals or birds and the *Torovirinae* comprises viruses in the *Torovirus* genus that infect horses, cattle and humans and in the *Bafinivirus* genus that infect fish (http://ictvonline.org/virusTaxonomy.asp, de Groot et al., 2012a). Viruses in the single *Arterivirus* genus of the *Arteriviridae* have been isolated from diverse mammalian species such as mice, monkeys, horses and pigs (Balasuriya and Snijder, 2008). Viruses in the single *Alphamesonivirus* 1 genus of the *Mesoniviridae* have only been detected in mosquitoes (Lauber et al., 2012) and viruses in the single *Okavirus* genus of the *Roniviridae* have only been detected in crustaceans (Cowley et al., 2000a, 2012; Sittidilokratna et al., 2008).

Depending on the family and genus, nidoviruses have diverse virion morphologies ranging from spherical to bacilliform, but despite this, they share common genome organization and transcription features (de Groot et al., 2012a). All possess a genome comprised of an infectious linear positive-sense (+) ssRNA, which for coronaviruses (26.4–31.7 kb) is the longest of any known RNA virus (de Groot et al., 2012b). While toroviruses (28.0–28.5 kb) (Snijder et al., 2013), bafiniviruses (26.6–27.3 kb) (Schütze et al., 2006; Batts et al., 2012) and roniviruses (26.2–26.6 kb) (Cowley et al., 2000a; Cowley and Walker, 2002; Sittidilokratna et al., 2002, 2008) possess similarly long genomes, those of mesoniviruses (20.2 kb) (Nga et al., 2011; Zirkel et al., 2011, 2013; Lauber et al., 2012) and arteriviruses (12.7–15.7 kb) (Snijder et al., 2013) are more compact. Despite being diverse in length and gene number, the 5′-genome region of all nidoviruses comprises two long genes encoding overlapping open reading frames (ORF1a and ORF1b) that can constitute up to three-fourths of the genome length (Cowley et al., 2000a; Cowley and Walker, 2002; Sittidilokratna et al., 2002, 2008). At the ORF1a/1b gene overlap, all nidovirus genomes possess a "slippery" heptanucleotide sequence, which in conjunction with a nearby downstream sequence capable of folding into a pseudoknot structure, functions as a translational −1 ribosomal frameshift (RFS) element (Brierley, 1995; Brierley et al., 1989, 1992; Cowley et al., 2002a; ten Dam et al., 1990). This −1 RFS element functions at between 20% and 30% efficiency in pausing polyprotein 1a (pp1a) translation from the ORF1a gene and then slipping the translational machinery back a single nucleotide to be in-frame with the ORF1b gene-coding sequence, thus allowing translation of pp1a to be continued though ORF1b to generate a pp1ab polyprotein. As the ORF1a and ORF1b genes encode nonstructural proteins (nsps) utilized in genome replication and mRNA transcription, the −1 RFS element appears to have been preserved in the evolution of nidoviruses by a need to regulate the abundance of proteins encoded in the ORF1b gene relative to those encoded in the ORF1a gene.

The pp1a polyprotein contains numerous functional motifs, including an ADP-ribose 1-phosphatase, papain-like proteases and a chymotrypsin-like serine protease (3C-like or main proteinase) involved in the site-specific cleavage the pp1a and pp1ab polyproteins into their function units, and the pp1ab polyprotein contains these enzymes in addition to ORF1b gene-encoded motifs, including a RNA-dependent RNA polymerase (RdRp), helicase, ribose methyltransferase, exoribonuclease and endoribonuclease involved in viral RNA transcription and genome replication (de Groot et al., 2012a; Ziebuhr et al., 2000, 2003; Ziebuhr, 2005, 2006; Cowley et al., 2000a; Sittidilokratna et al., 2002). Positioned downstream of the ORF1a/1b gene are as few as 2 genes for roniviruses (Cowley et al., 2002a; Cowley and Walker, 2002; Sittidilokratna et al.,

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00032-2
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2008) to as many as 12 genes for coronaviruses that encode either the virion structural proteins or accessory proteins with various family and genus-specific functions (de Groot et al., 2012a,b).

All nidoviruses also transcribe a nested set of 3′-coterminal capped and polyadenylated subgenomic (sg)mRNAs that encode the virus structural and accessory proteins (Pasternak et al., 2006; Cowley et al., 2002a, 2004a,b; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006, 2008). The mechanism by which these sgmRNAs are transcribed appears to have adopted complexity as nidovirus genomes have acquired greater numbers of genes, presumably to accommodate replication in more highly evolved hosts (Pasternak et al., 2006; Sawicki et al., 2007). In okaviruses, which replicate in crustacean hosts with ancient origins (Siveter et al., 2001; Zhang et al., 2007) and which have a simple genome organization comprising only the ORF1a/1b replicase genes and two genes (ORF2 and ORF3) encoding virion structural proteins, the genomic RNA and sgmRNAs used to translate these ORFs each begin with 5′-AC termini, with these sgmRNA termini mapping to a central position of highly conserved transcriptional regulatory sequences (TRSs) residing within the intergenic regions upstream of ORF2 and ORF3 (Cowley et al., 2002a; Sittidilokratna et al., 2008). Similarly to all but the longest sgmRNA of toroviruses (Snijder et al., 1990; van Vliet et al., 2002; Smits et al., 2005), these TRS elements appear to have roles in terminating negative-strand (−) RNA transcription in a lossy fashion that allows some transcripts to continue through to upstream TRSs or to the (+) RNA genome 5′-terminus. As evidenced by the presence of genomic-length and sgmRNA-length replicative-intermediate double-stranded (ds)RNAs in shrimp cells infected with gill-associated virus (GAV) (Cowley et al., 2002a), the type species okavirus (Cowley et al., 2012), it is speculated that transcription termination of the antisense RNAs might occur at precise positions, resulting in common 3′-termini, and that these then act directly as promoters for transcription initiation of the genomic and sgmRNAs. In all other nidoviruses, however, and for the longest of the sgmRNAs transcribed by toroviruses, the (−) and (+) strand sgmRNAs are transcribed using a far more complex discontinuous process involving the splicing of a common "anti-leader" sequence derived from the genome 5′-terminus to each (−) strand sgmRNA that then acts as a universal promoter for transcribing each (+) strand sgmRNA (Pasternak et al., 2006; Sawicki et al., 2007; Smits et al., 2005; van Vliet et al., 2002).

Nidoviruses of aquatic species include the rod-shaped okaviruses GAV and yellow head virus (YHV) that primarily infect Penaeid shrimp (Longyant et al., 2005; Lightner et al., 1998; Flegel, 2012; Flegel et al., 1997a; Spann et al., 1997; Cowley et al., 2000a, 2002a; Cowley and Walker, 2002; Sittidilokratna et al., 2008) and a morphologically similar virus with a ~22 kb ssRNA genome detected in diseased Chinese mitten crabs (Zhang and Bonami, 2007). They also include the rod-shaped bafiniviruses white bream virus (WBV) detected in white bream in Germany (Granzow et al., 2001; Schütze et al., 2006; Ulferts et al., 2011) and fathead minnow nidovirus (FHMNV) detected in diseased fathead minnows in the United States (Iwanowicz and Goodwin, 2002; Batts et al., 2012). A virus with a spherical coronavirus-like particle morphology has also been detected in color carp in Japan (Miyazaki et al., 2000, 2001), but it has not yet been demonstrated to possess a long (+) ssRNA genome with features characteristic of nidoviruses.

To aid clarity, the fish bafiniviruses and crustacean okaviruses will be described separately together with viruses not yet assigned formally as nidoviruses, but which share common structural features.

32.2 FISH BAFINIVIRUSES

Rod-shaped nidoviruses classified in the genus *Bafinivirus*, subfamily *Torovirinae*, family *Coronaviridae* (de Groot et al., 2012a) have been discovered in wild white bream (*Blicca bjoerkna* L.) in Germany (Granzow et al., 2001) and in diseased fathead minnows (*Pimephales promelas*) cultured as baitfish in the United States (Iwanowicz and Goodwin, 2002; Table 32.1 and Fig. 32.1). White bream support small wild freshwater fisheries in Europe and fathead minnows support a substantial freshwater baitfish culture industry in the United States (Meronek et al., 1997; McCann, 2012). WBV and

TABLE 32.1 Fish Viruses in the Genus *Bafinivirus*, Subfamily *Torovirinae*, Family *Coronaviridae*

Virus Name	Abbreviation	Natural Hosts	References
White bream virus	WBV	White bream (*Blicca bjoerkna L.*)	Granzow et al. (2001)
Fathead minnow nidovirus	FHMNV	Fathead minnow (*Pimephales promelas*)	Iwanowicz and Goodwin (2002)
Ctenopharyngodon idella virus Hungary 33/86[a]	CIVH 33/86	Grass carp (*Ctenopharyngodon idella*)	Ahne et al. (1987)

[a]*Virion morphology consistent with bafiniviruses, but its RNA genome not yet been sequenced to support inclusion in this genus.*

FHMNV possess an infectious 26.6–27.3 kb (+) ssRNA genome organized and transcribed similarly to other nidoviruses, and alignments of deduced amino acid sequences of their pp1ab and structural proteins have identified them to represent different bafinivirus species that appear to have diverged from a common ancestor early in their evolution (Batts et al., 2012; Schütze et al., 2006).

Another rod-shaped fish virus with similar virion morphology to bafiniviruses has been isolated in fish cell lines inoculated with tissue homogenates of healthy grass carp (*Ctenopharyngodon idella*) imported from Hungary into Germany in 1986 (Ahne et al., 1987). Like WBV and FHMNV, *Ctenopharyngodon idella* virus Hungary 33/86 (CIVH 33/86) also contains a ssRNA genome and infection causes syncytia formation in fish cell monolayers (Ahne et al., 1987). However, the genome of CIVH 33/86 must to be sequenced to confirm it is a bafinivirus (Table 32.1). While grass carp is a significant aquaculture species, particularly in China but also throughout Asia, Russia and Europe, with total production estimated to be in the order of 5 million metric tons in 2012 (http://www.fao.org/fishery/culturedspecies/Ctenopharyngodon_idella/en), no evidence has been reported for CIVH 33/86 impacting carp farming.

32.2.1 Virion Morphology and Morphogenesis

Negatively stained WBV particles purified from cell culture supernatants by sucrose density-gradient ultracentrifugation are bacilliform in shape (37–45 nm × 130–160 nm) and possess a lipid envelope decorated with prominent and regularly spaced fuzzy surface projections (20–25 nm in length) (Granzow et al., 2001; Fig. 32.1). Viral nucleocapsids observed in disrupted or partially disrupted virions appear as rigid rod-like structures (19–21 nm × 120–150 nm) with inner and outer sections indicative of their being cylindrical in morphology (Granzow et al., 2001). Negatively stained FHMNV particles semi-purified from cell culture supernatants are also bacilliform and similar in dimensions (31–47 nm × 130–180 nm) (Iwanowicz and Goodwin, 2002). Unlike WBV, however, the FHMNV particles appeared smooth and devoid of surface projections, possibly as a result of how they were purified and stained. Negatively stained rod-shaped virions of CIVH 33/86 are similar in dimensions (50–55 nm × 170–220 nm) and among some particles aligned in close proximity, regularly spaced surface projections like those of WBV have been observed (Ahne et al., 1987; Granzow et al., 2001).

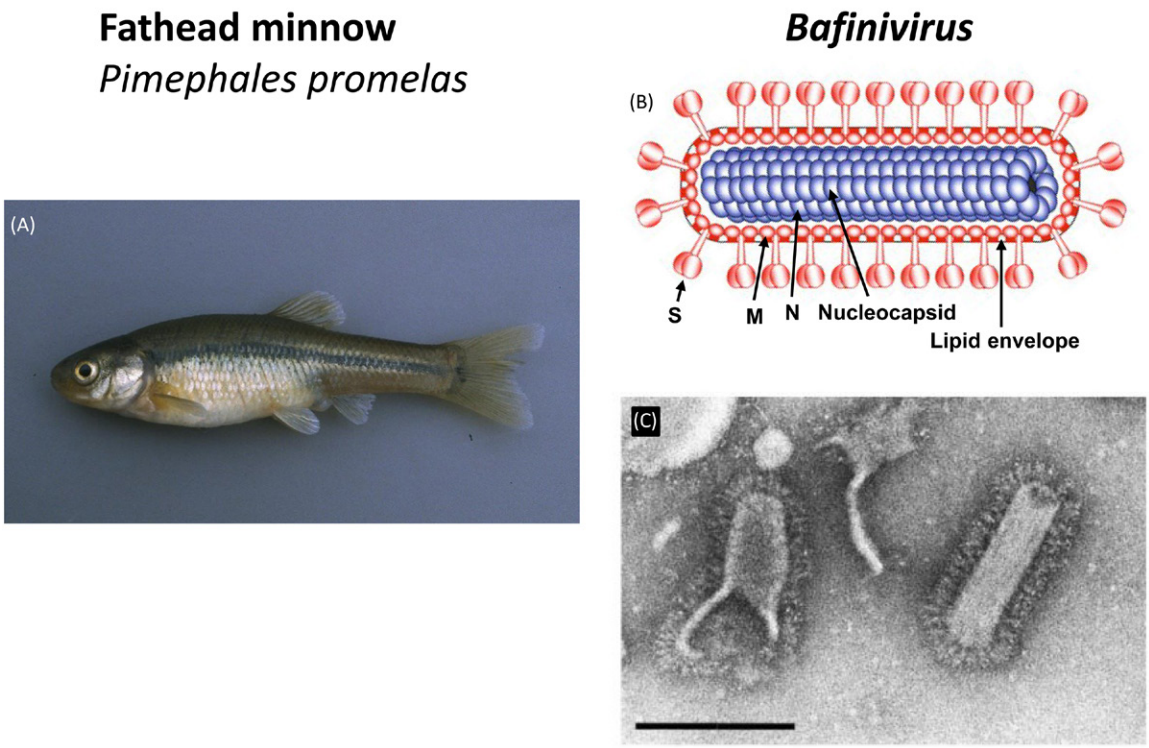

FIGURE 32.1 (A) Photograph of a fathead minnow (*Pimephales promelas*) (Lyons, 2015). (B) Schematic structure of a bafinivirus particle showing the positions of the envelope spike glycoprotein (S), membrane protein (M) and nucleoprotein (N) and (C) electron micrograph of negatively stained rod-shaped virions of WBV (Granzow et al., 2001), showing the presence of regularly spaced club-like spikes protruding from the lipid envelope as well as the underlying helical nucleocapsid (Bar = 150 nm).

32.2.2 Genome Organization and Transcription

The capped and polyadenylated (+) ssRNA genomes of WBV (Schütze et al., 2006) and FHMNV (Batts et al., 2012) each possess five genes encoding ORFs (ORF1a, 1b, 2, 3 and 4). The ORF1a/1b gene encodes pp1a and pp1ab polyproteins containing proteinase, polymerase and other replicase enzymes shared across all nidoviruses and ORF2, ORF3 and ORF4 encode a spike (S) glycoprotein, membrane (M) protein and nucleocapsid (N) proteins, respectively. Both viruses utilize −1 RFS elements comprised of an UUUAAAC "slippery" heptanucleotide sequence and a downstream RNA pseudoknot structure similar in predicted structure to facilitate translational read-through of the ORF1a/1b gene overlap, and possess intergenic nonanucleotide TRS elements near identical in sequence (Schütze et al., 2006; Batts et al., 2012). Subgenomic mRNAs transcribed for each gene possess 5′-genome leader sequences upstream of the conserved TRS, indicating the use of a discontinuous transcription strategy comparable to those used by nidoviruses other than roniviruses and for the longest sgmRNA of toroviruses (Pasternak et al., 2006; Sawicki et al., 2007; van Vliet et al., 2002; Smits et al., 2005; Snijder et al., 1988).

32.2.3 Disease and Pathology

WBV (virus isolate DF 24/00) was discovered in 2000 in fish cell cultures inoculated with a mixed tissue homogenate of white bream (*Blicca bjoerkna* L.) as part of a virus monitoring program of wild fish populations in Germany (Granzow et al., 2001). Although highly cytopathic for *Epithelioma papulosum cyprini* (EPC) cell cultures derived from carp (*Cyprinus carpio*) epidermal herpes virus-induced hyperplastic lesions, the health status of the white bream from which WBV was isolated was not reported, and there is no information on its pathogenic potential for this or other fish species.

FHMNV was isolated in 1997 from moribund wild-type and rosy red fathead minnows (*Pimephales promelas*) being reared as baitfish in ponds in central Arkansas (Iwanowicz and Goodwin, 2002). The minnows were a mixture of wild and farmed fish from various unspecified US locations and were experiencing chronic mortality associated with eye and skin hemorrhages. Like WBV, FHMNV is highly cytopathic for EPC cells characterized initially by cell rounding, with multifocal syncytia (fused multinucleated cells) then forming as the cell monolayer degrades. Among several others examined, rainbow trout gonad (RTG-2) and fathead minnow (FHM) cell lines were also highly susceptible to FHMNV-induced cytopathology (Iwanowicz and Goodwin, 2002). In FHMNV-infected EPC cells, histopathology is characterized by pyknotic nuclei displaying marginated chromatin, and in moribund FHMs, by lesions in the anterior kidney, liver and spleen, as well as severe multifocal necrosis of hepatocytes (Iwanowicz and Goodwin, 2002).

In FHMs challenged with FHMNV, morbidity becomes evident by behavioral changes characterized by fish initially swimming erratically in circles before becoming listless either at the tank bottom or with their head orientated upward at the water surface (Iwanowicz and Goodwin, 2002). In 19°C water, mortalities accumulate rapidly from 3 days post-challenge, but 17°C water delays mortality onset to 13 days post-challenge. Clinical signs consistent with the index case develop that include petechial hemorrhages in the eyes and skin (Iwanowicz and Goodwin, 2002). Also evident can be hemorrhaging of muscle, generally localized to the epaxial musculature anterior to the vent, and below the epidermis at the cranium apex. The kidneys in many fish also become visible due to musculature edema and darkening of the trunk kidney. Productive infections do not establish in challenged channel catfish, rainbow trout or goldfish or in golden shiners cohabitated with challenged FHMs, suggesting that FHMNV is highly host-specific (Iwanowicz and Goodwin, 2002).

32.2.4 Diagnostic Methods

FHMNV infection and disease can be diagnosed tentatively by behavioral changes and characteristic gross signs. Development of cytopathic effects in EPC and other fish cell lines that include syncytia formation can also assist diagnosis of FHMNV and WBV, with detection of rod-shaped virions by transmission electron microscopy (TEM) providing additional diagnostic confidence. Opportunities exist to develop specific and sensitive diagnostic reverse transcription-polymerase chain reaction (RT-PCR) tests because complete genome sequences are available for FHMNV (Batts et al., 2012) and WBV (Schütze et al., 2006). For FHMNV, PCR tests have been described that amplify either a relatively conserved region in the ORF1b gene helicase motif or an ORF2 gene sequence (Batts et al., 2012). Sequence analysis of the ORF2 gene region amplified by PCR has provided a means of differentiating FHMNV strains isolated in cell culture from FHMs being reared in Minnesota, Wisconsin and Illinois as well as present in jack pike (*Esox masquinongy*) fed live FHMs in Nebraska and a batch of creek chub (*Semotilus atromaculatus*) (Batts et al., 2012; McCann, 2012). A constant-temperature RT loop-mediated isothermal amplification (RT-LAMP) test with a sensitivity of ~5 RNA copies has also been developed for FHMNV diagnosis and surveillance (Zhang et al., 2014). While no molecular tests have been reported specifically

for WBV diagnosis, immunoblotting using a polyclonal rabbit antiserum to a fusion protein to the pp1a main proteinase (Mpro) motif expressed in bacteria has been used to detect predicted Mpro cleavage products of pp1a and pp1ab in WBV-infected fish cells (Ulferts et al., 2011). Opportunities thus exist to employ this or other WBV protein-specific antibodies in diagnostic immunotests.

32.2.5 Prevention and Control

There has been little impetus to control WBV, presumably because it has only been isolated once and not been identified to be pathogenic, because white bream is not an aquaculture species and as the wild fishery in Europe is small (Granzow et al., 2001). This contrasts to FHMNV, which, due to its pathogenicity for FHMs, poses a significant threat to the multi-million dollar baitfish industry in the midwestern United States (Meronek et al., 1997; McCann, 2012). Due to the industry value to this region and because FHMs are distributed widely in brooks, ponds and small lakes, the potential for virus dispersal via movements of live baitfish is currently regulated by state import policies and health certifications, as well as an overarching US Federal law called the Lacey Act (McCann, 2012). While these regulations rely primarily on observing fish for disease symptoms, a survey of the three primary baitfish species farmed in Wisconsin employing cell culture in conjunction with PCR testing has proved useful in isolating, identifying and determining the prevalence and species distribution of FHMNV (McCann, 2012). In Arkansas, it has been reported that destroying the farmed FHM population affected by FHMNV in 1997 in conjunction with high summer water temperatures might have been effective at eradicating the virus from this state (Batts et al., 2012). As for any pathogen of aquaculture species, FHMNV control will rely on robust surveillance, translocation regulations and farm biosecurity, including stock destruction and water decontamination protocols.

32.3 CRUSTACEAN RONIVIRUSES

The sequence and organization of the 26.2–26.6 kb (+) ssRNA genomes of GAV (Cowley et al., 2000a, 2004a; Cowley and Walker, 2002) and YHV (Sittidilokratna et al., 2002, 2008, 2009; Jitrapakdee et al., 2003), together with the transcription of a nested set of 3′-coterminal sgmRNAs used in protein translation (Cowley et al., 2002a; Sittidilokratna et al., 2008), has identified them to be related distantly to diverse vertebrate and invertebrate viruses classified in the order *Nidovirales* (Cowley et al., 2012; de Groot et al., 2012a). Due to observations of the viruses replicating and causing pathology in the lymphoid or "Oka" organ of shrimp (Oka, 1969; Spann et al., 1995), GAV was assigned as the type species of a genus called *Okavirus* in a family named *Roniviridae* (sigla, *ro*d-shaped *ni*dovirus) based on the evolutionary divergence of GAV and YHV from other nidoviruses (Gorbalenya, 2008; Gorbalenya et al., 2002, 2006) and on their rod-shaped enveloped virions (Spann et al., 1995, 1997; Spann and Lester, 1997; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Nadala et al., 1997b) being distinct from the spherical enveloped virions formed by viruses in either the *Coronaviridae* or the *Arteriviridae* families of the *Nidovirales* known to exist at that time (de Groot et al., 2012a).

In addition to GAV and YHV that have caused widespread disease in shrimp farmed throughout Eastern Hemisphere countries, a few viruses forming okavirus-like rod-shaped virions have been detected in crab species. Of these, *Eriocheir sinensis* ronivirus (*Es*RNV), which causes disease in the Chinese mitten crab, has been shown to possess a ~22 kb ssRNA genome (Zhang and Bonami, 2007) supportive of it being related to okaviruses, but no genome sequence information is yet available to confirm this relationship. The molecular and biological characteristics of GAV and YHV and the management of virus transmission and disease in farmed shrimp have been the subject of several comprehensive reviews (Dhar et al., 2004; Cowley and Walker, 2007; Lightner, 2011; Munro and Owens, 2007b; OIE, 2014; Walker and Sittidilokratna, 2008). For completeness, information available on okaviruses (Table 32.2) has also been reviewed and updated in the next sections, including more recent developments in diagnostic methods and potential antiviral therapies as well as brief descriptions of the crab viruses with virion morphologies suggestive of them being related to okaviruses.

32.3.1 YHV and GAV

32.3.1.1 Discovery, Disease Symptoms and Pathology

YHD was first reported as the cause mass mortality in Giant Tiger shrimp (*Penaeus monodon*) being cultured intensively in Thailand in the early 1990s (Limsuwan, 1991). The disease generally affected juvenile to subadult *P. monodon* 50–70 days into culture, and its pending onset was typically announced by a rapid abnormal increase in feed consumption followed 2–4 days later by an equally rapid cessation in feeding. Within 3–5 days of feeding cessation, moribund shrimp would amass at pond edges and die, often leading to almost complete pond losses. The name YHD was conceived because the bodies of

TABLE 32.2 Crustacean Viruses in the Genus *Okavirus*, Family *Roniviridae*

Virus Name	Abbreviation	Natural Hosts	References
Yellow head virus	YHV	Giant Tiger shrimp (*Penaeus monodon*) Pacific whiteleg shrimp (*Litopenaeus vannamei*)	Boonyaratpalin et al. (1993)
			Chantanachookin et al. (1993)
			Wongteerasupaya et al. (1995a,b)
			Nadala et al. (1997a,b)
			Sittidilokratna et al. (2002, 2008)
			Jitrapakdee et al. (2003)
Gill-associated virus	GAV	*Penaeus monodon*	Spann et al. (1995, 1997)
			Cowley et al. (1999, 2000a, 2002a)
			Cowley and Walker (2002)
Eriocheir sinensis ronivirus[a]	*Es*RNV	Chinese mitten crab (*Eriocheir sinensis*)	Zhang and Bonami (2007)

[a]*Virion morphology and ~22 kb ssRNA genome consistent with okaviruses but its genome not yet sequenced to support inclusion in this genus.*

many diseased shrimp had a bleached appearance often with a yellowed cephalothorax due to the gills developing a brownish color and the hepatopancreas becoming swollen and yellowed rather than dark brown (Limsuwan, 1991; Flegel et al., 1995b; Fig. 32.3). Transmission trials, histology and TEM undertaken to identify the cause of YHD identified a rod-shaped enveloped virus (40–50 nm × 150–200 nm) speculated at the time to be an atypical baculovirus and accordingly given the name yellow-head baculovirus (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). However, when purified virions were identified to contain a genome comprised of a long (>22 kb) ssRNA, its name was revised to YHV (Wongteerasupaya et al., 1995b; Nadala et al., 1997a,b). While thought to have emerged in Thailand, a review of histopathology and the morphology and morphogenesis of a virus cultured in primary shrimp lymphoid organ (LO) cells inoculated with a tissue extract of diseased *P. monodon* investigated at the time when shrimp aquaculture collapsed in Taiwan in the late 1980s suggests that YHV was the likely cause of that collapse (Chen and Kou, 1989).

Soon after YHV was identified in Thailand, virions indistinguishable in morphology were detected in cells with hypertrophied nuclei sequestered within LO spheroids of overtly healthy *P. monodon* being farmed in eastern Australia (Spann et al., 1995). The virus was named lymphoid organ virus (LOV) because it was not associated with disease and virus particles were only evident in LO spheroids; in addition no genome sequence information was available to determine its relationship to YHV (Spann et al., 1995; Spann and Lester, 1997). Around the same time LOV was discovered in 1994, mass mortalities of subadult *P. monodon* were reported at four farms in North Queensland, Australia (Anderson and Owens, 2001; Spann et al., 1995, 1997; Spann and Lester, 1997; Walker et al., 1998; Munro et al., 2011). As with YHD, the disease was accompanied by rapid feeding increases and cessation, shrimp swimming erratically at the water surface and as morbidity became acute, shrimp congregating in large numbers at pond edges before death and mortality rates of up to 80% at pond harvest. As more widespread disease outbreaks occurred during the 1996/97 season generally around 90–120 days into grow-out, the name 'mid-crop mortality syndrome' (MCMS) was adopted to describe the disease (Owens et al., 1998; Anderson and Owens, 2001; Walker, 2000; Walker et al., 2001; Cullen and Owens, 2004). While gills of affected shrimp would typically become pink to brownish in color like YHD, disease gross signs were typified by a generalized reddened of the body and particularly at pereiopod, pleopod and tail fin extremities of experimentally challenged shrimp (Spann et al., 1997).

While up to four virus particle types were detected in MCMS-affected shrimp (Owens et al., 1998), a rod-shaped enveloped virus particle morphologically indistinguishable from LOV and YHV was demonstrated to be the primary disease agent (Spann et al., 1997; Oanh et al., 2011). Due to the lack of genome sequence data to confirm their evolutionary relatedness, and as gill cells of diseased *P. monodon* possessed large numbers of virus particles in addition to LO cells, the virus was named GAV (Spann et al., 1997). However, following subsequent genome sequence comparisons of LOV and GAV (Cowley et al., 2000b) together with shrimp challenge data showing that an inoculum prepared from LO tissue of LOV-infected healthy shrimp could induce disease and mortality typical of GAV and that infection in the absence of disease could be induced using a diluted GAV inoculum (Walker et al., 2001), LOV and GAV were considered the same virus and the name LOV was abandoned.

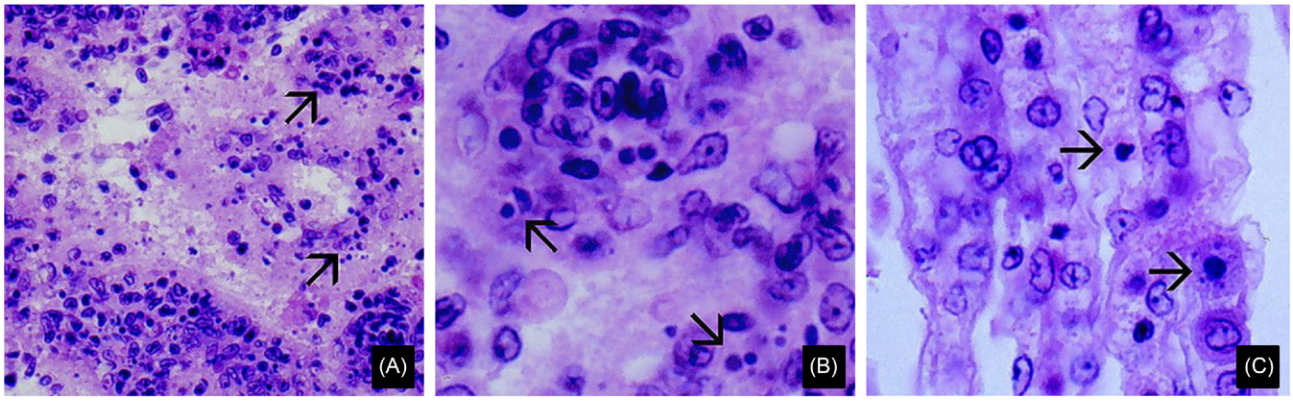

FIGURE 32.2 Histopathology evident in tissue sections stained with H&E. Juvenile *Penaeus monodon* with acute YHD displaying marked cellular necrosis in the lymphoid organ, (A) 525×, (B) 1700× magnification, characterized by affected cells displaying pyknotic and karyorrhectic nuclei, with single or multiple perinuclear inclusion bodies ranging from pale to darkly basophilic (light to dark gray) apparent in some cells (*arrows*) as well as in (C) gill lamellae cells (1000×) displaying similar pyknotic and karyorrhectic nuclei (*arrows*) with some enlarged and generally spherical cells possibly representing immature hemocytes released in response to disease displaying a basophilic cytoplasm (Lightner, 1996).

In acute-stage infections associated with disease, YHV and GAV can invade all tissues of mesodermal and ectodermal origin. These include the LO, circulating hemocytes as well as developing hemocytes in hemopoietic tissues and fixed phagocytes in the heart, gill lamellae and epithelial pillar cells, and spongy connective tissues of various organs including the heart, stomach subcuticulum, antennal gland, gonads and nerve tissues, including neural ganglia, nerve fibers, neurosecretory and glial cells (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lightner, 1996; Spann et al., 1997, 2000; Tang and Lightner, 1999; Soowannayan et al., 2002), as well as the fasciculate zone of eye (Smith, 2000; Callinan et al., 2003). Histopathology evident in hematoxylin and eosin (H&E) stained cephalothorax tissue sections is typified by severe necrosis in which cells display prominent nuclear pyknosis and karyorrhesis, as well as intense basophilic perinuclear inclusions in the cytoplasm resulting from nuclear phagocytosis and virus inclusions (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lightner, 1996; Lu et al., 1994; Nash et al., 1992; OIE, 2014; Spann et al., 1997, 2000; Wang and Chang, 2000). Typical histopathology seen in the LO and gill lamellae of *P. monodon* with acute YHD are shown in Fig. 32.2.

Both YHV and GAV can also persist over the life of wild shrimp in a subclinical or chronic infection state (Spann et al., 1995, 2003; Cowley et al., 2000b; Walker et al., 2001; Pasharawipas et al., 1997). Infection can also persist in *P. monodon* and Pacific whiteleg shrimp (*Litopenaeus vannamei*) that survive experimental YHV challenge (Longyant et al., 2005, 2006; Anantasomboon et al., 2007) and in farmed *L. vannamei* that survive a YHD outbreak (Senapin et al., 2010). The only histopathology observed consistently in asymptomatic shrimp is LO spheroids comprised of partitioned aggregations of cells with hypertrophied nuclei (Spann et al., 1995; Anantasomboon et al., 2007). Cells within these spheroids are often apoptotic and there is evidence for them being granular hemocytes exocytosed due to them having phagocytosed virus-infected cells or cell debris recognized to be foreign (Anggraeni and Owens, 2000; van de Braak et al., 2002; Duangsuwan et al., 2008).

32.3.1.2 Particle Morphology and Morphogenesis

In ultrathin sections of infected shrimp tissue, mature virions of YHV appear bacilliform in shape (40–60 nm × 150–200 nm) and possess a lipid envelope decorated with regularly spaced diffuse surface projections (8 nm dia. × 11 nm long) (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Wang and Chang, 2000; Wongteerasupaya et al., 1995a,b). The morphology of negatively stained virions purified from shrimp hemolymph by sucrose density-gradient ultracentrifugation has been reported to be similar (Wang and Chang, 2000; Wongteerasupaya et al., 1995a,b; Fig. 32.3), except for the presence of narrowed envelope appendages extending from particle ends that can join the ends of curled particles to form donut-like structures (Nadala et al., 1997b). The origin and purpose of the envelope extensions are unknown. However, the donut-shaped YHV particle structures quite closely resemble the virions (120–140 nm dia.) containing curled nucleocapsids formed by viruses classified within the *Torovirus* genus of the *Torovirinae* (de Groot et al., 2012b), with the rod-shaped YHV particles being similar in dimensions and morphology to those of the fish viruses classified within the *Bafinivirus* genus of the *Coronavirinae* (Granzow et al., 2001; Iwanowicz and Goodwin, 2002).

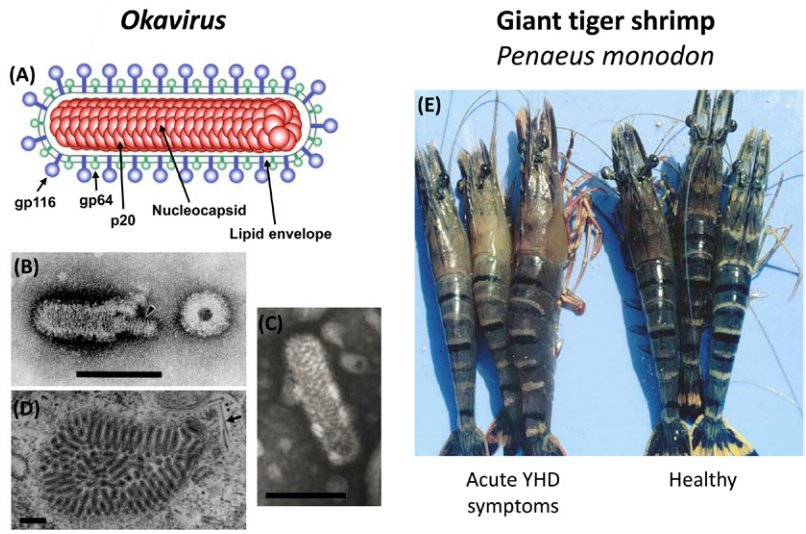

FIGURE 32.3 (A) Schematic structure of an Okavirus particle showing the positions of the envelope glycoproteins (gp116, gp64) and the nucleoprotein (p20) and electron micrographs of negatively stained rod-shaped virions of (B) YHV (Nadala et al., 1997a,b) and (C) GAV (Spann et al., 1995) showing the helical nucleocapsid and diffuse regularly spaced envelope projections as well as (D) GAV particles clustered tightly within a cytoplasmic vesicle surrounded by filamentous nucleocapsid precursors (*arrow*) in a lymphoid organ cell (Spann and Lester, 1997) (Bars = 150 nm). (E) Photograph of Giant tiger shrimp (*Penaeus monodon*) showing yellow-brown (bleached) discoloration of the cephalothorax and gills (left side) as observed in early disease outbreaks in Thailand caused by YHV (Lightner, 1996).

The nucleocapsid component of YHV virions appears as a coiled filament (16–30 nm dia.) with a 5–7 nm periodicity (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Nadala et al., 1997b; Wang and Chang, 2000). Free nucleocapsid precursors (15-nm diameter) that occur abundantly in the cytoplasm of infected cells appear as flexible helical filaments up to 450 nm in length. During virion morphogenesis, nucleocapsids generally bud internally through membranes of the Golgi-endoplasmic reticulum (ER) complex to generate enveloped virions, which sometimes are observed abutted end-to-end, as if formed consecutively from multiple linked nucleocapsids that appear to comprise the long filaments (Chantanachookin et al., 1993; Spann et al., 1997). This consecutive mode of nucleocapsid and virion morphogenesis is also supported by purified virions being identified joined end-to-end (Wongteerasupaya et al., 1995a,b). Virions often occur in the vicinity of nucleocapsid filaments between the outer and inner membranes of the nuclear envelope (Chantanachookin et al., 1993; Wang and Chang, 2000), suggesting that the outer nuclear membrane can serve as an alternative to Golgi-ER membranes for virion maturation. Virions also bud infrequently from the cell plasma membrane (Boonyaratpalin et al., 1993; Spann et al., 1995, 1997). Nucleocapsid precursors and virions accumulate throughout the cytoplasm, with virions commonly seen congregated and sometimes densely packed within cytoplasmic vesicles (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). Exocytosis of these vesicles during cell degeneration appears to be the primary mode of virion release.

32.3.1.3 Genome Organization, RNA Transcription and Viral Proteins

Initial analyses of the YHV genome showed it to be long (~22 kb), sensitive to RNase A, but not DNase 1 digestion (Wongteerasupaya et al., 1995b) and while preliminary in vitro translation analysis suggested it might be negative in polarity (Nadala et al., 1997b), in situ hybridization (ISH) using RNA probes of either polarity confirmed it had positive polarity (Tang and Lightner, 1999). Further characterization of the (+) ssRNA genomes of GAV (Cowley et al., 2000a, 2001; Cowley and Walker, 2002) and YHV (Sittidilokratna et al., 2002, 2008; Jitrapakdee et al., 2003) showed them to be 26,235 and 26,662 nt in length, respectively, to possess a 5′-7-methylguanosine cap structure and a 3′-poly(A) tail and to be organized similarly to coronaviruses and arteriviruses (Fig. 32.4).

Downstream of a short 5′-terminal untranslated sequence, the genome of okaviruses contains a ~20 kb ORF1a/ORF1b replicase gene proceeded by an ORF2 gene that encodes the p20 nucleoprotein, an ORF3 gene that encodes a pp3 polyprotein from which the gp116 and gp64 envelope glycoproteins are derived and, in the case of GAV, a short ORF4 gene encoding a protein of unknown function that is not translated in abundance and that is truncated in YHV (Cowley et al., 2012). Similarly to other nidoviruses, a slippery heptanucleotide (AAAUUUU) in conjunction with a downstream 131-nt sequence predicted to fold into a complex pseudoknot structure resides in the ORF1a/ORF1b gene overlap and functions as −1 RFS

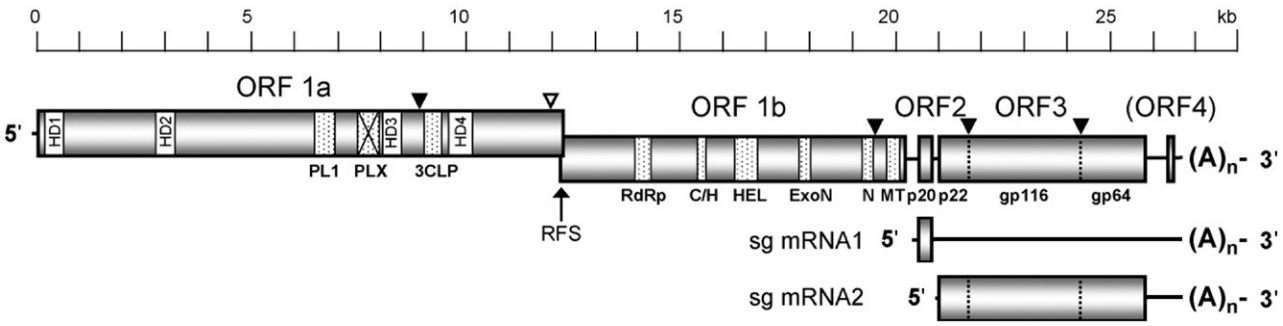

FIGURE 32.4 Schematic representation of the 26,662 nt (+) ssRNA genome of YHV and (subgenomic) sgmRNA1 and sgmRNA2 that translate ORF2 and ORF3, respectively. Each RNA contains a 5′-7-methylguanosine cap and a 3′-poly(A) tail. Functional domains identified in ORF1a include four hydrophobic regions (HD1-HD4), a 3C-like protease (3CLpro), a papain-like protease (PL1) and a domain with homology to PL1, but lacking the canonical α + β fold of papain-like proteases (PLX) and in ORF1b include a RNA-dependent RNA polymerase (RdRp), cysteine- and histidine-rich domain (C/H) Zn fingers, a helicase (HEL), an exoribonuclease (ExoN), a uridylatespecific endoribonuclease (N) and a ribose-*O*-methyl transferase (MT). The −1 ribosomal frame-shift (RFS) element allows translational read-through of ORF1a polyprotein pp1a into ORF1b to generate polyprotein pp1ab. ORF2 encodes the nucleoprotein (p20). ORF3 encodes a polyprotein (pp3) containing six transmembrane (TM) domains that undergoes posttranslational cleavage at the C-terminal side of TM3 and TM5 to generate the envelope glycoproteins gp116 and gp64, respectively, as well as a 22 kDa N-terminal triple-membrane-spanning fragment of unknown function. Known (▼) and likely (▽) sites of proteolytic cleavage in pp1a, pp1ab and pp3 are indicated (Sittidilokratna et al., 2008).

element to facilitate read-through translation of the ORF1a gene pp1a polyprotein into the ORF1b gene at approximately 23% efficiency to generate a pp1ab polyprotein (Cowley et al., 2000a, 2012; de Groot et al., 2012a; Sittidilokratna et al., 2002).

The ORF1a gene coding sequence contains four hydrophobic domains (HD1-HD4), each predicted to comprise multiple transmembrane (TM) sequences (Cowley et al., 2000a, Sittidilokratna et al., 2002). A chymotrypsin-like cysteine proteinase (3CLpro) flanked by HD3 and HD4 and identified tentatively to use a VxHE↓(L,V) consensus cleavage-site motif functions in autocatalytic processing of the pp1a/pp1ab polyproteins into functional nsp units (Ziebuhr et al., 2003). However, the okavirus 3CLpro appears to combine the Cys–His catalytic dyad of the coronavirus 3CLpro with a substrate-binding pocket representative of the 3C-like proteinase of plant potyviruses, thus distinguishing it from the cognate enzymes of other nidoviruses (Ziebuhr et al., 2003). ORF1a also contains two putative papain-like proteinase motifs (PLP1 and PLPx) that lack a Zn^{2+}-binding finger, as in the papain-like proteases (PLpro) of toroviruses, and share only remote structural similarity to PLpro motifs present in similar ORF1a gene positions of other nidoviruses (Sittidilokratna et al., 2002). While shown to cleave consensus sites immediately to the N-terminal side of the 3CLpro domain and near the pp1ab C-terminus (Ziebuhr et al., 2003), the number and nature of okavirus nsp units cleaved from the pp1a/pp1ab polyproteins by the 3CLpro and PLPs have yet to be determined.

The okavirus ORF1b gene-coding sequence component of the pp1ab replicase polyprotein contains homologs of a RdRp polymerase with a "SDD" active site motif characteristic of nidoviruses (Koonin, 1991; Gorbalenya et al., 2002), as well as putative multinuclear Zn^{2+}-binding finger-like domains preceding a 5′→3′ helicase domain (Zn-HEL), a 3′→5′exoribonuclease (ExoN), a *N*idoviral *end*oribonuclease specific for *U*ridylate (NendoU) and a ribose-2′-O-methyltransferase (O-MT) present in the cognate pp1ab polyproteins of other nidoviruses (de Groot et al., 2012a,b; Faaberg et al., 2012; Cowley et al., 2000a, 2012; Lauber et al., 2012; Sittidilokratna et al., 2002; Ziebuhr, 2005, 2006, 2008; Ziebuhr et al., 2000, 2003; Gorbalenya, 2008; Gorbalenya et al., 2006).

Unlike vertebrate nidoviruses in which the viral nucleoprotein gene resides close to the 3′-end of the genome, and more like the mosquito mesoniviruses in which the nucleoprotein appears to be encoded in an alternative reading frame in the N-terminal signal peptidase cleavage product of the ORF2a polyprotein from which the mature spike (S) envelope glycoprotein is also cleaved (Zirkel et al., 2013; Vasilakis et al., 2014), the okavirus p20 nucleoprotein gene (ORF2) resides upstream of the 5 kb ORF3 gene, encoding the gp116 and gp64 envelope glycoproteins (Cowley et al., 2004a; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006). Positioning of the nucleoprotein gene at the 3′-genome end ensures its translation in high abundance because the 3′-coterminal sgmRNA transcription process that is utilized by vertebrate nidoviruses containing larger numbers of genes results in their abundance decreasing relative to their length (de Groot et al., 2012a). However, as ~6 and ~5.5 kb sgmRNAs from which the p20 nucleoprotein and gp116/gp64 envelope glycoproteins are translated, respectively, are transcribed in similar relative abundance (Cowley et al., 2002a; Sittidilokratna et al., 2008), okaviruses appear to have no need to order these genes similarly to coronaviruses or arteriviruses (de Groot et al., 2012a,b;

Faaberg et al., 2012). The mesoniviruses have accommodated this need by having the nucleoprotein and S glycoprotein translated from the same sgmRNA (Lauber et al., 2012). Similar to the mosquito mesoniviruses, the okaviruses also have no gene-encoding a membrane (M) protein integral to the virion structures formed by the vertebrate nidoviruses. Moreover, the presence of only two major genes (ORF2 and ORF3) in addition to the ORF1a/1b gene appears to have allowed okaviruses to preserve use of a sgmRNA transcription mechanism like that used for all but the longest sgmRNA of toroviruses (Snijder et al., 1990; Smits et al., 2005; Van Vliet et al., 2002) and less complex than that used by viruses in the *Coronaviridae* and *Arteriviridae*, and appear to have acquired greater gene numbers to accommodate replication in more highly evolved hosts (de Groot et al., 2012a,b; Faaberg et al., 2012). In addition, YHV particles comprise three structural proteins, a p20 (20–22 kDa) nucleoprotein that binds viral RNA to form the nucleocapsid and two envelope glycoproteins, gp116 (110–135 kDa) and gp64 (63–67 kDa), that form the envelope spikes visible on virions and are utilized in cell attachment and entry (Cowley and Walker, 2002; Cowley et al., 2004a; Nadala et al., 1997b; Jitrapakdee et al., 2003; Sittidilokratna et al., 2006; Soowannayan et al., 2003, 2011; Fig. 32.4). In addition to p20-specific antibodies binding to precursor and virion nucleocapsids (Cowley et al., 2004a; Sittidilokratna et al., 2006), RNA binding analyses using recombinant proteins expressed to various portions of the p20 coding sequence as well as to a synthetic p20 peptide sequence have localized its RNA binding domain to an N-terminal proline/arginine-rich sequence (Met^{11}-Ile^{29}) (Soowannayan et al., 2011). The mature gp116 and gp64 glycoproteins originate from posttranslational cleavage of the pp3 polyprotein encoded by the ORF3 gene (Cowley and Walker, 2002; Jitrapakdee et al., 2003; Soowannayan et al., 2011). Of the six predicted TM domains in pp3, TM3 and TM5 appear to act as signal peptidase type 1-like sites (Fig. 32.4). Cleavage at the C-terminal side of these sites generates the N-terminus of gp116 anchored to the lipid membrane by TM4 and TM5, and the N-terminus of gp64 anchored by TM6. The fate and function of the 25.2 kDa N-terminal pp3 fragment, which contains three TM domains and a N-linked glycosylation site in its predicted ectodomain, have yet to be identified. Of the potential N-linked glycosylation sites in gp116 and gp64, six in gp116 and three in gp64 have been identified to possess mannose-rich glycans, with those in gp116 also possessing *N*-acetylgalactosamine and *N*-acetylglucosamine terminal-type sugars (Soowannayan et al., 2011). Injection of shrimp with tunicamycin to inhibit N-linked glycosylation prior to YHV challenge results in reduced viral RNA and protein levels in hemolymph, numbers of mature virions in cells and mortality rate, confirming that the gp116 and gp64 glycosylation process is critical to YHV replication and thus disease (Soowannayan et al., 2013). In variants of YHV1 (YHV1b) as well as a potential recombinant virus derived from ancestral YHV1b and YHV5 genotypes, a 54 amino acid deletion occurs at the N-terminus of gp116 just downstream of the pp3 TM3 cleavage site, but this deletion appears to have little if any impact on the pathogenic potential of these variants relative to YHV1a (Sittidilokratna et al., 2009; Gangnonngiw et al., 2009; Senapin et al., 2010).

32.3.1.4 Genotypic Variants and Recombinant Viruses

Initial comparisons of three ORF1b gene sequences of GAV from Australia with those reported or determined for YHV samples from Thailand identified 83.0% identity in a 135-nt region (Wongteerasupaya et al., 1997), 80.9% identity in an adjoining 1068-nt region (Tang and Lightner, 1999) and 85.1% identity in a downstream 577-nt region including the C-terminus of the Zn-HEL motif (Cowley et al., 1999, 2000b). These data showed YHV and GAV to be genotypic variants of a YHV complex and were assigned to genotypes YHV1 and YHV2, respectively (Walker et al., 2001; Wijegoonawardane et al., 2008a). Moreover, recent reanalysis of the 577-nt YHV sequence originated from healthy *P. monodon* broodstock sampled from a hatchery in Thailand (Cowley et al., 1999; J.A. Cowley, unpublished) has revealed it to be related mostly closely to YHV genotype 5 (YHV5) strains (Wijegoonawardane et al., 2008a; Gangnonngiw et al., 2009) than the virulent YHV1 genotype that was only type known to exist at the time. Based on a 423-nt ORF1b gene sequence amplified using a commercial YHV/GAV IQ2000 PCR test (Tang and Lightner, 1999; Cowley et al., 2004b) displaying 92.7% and 79.0% nt identity, respectively, to the reference GAV and YHV genome sequences, a third unique YHV genotype (YHV3) was identified in healthy wild *P. monodon* broodstock originating from the Andaman Sea in southern Thailand (Soowannayan et al., 2003).

To identify whether other YHV genotypes might exist in geographically isolated populations of *P. monodon*, a consensus nested (n)PCR was used to amplify an ORF1b gene sequence from large numbers of juveniles, broodstock and postlarvae (PL) sampled during the early 2000s either from YHD outbreaks or from hatcheries in countries distributed widely across the natural Indo-Pacific distribution range of this species from Mozambique in eastern Africa to Fiji in the South Pacific (Wijegoonawardane et al., 2008a). Phylogenetic analysis of the 671-nt sequence determined for 57 strains, including the reference YHV1 and GAV (=YHV2) strains, clustered the viruses into six discrete clades designated as genotypes 1-6 (YHV1-YHV6) (Fig. 32.5). YHV1 strains were associated solely with YHD episodes in Thailand and YHV2, YHV4 and YHV6 were the only genotypes detected in *P. monodon* from eastern Australia, India

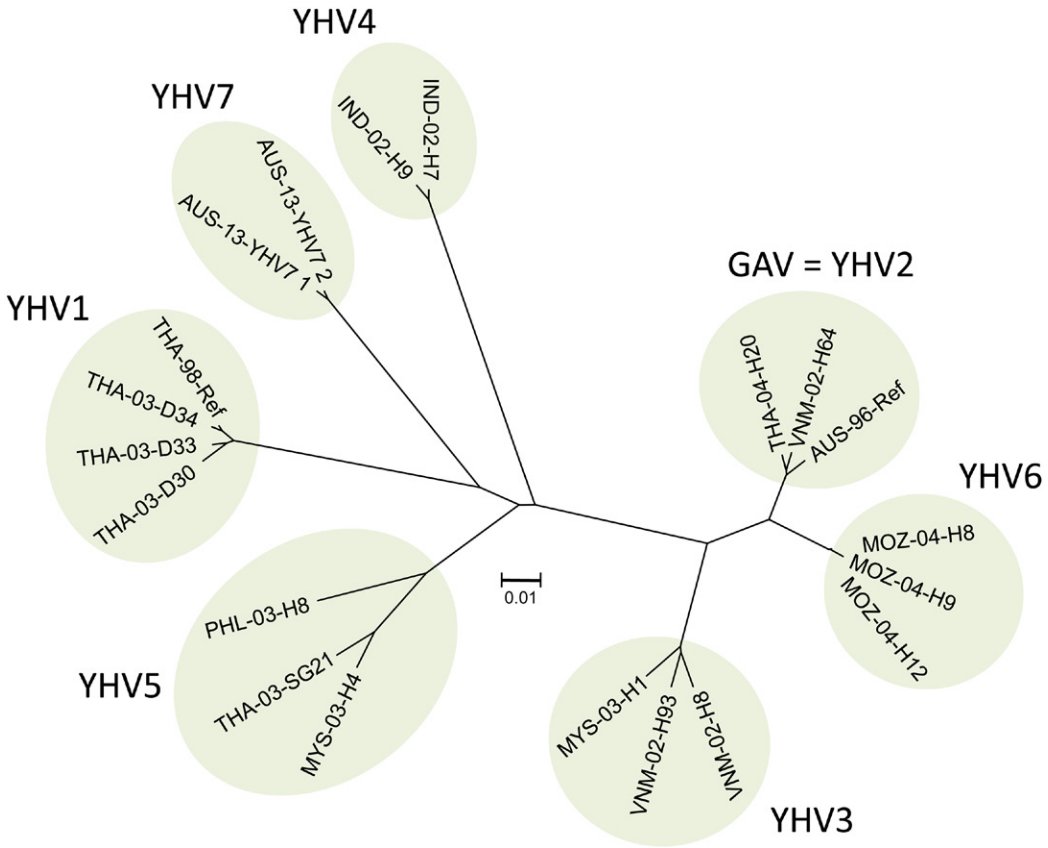

FIGURE 32.5 Phylogenetic analysis of a 646-nt sequence including the 3′ region of the Zn-HEL domain of the ORF1b gene showing clustering and the relative genetic divergence of okaviruses representative of YHV genotypes 1–6 (Wijegoonawardane et al., 2008a) and YHV7 (Mohr et al., 2015). (Bar length = 1% nt difference).

and Mozambique, respectively. Together with some country-specific inter-genotype branches also being evident, the genotype-country associations support the hypothesis of YHV existing as a complex of different genotypes that evolved in isolated shrimp populations, with intensive aquaculture and loosely regulated movements of wild *P. monodon* broodstock in Southeast Asia likely contributing to the mixed genotypes detected in countries like Vietnam (Walker et al., 2001; Wijegoonawardane et al., 2008a).

In support of the YHV complex hypothesis, sequence comparisons of ORF1b gene regions have confirmed the existence of a unique seventh genotype (YHV7) in *P. monodon* originating from the remote Joseph Bonaparte Gulf in northern Australia (Mohr et al., 2015, Fig. 32.5), and based on another ORF1b gene sequence being found to be most closely related (87.6% nt identify) to reference YHV1 sequences (Tang and Lightner, 1999; Sittidilokratna et al., 2002), a unique eighth genotype (YHV8) has been identified in *Penaeus chinensis* farmed in China's Hebei Province (Liu et al., 2014; Mohr et al., 2015).

In addition to YHV existing as diverse genotypes, phylogenetic comparisons of ORF1b and ORF3 gene sequences identified 10 putative recombinant viruses among 28 strains selected to be representative of the YHV-YHV6 genotypes from the different Indo-Pacific regions (Wijegoonawardane et al., 2009). The recombinants originated from Vietnam, Taiwan, Indonesia, Malaysia and the Philippines and represented ORF1b:ORF3 genotype permutations 3:2, 3:5, 5:2 and 5:3, with the 3:2 permutation detected in 6 of the 10 viruses (Fig. 32.6). Maximum-likelihood breakpoint analysis of the ~4.6 kb sequence joining the ORF1b-ORF3 gene regions of representative recombinant viruses from Indonesia (3:2), Vietnam (3:5) and the Philippines (5:2) localized the crossover sites to an identical position just upstream of the ORF1a gene-stop codon in the Indonesian and Vietnamese recombinants and to a position 12-nt further upstream in the recombinant from the Philippines (Wijegoonawardane et al., 2009). The ability of virus recombinants to be generated at high frequency at this crossover hotspot was confirmed by PCR analysis of progeny viruses produced in shrimp co-infected with YHV1 and GAV (Wijegoonawardane et al., 2009). These findings together with reports of other naturally occurring recombinant

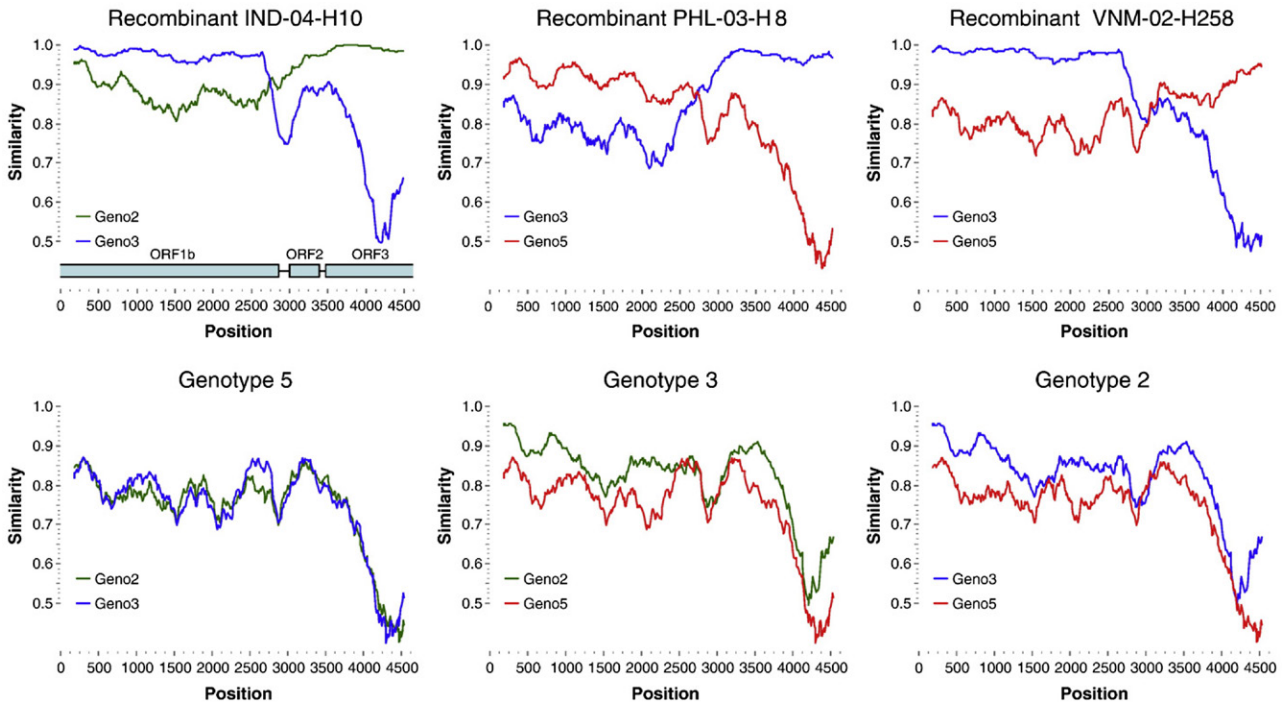

FIGURE 32.6 SimPlot analysis of YHV recombinants IDN-04-H10, VNM-02-H258 and PHL-03-H8 derived from representative parental genotypes 2, 3 and 5 showing the recombination crossover point located near to the ORF1b gene 3′-terminus. Pair-wise divergence between the recombinant and each parental virus or between different genotypes over the ~4.6 kb ORF1b-ORF3 region employed a 350 nt sliding window analyzed at 10 nt intervals and the default Kimura (two-parameter) Distance Model. Percentage identities at each analysis point were plotted on a line chart. The relative positions of the ORF1, ORF2 and ORF3 genes are shown in the first panel (Wijegoonawardane et al., 2009).

YHV strains (Gangnonngiw et al., 2009) indicate that genetic exchange amongst genotypes is occurring commonly in *P. monodon* in southeast Asia, which has profound implications for genotype assignment, disease diagnosis and the potential generation of viruses with increased pathogenic potential.

32.3.1.5 Distribution and Host Range

Within a decade of YHD being discovered in Thailand (Limsuwan, 1991; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993), disease symptoms consistent with acute YHV infection had been reported in most Asian countries with substantial *P. monodon* aquaculture industries, including China, Indonesia, Malaysia, the Philippines, Taiwan, Vietnam, India and Sri Lanka (Albaladejo et al., 1998; Natividad et al., 1999, 2002; Mohan et al., 1998; Wang and Chang, 2000; Walker et al., 2001; Siriwardena, 2000; Subasinghe et al., 2000; Rukyani, 2000; Khoa et al., 2000; Yang et al., 2000). Viruses morphologically indistinguishable from YHV were also detected in Kuruma shrimp (*Penaeus japonicus*) farmed in Taiwan (Wang et al., 1996) and, although described as GAV, in healthy and diseased *P. monodon* farmed in Australia (Spann et al., 1995, 1997; Cowley et al., 1999; Callinan et al., 2003; Callinan and Jiang, 2003; Munro et al., 2011) as well as in wild-caught *P. monodon* broodstock sampled from hatcheries (Cowley et al., 2000b; Walker, 2000; Walker et al., 2001).

In southeast Asia, YHV has been detected in brackish water crustacean species, such as mysid shrimp (*Palaemon styliferus*) and krill (*Ascetes* sp.), as well as shrimp species such as the white banana prawn (*Penaeus merguiensis*) and red endeavor prawn (*Metapenaeus ensis*) that commonly co-inhabit aquaculture ponds growing *P. monodon* (Flegel et al., 1995a, 1997a). Although *P. merguiensis* and *M. ensis* can develop disease and die following experimental challenge (Chantanachookin et al., 1993; Flegel et al., 1995a), these species appear more resilient to developing acute YHV infection in commercial ponds of *P. monodon* dying due to YHD. Similarly in Australia, GAV has been detected at low infection loads in Brown tiger shrimp (*Penaeus esculentus*) inhabiting *P. monodon* ponds affected by GAV disease (Walker et al., 2001). However, when challenged experimentally, *P. esculentus* as well as *P. merguiensis* and *P. japonicus* are susceptible to GAV disease and mortality, although in *P. japonicus*, disease susceptibility was lower in >20 g shrimp compared to 6–13 g

shrimp (Spann et al., 2000, 2003). Following injection challenge, Barred estuarine shrimp (*Palaemon serrifer*), Pacific blue prawn (*Palaemon styliferus*) and the freshwater prawn *Macrobrachium sintangense* are also susceptible to developing mild YHV symptoms that do not necessarily progress to cause mortality, while *Macrobrachium rosenbergii* and *Macrobrachium lanchesteri* are highly refractive to infection, the reasons for which remain to be determined (Longyant et al., 2005). YHV infection in the absence of disease has similarly been induced experimentally in Jinga shrimp (*Metapenaeus affinis*) and the Yellow prawn (*Metapenaeus brevicornis*), but not in any of 16 different crab species challenged by injection (Longyant et al., 2006). Australian red claw crayfish (*Cherax quadricarinatus*) also appear capable of supporting low-level YHV infection following experimental challenge (Soowannayan et al., 2015).

Among shrimp species indigenous to the Americas, such as *L. vannamei* and blue shrimp (*Penaeus stylirostris*) (Lightner et al., 1998; Lu et al., 1994, 1997) as well as white shrimp (*Litopenaeus setiferus*), brown shrimp (*Penaeus aztecus*) and northern pink shrimp (*Penaeus duorarum*) (Lightner et al., 1998), juveniles but not PL stages up to PL20 develop systematic lethal YHV infections when fed carcasses of diseased shrimp (Lightner, 1996). As in these species, *P. monodon* only become susceptible to YHD beyond approximately PL15 (Khongpradit et al., 1995). Injection challenge of Daggerblade grass shrimp (*Palaemonetes pugio*) and the blue crab *Callinectes sapidus* have shown these species to also be susceptible to YHV infection (Ma et al., 2009).

Based on the potential susceptibility of western hemisphere shrimp species, the discovery of infectious YHV together with white-spot syndrome virus (WSSV) in frozen, uncooked commodity shrimp imported into the United States from Asia (Nunan et al., 1998; Durand et al., 2000), and the occurrence of severe LO histopathology thought at the time to be exclusively indicative of YHD in farmed shrimp sampled during outbreaks of white-spot disease (WSD) in the late 1990s, it was speculated that YHV had been introduced into US shrimp farms together with WSSV (Lightner, 1996; Lightner et al., 1998; Lightner and Redman, 1998). However, its presence could not be substantiated by other molecular diagnostic methods, and it was discovered subsequently that in shrimp with severe WSD, LO histopathology can be confused for that caused by acute YHV infection (Pantoja and Lightner, 2003; Lightner, 2011).

Despite the erroneous early reports of YHV occurring in US shrimp farms, a virus consistent in genome sequence to YHV1 was identified subsequently in *L. vannamei* and *P. stylirostris* farmed in northwest Mexico from at least 1999 (de la Rosa-Vélez et al., 2006; Sánchez-Barajas et al., 2009), wild *P. stylirostris* sampled in 2003 from a Gulf of California region nearby to extensive shrimp farms (Castro-Longoria et al., 2008) and *L. vannamei* sampled from low-salinity inland aquaculture enterprises further south in the state of Colima (Sánchez-Barajas et al., 2009). In none of these detections was YHV associated with disease, suggesting it might be a low-virulence strain. Contrary to this assumption, however, *L. vannamei* injected with a high dose of inoculum prepared from healthy YHV-infected *L. vannamei* (YHVLv) developed acute infection resulting in 50% accumulated mortality within 14 days (de la Rosa-Vélez et al., 2006; Cedano-Thomas et al., 2010). Moreover, injection challenge of *L. vannamei* with the Mexican YHV1 strain identified them to be susceptible to disease and mortality and particularly so at seawater salinity extremes (5 and 40 ppt) sometimes occurring in farms along the Mexican Pacific coast (Navarro-Nava et al., 2011).

While the introduction of *P. monodon* into non-native regions might explain how YHV has been introduced into the Gulf of Mexico, it has only been identified and become established as an invasive species in Atlantic coastal regions of the Americas, including the Caribbean (Gómez-Lemos and Campos, 2008), Venezuela (Aguado and Sayegh, 2007) and Brazil (Coelho et al., 2001; Cintra et al., 2011) and in the South Atlantic Bight and Gulf of Mexico regions of the United States (Fuller et al., 2014).

Unlike in Mexico, YHV has been established as a problematic disease of *L. vannamei* since its introduction in the early-2000s and subsequent rapid expansion to become the primary aquaculture species in Thailand and other countries in southeast Asia (Anantasomboon et al., 2007; Senapin et al., 2010). To evaluate the potential for YHV to be translocated and become established in different regions, a comprehensive list of crustacean species capable of carrying infection has been compiled from a meta-analysis of available literature (Stentiford et al., 2009).

32.3.1.6 Transmission

YHV infection can be transmitted horizontally to susceptible crustacean species by several natural challenge routes. These include cannibalization of moribund live shrimp, carcasses or small pieces of flesh from diseased shrimp, cohabitation with infected shrimp and exposure to waterborne virus particles (Flegel et al., 1995a; Lightner, 1996; Lightner et al., 1998; Lu et al., 1997; Walker et al., 2001; Hamano et al., 2015; Soowannayan et al., 2015). Shrimp ingestion of carrier species such as *Acetes* sp. and *Palaemon styliferus* can also transmit YHV infection and disease (Flegel et al., 1995b, 1997a). However, such oral transmission routes only appear to be effective in establishing productive infections and disease in shrimp from late PL life stages (PL17-PL20) onward (Flegel et al., 1995b; Khongpradit et al., 1995; Lightner et al., 1998). Muscle

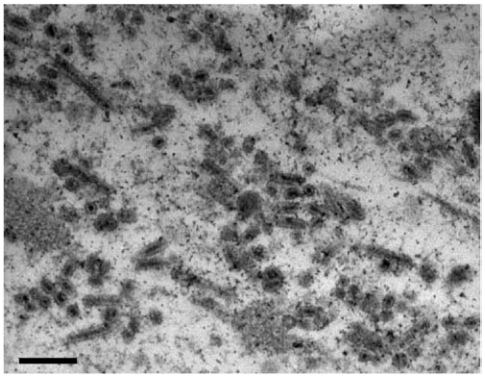

FIGURE 32.7 Transmission electron micrograph showing GAV particles within the seminal fluid of a spermatophore sampled from an adult male *Penaeus monodon* (Cowley et al., 2002b). Bar = 200 nm.

injection of hemolymph or filtered cephalothorax tissue homogenates from diseased shrimp is also very effective at transmitting infection at a defined dose and thus has been used extensively as an experimental challenge method (Boonyaratpalin et al., 1993; Spann et al., 1997; Longyant et al., 2006).

YHV transmission from broodstock to progeny in hatcheries was speculated early on to be the source of infection leading to widespread disease in *P. monodon* being farmed in Thailand (Chantanachookin et al., 1993; Pasharawipas et al., 1997). Initially this assumption was poorly supported by data indicating a low prevalence of YHV infection in wild-caught broodstock sampled from Thai hatcheries (Flegel et al., 1997b) and difficulty in detecting YHV in broodstock reproductive organs. However, accumulated subsequent data now strongly supports vertical transmission being a primary route through which infection is perpetuated in wild populations and enters aquaculture ponds. Such data includes the widespread detection of YHV at high prevalence among *P. monodon* broodstock and PL sampled from hatcheries (Phan, 2001; Cowley et al., 2000b, 2002b; Spann et al., 1995; Walker et al., 2001; Wijegoonawardane et al., 2008a) as well as juveniles sampled from farm ponds (Spann et al., 1995; Pasharawipas et al., 1997).

ISH and PCR data have also provided evidence for GAV occurring in male *P. monodon* spermatophores and spermatophore secretions as well as female ovaries (Walker et al., 2001). The presence of mature virions in spermatophore seminal fluid but not in sperm per se has been substantiated by TEM and nested (n)PCR and nPCR has also confirmed the presence of low GAV RNA amounts associated with unwashed eggs, but not newly hatched nauplii (Cowley et al., 2002a; Fig. 32.7). In *P. monodon*, egg activation and fertilization requires a multitude of egg surface alterations that initiate immediately upon contact with seawater (Pongtippatee-Taweepreda et al., 2004). Although the mechanism by which GAV/YHV are transmitted vertically remains unknown, opportunities for virus entry into eggs might present themselves during the extraordinary egg surface ultrastructural changes that occur as a result of the cortical reaction and egg-sperm interaction processes activated within the first few seconds after release from the female (Pongtippatee-Taweepreda et al., 2004).

To directly examine the capacity of either gender of *P. monodon* to transmit GAV infection vertically, female broodstock captured from one location in north Queensland were inseminated artificially with the spermatophores of males captured at a disparate location. GAV strains present as a subclinical infection in each parent as well as in progeny batches reared to an early PL stage could thus be identified clearly by sequence analysis of a more variable region of the ORF3 gene (Cowley et al., 2002a). GAV strain sequences identified in progeny demonstrated that virus could be transmitted from the male or female parent or both, with the predominant strain detected in progeny generally correlating with that existent in either parent at the highest infection load.

32.3.1.7 Diagnostic Methods

32.3.1.7.1 Gross Signs and Light and Electron Microscopy

Gross signs such as erratic swimming and shrimp congregating at pond edges with a yellowed cephalothorax, bleaching/reddening general body appearance and pink discolored gills and appendage extremities can alert to disease caused by YHV. For rapid presumptive diagnosis of YHD, gill squashes or hemocyte smears from moribund shrimp can be observed either directly by phase-contrast microscopy or by normal light microscopy after Wright-Giemsa staining (Nash et al., 1992; Lightner, 1996; FAO Fisheries Technical Paper 402/2 2001). Gill filaments or subcuticular tissue fixed in Davidson's

AFA fixative and stained with H&E can also be observed by light microscopy without wax embedding and tissue sectioning (Lightner, 1996; OIE, 2014). Pathologies predictive of YHD include evidence of nuclear pyknosis and karyorrhexis as well as medium-to-large numbers of evenly stained and deeply basophilic spherical cytoplasmic inclusions ≤2 mm in diameter in cells of mesodermal and ectodermal origins (Lightner, 1996; Flegel et al., 1997a). Histology on wax-embedded cephalothorax tissue sections stained with H&E will typically delineate the nature of the cellular pathologies more precisely as well as assist in determining YHD severity based on the nature and numbers of LO spheroids (Chantanachookin et al., 1993; Boonyaratpalin et al., 1993; Nash et al., 1992; Lightner and Redman, 1998; Spann et al., 1997; Flegel et al., 1995b, 1997a; OIE, 2014; Fig. 32.2).

Despite the diagnostic value of histology, care must be taken when interpreting data because pathology evident in the LO of shrimp with severe WSD can be confused for that caused by YHV (Pantoja and Lightner, 2003; Lightner, 2011). Histology alone is thus insufficient to diagnose YHV infection or disease with confidence unless supported by other methods. TEM on LO or gill tissue can also be used to confirm the presence of variable length (80–450 nm), filamentous nucleocapsid precursors and rod-shaped enveloped particles characteristic of YHV/GAV (Chantanachookin et al., 1993; Boonyaratpalin et al., 1993; Spann et al., 1995, 1997; Fig. 32.3). Like histology, however, TEM is time consuming, requires specialized equipment and, for surveillance and diagnostic purposes, has generally been superseded by more rapid molecular diagnostic methods.

32.3.1.7.2 Shrimp Bioassay, Cell Culture and Hemagglutination Assays

Primary cultures of shrimp LO cells support YHV replication (Chen and Kou, 1989; Chen and Wang, 1999), and assays based on cytopathic effects and cell monolayer disintegration have been used to quantify virus infectivity (Lu et al., 1995a,b; Assavalapsakul et al., 2003). However, because no continuous cell lines are yet available to easily culture YHV, bioassay in naïve shrimp remains the most common means of confirming the presence of infectious YHV (Lightner et al., 1998; Longyant et al., 2006; Lu et al., 1995b; Spann et al., 1997; McColl et al., 2004). While culture methods are useful in amplifying infectious YHV, definitive diagnosis relies on TEM to detect virus nucleocapsids and/or virions or on molecular methods to detect YHV protein or RNA (OIE, 2014).

Hemagglutination (HA) of chicken erythrocytes by YHV (Nadala et al., 1997b) or GAV particles (Munro and Owens, 2005, 2006) in titrations of shrimp hemolymph or gill tissue extracts has also been investigated as a potential pond-side diagnostic method. While the HA method is not particularly sensitive and provides no specificity, there are circumstances in which it could be applied to guide disease management decisions at hatcheries and farms.

32.3.1.7.3 Molecular Diagnostic Methods—Viral Proteins

To diagnose YHV infection unequivocally, various molecular methods have been developed to detect either viral proteins or viral RNA. Methods to detect YHV proteins have employed polyclonal antiserum prepared to purified whole virus (Lu et al., 1996; Munro and Owens, 2007a), monoclonal antibodies (MAbs) generated to purified whole virus and selected for specificity to the gp116 and gp64 envelope glycoproteins or the p20 nucleoprotein (Sithigorngul et al., 2000, 2002; Munro and Owens, 2007a; Sittidilokratna et al., 2006), monospecific antiserum prepared to a p20 peptide sequence or to a p20-fusion protein expressed in *Escherichia coli* (Cowley et al., 2004a) and a recombinant single-chain variable fragment antibody to gp116 expressed in *E. coli* and constructed by PCR from a hybridoma clone generated to purified YHV particles (Intorasoot et al., 2007). The range of antibody-based methods described for detecting YHV or GAV proteins includes dot-blot nitrocellulose enzyme immunoassays (DB-NC-EIA) (Intorasoot et al., 2007; Lu et al., 1996; Loh et al., 1998; Nadala and Loh, 2000; Sithigorngul et al., 2000) and Western blots (Cowley et al., 2004a; Intorasoot et al., 2007; Nadala et al., 1997a; Sithigorngul et al., 2000; Soowannayan et al., 2003; OIE, 2014), a solid-phase noncompetitive enzyme-linked immunosorbent assay (ELISA) (Intorasoot et al., 2007), as well as a sandwich ELISA employing polyclonal chicken antibodies raised against GAV proteins for protein capture and a p20 nucleoprotein-specified MAb for GAV protein detection (Munro and Owens, 2006, 2007a), immunohistochemistry (IHC) for detecting infected cells and tissues in histological sections (Sithigorngul et al., 2000; Soowannayan et al., 2003) and immunoelectron microscopy for detecting viral nucleocapsids and virions in ultrathin tissue sections or protein components of purified and negatively stained virions (Cowley et al., 2004a; Soowannayan et al., 2003).

Of these methods, the DB-NC-EIA protocol, using gill tissue homogenates, provides a rapid and simple potential method to diagnose YHV infection (Nadala and Loh, 2000). However, an even more simple-to-implement lateral-flow immunochromatographic test strip (ICTS) method has been developed for the pond-side diagnosis that employs either shrimp hemolymph or gill/appendage tissue extracts as a protein source, a polyclonal rabbit antiserum prepared to a recombinant YHV p20 protein to capture this viral protein and a p20-specific MAb (Y19) conjugated to colloidal gold as a detector

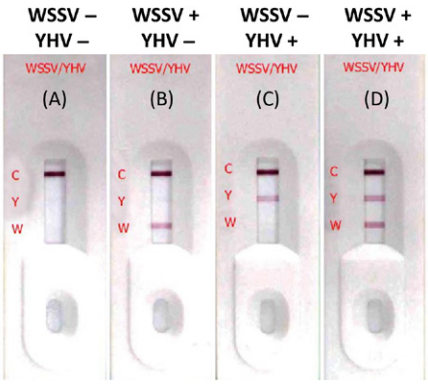

FIGURE 32.8 Dual immunochromatographic strip test showing bands indicating the detection of YHV (Y) and/or WSSV (W) using pleopod tissue homogenates from (A) uninfected shrimp, (B) WSSV-infected shrimp, (C) YHV-infected shrimp and (D) a combination of WSSV- and YHV-infected shrimp, C = control line confirming test performance (Sithigorngul et al., 2011).

antibody (Sithigorngul et al., 2007; Chaivisuthangkura et al., 2014). An ICTS method to co-detect YHV and WSSV in the same strip has also been described (Sithigorngul et al., 2011, Fig. 32.8).

Antibody-based diagnostic methods can offer advantages in speed, simplicity and cost in detecting GAV/YHV compared with molecular methods to detect viral RNA such as PCR (Munro and Owens, 2006; Sithigorngul et al., 2007). However, their analytical sensitivity is generally lower. For example, the lateral-flow ICTS developed to detect YHV is approximately 500-fold less sensitive in detecting YHV p20 protein compared with a one-step PCR test designed to detect YHV RNA (Wongteerasupaya et al., 1997; Sithigorngul et al., 2007). YHV detection sensitivity was reduced further in an ICTS designed to co-detect YHV and WSSV (Sithigorngul et al., 2011). Antibody specificity also needs to be considered. For example, while polyclonal antiserum to recombinant GAV p20 protein cross-detects the YHV p20 protein (Cowley et al., 2004a) and MAbs to the YHV p20 nucleoprotein (Y19) and gp64 glycoprotein (Y18) cross-detect the cognate GAV proteins, MAb V-3-2B specific to the YHV gp116 glycoprotein does not detect gp116 of GAV (Sithigorngul et al., 2000, 2007; Soowannayan et al., 2003). Despite these limitations, protein-based virus detection methods provide practical and relatively cheap diagnostic options for managing YHV infection and disease risks at shrimp hatcheries and farms.

32.3.1.7.4 Molecular Diagnostic Methods—Viral RNA

Molecular methods designed to detect YHV RNA generally provide the highest analytical sensitivity. ISH using digoxigenin (DIG)-labeled DNA or RNA probes is useful, like the IHC method, in associating YHV with specific tissue types and histopathology and thus in diagnosing infection as well as assessing disease severity (Spann et al., 2003; Tang and Lightner, 1999; Tang et al., 2002; OIE, 2014). However, due to its complexity, ISH has been used primarily as a research tool to investigate YHV infection processes and disease progression.

Numerous PCR tests have been reported for either YHV and GAV diagnosis or research (Cowley et al., 2000b, 2004b; Wongteerasupaya et al., 1997; Wijegoonawardane et al., 2008b, 2010; Dhar et al., 2002; Mouillesseaux et al., 2003; de la Vega et al., 2004; Tang and Lightner, 1999; Aranguren et al., 2012; de la Rosa-Vélez et al., 2006). Of these, the one-step RT-PCR first reported for YHV (Wongteerasupaya et al., 1997), a two-step multiplexed nPCR utilizing PCR primers to amplify both YHV and GAV and multiplexed virus-specific nPCR primers to amplify and differentiate YHV from GAV based on amplicon size (Cowley et al., 2004b) and a two-step multiplexed PCR test utilizing combined degenerate primers designed to have broad specificity for YHV genotypes 1–6 and to assist in detecting other genotypic variants that might exist (Wijegoonawardane et al., 2008b) are endorsed for infection/disease diagnosis in the Yellowhead Disease chapter of the OIE Manual of Diagnostic Tests for Aquatic Animals 2014 (OIE, 2014). Each of these tests targets regions in the okavirus ORF1b gene, and are likely to now require revision or caveats to their applicability as new YHV genotypes are discovered such as YHV7 (Mohr et al., 2015) and YHV 8 (Liu et al., 2014) because they were designed to sequences of YHV genotypes known at the time.

The inclusion of a nPCR step can improve YHV/GAV RNA detection efficiency up to 100-fold or more compared with one-step PCR (Cowley et al., 2000b, 2004b). Various real-time RT-PCR tests that provide viral RNA detection sensitivities further improved compared with nPCR as well as improved throughput capacity and linear dynamic range over which viral RNA loads and thus infection severity can be quantified accurately, have been described for detecting

YHV, GAV or YHV genotypes 1–6 that utilize either SYBR-Green chemistry (Dhar et al., 2002; Mouillesseaux et al., 2003) or a TaqMan probe to generate fluorescence (de la Vega et al., 2004; Aranguren et al., 2012; Panichareon et al., 2011; Wijegoonawardane et al., 2010). The detection limits of these real-time PCR tests can approach a single RNA copy. A modified version of the nPCR designed to differentiate YHV from GAV (Cowley et al., 2004b) is also available commercially (IQ2000 YHV/GAV PCR test) together with commercial real-time SYBR-Green PCR and RT-LAMP tests for YHV (GeneReach Biotechnology Corp.).

In addition to the PCR designed to detect YHV and/or GAV (Cowley et al., 2004b), a test employing multiplexed PCR primer sets to detect any of six different shrimp viruses and a TaqMan real-time PCR employing multiplexed PCR primers and probes labeled with different fluorophores to co-detect and quantify YHV and Taura syndrome virus (TSV) have been described (Khawsak et al., 2008; Aranguren et al., 2012). As an alternative to gel electrophoresis to detect and confirm the expected size of PCR amplicons, hybridization to biotin-labeled virus-specific oligonucleotides followed by avidin capture and detection using anti-DIG-peroxidase has been used for ELISA-based visualization of DNA amplified in the presence of DIG-dUTP (Khawsak et al., 2008). Hemolymph spotted onto specialized filter papers such as IsoCode (Schleicher and Schuell Inc.) has proved useful for field collection and preservation of YHV RNA for subsequent RT-PCR analysis (Kiatpathomchai et al., 2004). The method avoids the complexities of tissue dissection and collection into liquid-based preservatives, and/or the need to refrigerate or freeze tissues, and can maintain RNA integrity in samples stored for periods of up to 9 months at ambient temperature (Kiatpathomchai et al., 2004). Hemolymph stored as a liquid at either 4°C of 25°C for up to 5 days has also been found show little sensitivity drop-off in real-time PCR detection of YHV RNA (Ma et al., 2008).

While RNA detection by PCR methods can provide exquisite sensitivity, most tests employ primers designed to genome sequences determined for a single strain/genotype and with no regard for them targeting gene regions constrained by their requirement to encode more highly conserved functional domains within YHV proteins. Based on these shortcomings in combination with YHCVs existing as a complex of genotypes that appear to have evolved in geographically isolated populations of *P. monodon* (Walker et al., 2001; Soowannayan et al., 2003; Wijegoonawardane et al., 2008a), extreme care is needed in interpreting data generated by any one particular PCR test. For example, the original RT-PCR test described to detect YHV (Wongteerasupaya et al., 1997) has retained good specificity for the YHV1 genotype due to the 3′-terminus of 10R PCR primer serendipitously targeting a sequence that has diverged in other identified genotypes (Wijegoonawardane et al., 2008b). However, other tests, including the RT-nPCR designed to differentiate YHV1 from GAV (Cowley et al., 2004b) and the TaqMan real-time PCR to detect GAV (de la Vega et al., 2004), have been identified to cross-detect other genotypes at variable efficiency depending on their sequence relatedness to GAV in the genome regions targeted by the PCR primers (Wijegoonawardane et al., 2008b; J.A. Cowley et al., unpublished).

To detect YHV genotypes 1–6 as well as other genotypes that might exist, consensus RT-nPCR and TaqMan real-time RT-PCR tests have been described that use degenerate PCR primers designed to accommodate genotype sequence variations identified in an ORF1b gene region (Wijegoonawardane et al., 2008a,b, 2010). Phylogenetic analyses of sequences amplified from this and another ORF1b gene region have proved useful in assigning YHV genotypes and in identifying new genotypes such as YHV7 found recently in *P. monodon* originating from the previously unstudied Joseph Bonaparte Gulf region of northern Australia (Mohr et al., 2015).

While phylogenetic comparisons of ORF1b gene sequences have proved useful in classifying YHV genotypes, PCR tests designed to amplify sequences within the ORF2/ORF3 genes encoding the virus structural proteins have proved useful in identifying YHV1 subtypes YHV1a and YHV1b (Sittidilokratna et al., 2008, 2009; Wijegoonawardane et al., 2008a) as well as viruses representing recombinants (Wijegoonawardane et al., 2009; Gangnonngiw et al., 2009). Based on the high frequency at which recombinants comprising various permutations of the 5′-ORF1a/1b and the ORF2-3 3′-genome regions of parental YHV genotype 2, 3 and 5 viruses have been detected in *P. monodon* collected from various countries in southeast Asia (Wijegoonawardane et al., 2009), genotype assignments based on ORF1a/1b gene sequences alone need to be viewed with caution, particularly in regions where hatcheries are densely colocated and/or commonly source wild broodstock from diverse locations. In cases when a YHV genotype needs to be defined definitively, it is recommended that regions in both the ORF1a/1b and ORF2/ORF3 genes be amplified, sequenced and compared, as was done to identify the nature of a YHV genotype when first detected in shrimp farmed in Mexico (de la Rosa-Vélez et al., 2006).

As an alternative to PCR, RT-LAMP methods have been described to detect YHV (Mekata et al., 2006, 2009; Jaroenram et al., 2012; Khunthong et al., 2013). A standard RT-LAMP test employing agarose gel electrophoresis to detect the amplified DNA ladder was found to be about 10-fold less sensitive than a commercial IQ2000 YHV/GAV nPCR test (Mekata et al., 2006). In contrast, a real-time RT-LAMP test employing a photometer to quantify turbidity increases in the magnesium pyrophosphate by-product of DNA amplification was found to be about 10-fold more sensitive than the same commercial nPCR test (Mekata et al., 2009). To avoid the need for either gel analysis or a photometer to detect amplified

DNA, a RT-LAMP method modified to amplify biotin-labeled DNA ladder products has also been developed to detect YHV (Khunthong et al., 2013). By hybridizing amplified DNA products to a fluorescein isothiocyanate-labeled DNA oligonucleotide, a simple lateral-flow dip (LFD) stick system was employed to trap and visualize the DNA duplexes as a dark-colored band (Khunthong et al., 2013). A similarly simple tube-based colorimetric method has been described that utilizes gold nanoparticles (AuNP) to bind and detect YHV DNA ladder products amplified by RT-LAMP (Jaroenram et al., 2012). These rapid, specific and highly sensitive methods that only require a constant temperature apparatus and simple LFD stick or tube-based colorimetric detection systems, such as LAMP, offer substantial potential for field diagnosis of YHV. However, due to the complexity of primers required for LAMP, the specificity they provide is likely to limit the applicability of any test to a single genotype.

32.3.1.8 Prevention and Control

32.3.1.8.1 Hatchery and Farm Biosecurity and Management Practices

No commercial treatments are yet available to protect farmed shrimp against YHD. Disease prevention thus relies on the regulatory oversight and surveillance by government quarantine and agricultural agencies (AQIS, 2000; Stentiford et al., 2009; Lightner et al., 1997; McColl et al., 2004), on hatcheries and farms employing robust biosecurity systems that exclude virus entry via water, carrier species, equipment or personnel, as well as infection surveillance in ponds, pond bottom and pond water disinfection systems and farm management practices that avoid exposing shrimp to adverse stressors, such as rapid changes in water pH, pH levels >9 or prolonged periods of low (<2 ppm) dissolved oxygen (Limsuwan, 1996; Lightner, 1999, 2003, 2005; Walker and Mohan, 2009).

32.3.1.8.2 Broodstock Screening and Use of Specific Pathogen-Free or Specific Pathogen-Resistant Broodstock

Since wild shrimp often carry a subclinical YHV or GAV infection (Spann et al., 1995; Pasharawipas et al., 1997; Cowley et al., 2000b; Anantasomboon et al., 2007; Castro-Longoria et al., 2008) and the virus can be transmitted vertically from both male and female broodstock (Cowley et al., 2002b), infected progeny represent a major risk factor in farm disease. PCR screening of hemolymph, gill or pleopod biopsies collected nonsacrificially can provide a means of selecting broodstock that are either free of infection or infected at low levels, posing a lowered risk of virus transmission (Nadala et al., 1997a,b; Cowley et al., 2002b; Pasharawipas et al., 1997). While less ideal, PL batches can also be screened and discarded if deemed to pose an unacceptable disease risk.

Progress made in domesticating and selectively breeding shrimp species of aquaculture importance that are specific pathogen-free (SPF) for YHV and other problematic viruses (Wyban, 1992; Wyban et al., 1992; Lotz, 1992, 1997; Lightner, 2003, 2005; Lightner et al., 2009). However, unlike for infectious hypodermal and hematopoietic necrosis virus (IHHNV) and TSV, in which genetic lines of shrimp have been repeatedly challenged and selected to be specific pathogen-resistant (SPR) of disease caused by these viruses (Tang et al., 2000), as yet only shrimp breeding stocks SPF for YHV are available commercially (Lightner, 2005, 2011). While the farming of virus-free seed stock spawned from SPF *L. vannamei* has assisted in reducing YHV disease impacts in Asian regions that previously farmed *P. monodon* or *P. chinensis* (Lightner, 2011; Lightner et al., 2009), it has not provided a panacea because shrimp remain highly susceptible to YHD and other diseases originating from exogenous sources (Senapin et al., 2010).

In Australia, rearing of domesticated *P. monodon* broodstock over several generations has resulted in profound improvements in production yields due to increased growth rates and survival (Preston et al., 2007, 2010). A strategy employing early rearing of *P. monodon* broodstock in dedicated farm ponds, rather than solely in biosecure facilities, and PCR screening to remove individuals infected with GAV at high loads prior to each breeding cycle has proved useful in establishing breeding populations in which subclinical GAV infection is prevalent, but in which tolerance against GAV-induced disease has developed (J.A. Cowley and M.J. Sellars, unpublished). Disease tolerance among *P. monodon* that have developed a capacity to accommodate YHV/GAV infection in a subclinical state is similar to that observed to have occurred within a few years of YHD emerging in Thailand (Pasharawipas et al., 1997), with some "viral accommodation" process associated with the suppression of cell apoptosis (Flegel and Pasharawipas, 1998; Flegel, 2007; Khanobdee et al., 2002) and/or suppression of YHV gp116 envelope glycoprotein synthesis (Chaivisuthangkura et al., 2008) speculated to be involved. As mortalities in *L. vannamei* exposed *per os* to TSV before challenge with YHV also accumulate more slowly, preexisting infection by some viruses appears able to interfere or compete with YHV infection (Aranguren et al., 2012). In the Americas where subclinical TSV infection occurs commonly in farmed *L. vannamei*, it has been speculated that such infections might have helped prevent YHV infection and disease from becoming established in this region (Aranguren et al., 2012).

32.3.1.8.3 Therapeutic Feed Ingredients

Extracts of various plants, herbs and seaweeds with immunostimulant or antimicrobial activities have been investigated as feed supplements to protect shrimp against viruses including WSSV and YHV (Sivasankar et al., 2015). For example, a feed produced to contain a leaf ethanol extract of the Thai medicinal plant *Clinacanthus nutans* decreased mortalities in shrimp challenged with YHV by water immersion (Direkbusarakom et al., 1998). Leaf extracts of the medicinal herbs *Phyllanthus amarus* and *Phyllanthus urinaria* also neutralize YHV infectivity, but their protective efficacy as a feed supplement was not assessed (Direkbusarakom et al., 1995). An ethanol leaf extract of Guava (*Psidillm guajava*) also neutralizes YHV infectivity, but its addition to feed provides no protection against YHV challenge (Direkbusarakom et al., 1997). While the feeding of such medicinal plant extracts has thus shown promise in protecting shrimp against YHD and other virus diseases, there appears to have been little interest from shrimp feed manufacturers in including such extracts in commercial feed pellets.

When processed and added to shrimp feed pellets, an ingredient produced from flocks of microalgae and bacteria grown in seawater ponds seeded with low-value carbon by-products, such as sugarcane bagasse or rice husks, and patented as Novacq can improve the growth performance of farmed *P. monodon* and *L. vannamei* by 30% and 50%, respectively (Glencross et al., 2012, 2014). Juvenile *P. monodon* fed on pellets containing Novacq have also displayed lowered mortality rates following GAV challenge (Sellars et al., 2015). Thus ingredients like Novacq that will be included in commercial feeds for the primary reason of improving shrimp growth rates and general robustness might provide secondary benefits in reducing farm disease impacts by enhancing shrimp resilience against disease caused by GAV/YHV and other viruses.

32.3.1.8.4 RNA Interference

RNA interference (RNAi) strategies based on the use of short interfering (si)RNA or either long dsRNA or hairpin dsRNA are being explored as a means of preventing diseases caused by most viruses problematic to shrimp aquaculture (Shekhar and Lu, 2009; Sagi et al., 2013). Long dsRNA was identified early to direct a RNAi response more specific than siRNA (Robalino et al., 2004, 2005; Westenberg et al., 2005), presumably through mobilizing the Dicer RNase III dsRNA cleavage enzyme (Chen et al., 2011; Li et al., 2013; Su et al., 2008; Yao et al., 2010) as well as the Argonaut enzyme and other minor components of the RNA-induced silencing complex that mediates RNAi-based gene silencing in shrimp and other organisms (Unajak et al., 2006; Dechklar et al., 2008; Chen et al., 2011). Gene silencing in shrimp mediated by long dsRNA is thus being investigated intensively as a potential therapeutic to combat YHD.

In an initial study using cultures of shrimp LO cells, transfection of in vitro transcribed dsRNA targeted to the YHV ORF1b gene and thus the YHV genomic and antigenomic length RNAs inhibited YHV replication more effectively than dsRNAs targeted to ORF3 gene sequences encoding the gp116 or gp64 glycoproteins (Tirasophon et al., 2005). As okaviruses transcribe 3′-coterminal ORF2 and ORF3 sgmRNAs in greater abundance than genomic-length RNA (Cowley et al., 2002a; Cowley and Walker, 2002; Sittidilokratna et al., 2006), dsRNA targeted to the ORF3 gene would interact with all three RNAs. It is not unexpected, therefore, that dsRNAs targeting the ORF1a/1b gene region of the low-abundance genomic and antigenomic RNAs are more effective in inhibiting YHV replication, and particularly because the genomic-length RNA is responsible for translating the pp1a and pp1ab polyproteins that contain the various enzymes critical for viral RNA transcription and replication.

In *P. monodon* challenged with YHV, an ORF1a/1b gene dsRNA inhibited virus replication specifically when injected up to 12 h post-challenge, and also reduced shrimp mortality when injected earliest at 3 h post-challenge (Tirasophon et al., 2007). Using different methodology, the same ORF1a/1b gene dsRNA injected into hemolymph of *P. monodon* just prior to YHV challenge completed protected the shrimp for 10 days, at which time 90% of noninjected shrimp had died (Yodmuang et al., 2006). Similarly, injection of a hairpin YHV ORF1a/1b gene dsRNA expressed and purified from bacteria into the hemolymph of *L. vannamei* as a therapeutic agent prior to YHV challenge inhibited mortalities over a 16-day period, with no evidence of YHV replication evident by one-step PCR analysis (Assavalapsakul et al., 2009). Consistent with earlier findings, when dsRNA was injected as a prophylactic agent at different times post-challenge, YHV replication levels were suppressed most significantly at the earliest dsRNA injection time points (Assavalapsakul et al., 2009). Injection of an ORF1a/1b gene hairpin dsRNA expressed in bacteria and purified using a cheap and simple process involving phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by RNase A and DNase digestion also suppresses YHV replication and mortalities after *L. vannamei* challenge with YHV (Saksmerprome et al., 2009). Injection of *P. monodon* with a pool of five dsRNAs targeted to different ORF1a/1b gene regions and expressed similarly in bacteria but purified differently, has also proved highly protective against subsequent GAV challenge (Sellars et al., 2011, Fig. 32.9). Moreover, delayed and slowed mortality rates in *P. monodon* challenged by GAV injection has also been provided by injection immediately after challenge of either of two in vitro-transcribed dsRNAs targeted to different ORF1b gene sequences (Oanh et al., 2011).

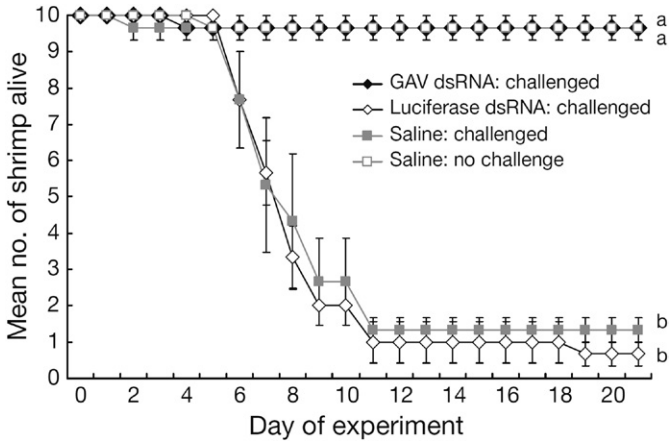

FIGURE 32.9 High survival over time of juvenile *Penaeus monodon* injected with a pool of five dsRNAs targeted to the GAV ORF1b gene, but not with either a nonspecific luciferase dsRNA or saline immediately prior to injection challenge with GAV. Significant differences ($p < 0.001$) are indicated by different letters (Sellars et al., 2011).

In addition to virus-specific dsRNA, dsRNA-mediated RNAi of a *P. monodon* gene (PmYRP65) expressing a protein identified to be a cell-surface receptor for YHV has also been identified to suppress YHV replication in primary LO cell cultures (Assavalapsakul et al., 2006). The same dsRNA injected into shrimp also completely abrogates mortalities following experimental YHV challenge (Assavalapsakul et al., 2014). Muscle injection of shrimp with a dsRNA targeted to another endogenous *P. monodon* gene (PmRab7) encoding a small GTPase has similarly been found to inhibit replication of YHV and WSSV following challenge (Ongvarrasopone et al., 2008). Suppression of its expression might interfere generally with virus entry or the transport of viral proteins into the cytosol because the PmRab7 protein binds to the VP28 envelop glycoprotein of WSSV (Sritunyalucksana et al., 2006) and has been suggested to function in endosomal membrane trafficking (Ongvarrasopone et al., 2008).

The oral delivery of dsRNA expressed in *E. coli* following brief fixation of the bacteria in 0.5% formaldehyde before incorporation into feeds has also been examined for its ability to induce RNAi responses projective against GAV (Sellars et al., 2011) or YHV (Sanitt et al., 2014). When processed into a wet feed, a pool of five dsRNA targeted diverse regions of the GAV ORF1a/1b gene in an attempt to amplify its efficacy did not reduce virus replication or morality rates in shrimp challenged by muscle injection of a minimal lethal dose of GAV (Sellars et al., 2011). In contrast, fixed bacteria containing expressed dsRNA targeted to the Rab7 gene and/or the YHV ORF1a/1b gene and concentrated into small agarose blocks suitable as a feed for juvenile (250–300 mg) *L. vannamei* reduced mortalities following YHV challenge by water immersion, with feeding of the Rab7 dsRNA generating the highest mortality reductions followed by the Rab7 + YHV dsRNA and the YHV dsRNA (Sanitt et al., 2014). Inexpensive and biodegradable polymer nanoparticles derived from chitosan and its quarternized derivative, QCH4, have also been assessed for their ability to bind and deliver YHV ORF1a/1b gene hairpin dsRNA expressed in and purified cheaply from *E. coli* (Saksmerprome et al., 2009; Theerawanitchpan et al., 2012). Based on reduced YHV replication occurring in treated caterpillar (*Spodoptera frugiperda*, Sf9) cells, it was speculated that nanoparticle binding or encapsulation of dsRNA might offer advantages in dsRNA stability and/or delivery efficiency for feed-based dsRNA delivered via the shrimp gut (Theerawanitchpan et al., 2012). Despite these promising data, serious investment in feed-based delivery of dsRNA to prevent YHD via commercial feed pellets seems improbable.

Injection of GAV-infected juvenile *P. monodon* with single or pooled dsRNAs targeted to five different ORF1a/1b gene regions has been identified to reduce GAV infection levels (Sellars et al., 2014). Of the single dsRNAs tested, one targeted to the extreme 5′-end of the GAV genome reduced infection loads most profoundly followed by the dsRNA pool. Injection of the dsRNA pool into GAV-infected female *P. monodon* broodstock has confirmed its ability to reduce virus infection loads without impairing female reproductive capacity (M.J. Sellars et al., unpublished). Thus, if dsRNA injection can be optimized to reduce viral infection loads to levels below which vertical transmission is unlikely to occur, this RNAi strategy might find application in hatcheries reliant on wild *P. monodon* broodstock captured from regions in which GAV or YHV are endemic or in cases when it might be desirable to eliminate viral infection from valuable domesticated breeding populations.

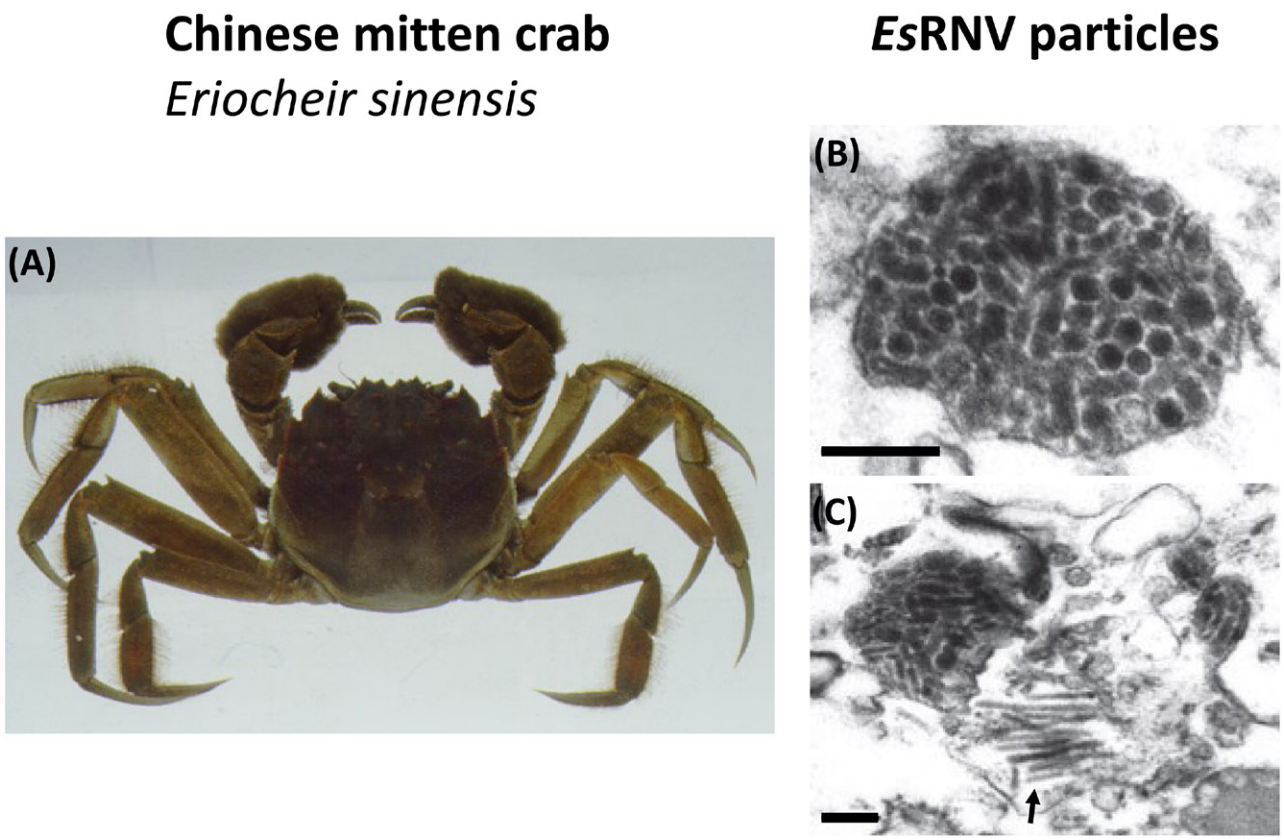

FIGURE 32.10 (A) Photograph of the freshwater Chinese mitten crab (*Eriocheir sinensis*). Transmission electron micrographs of *Eriocheir sinensis* ronivirus (*Es*RNV) particles (B) clustered with cytoplasmic vesicles and (C) filamentous nucleocapsid precursors (*arrow*) in the vicinity of virion clusters in connective tissue cells within the hepatopancreas of diseased *Eriocheir sinensis* (Bars = 120 nm) (Zhang and Bonami, 2007).

32.3.2 *Eriocheir sinensis* Ronivirus

The freshwater Chinese mitten crab (*Eriocheir sinensis*) has become an important aquaculture species in many inland provinces in China (Zeng et al., 2012; Fig. 32.10). With the increasingly intensive aquaculture of *E. sinensis*, several diseases have emerged. Among these, black gill syndrome (BGS) emerged in 1996 as a cause of substantial production losses (Zhang and Bonami, 2007). As other symptoms associated with BGS are generalized, such as sluggishness and anorexia, and as affected crabs display respiratory difficulties manifest by them audibly sounding to "sigh" in the quiet of night as they respire bubbles, the disease is also referred to colloquially as "sighs" disease (SD) (Zhang and Bonami, 2007).

In investigating the cause of SD in crabs collected from a farm in Hubei Province, filamentous viral nucleocapsid precursors (16–18 nm × 150–250 nm) up to 400 nm in length, sometimes closely aligned and amassed adjacent to ER membranes, and enveloped rod-shaped virions (24–42 nm × 60–170 nm) either scattered throughout the cell cytoplasm or packed densely within cytoplasmic vesicles, were identified by TEM in cells of connective tissues (Zhang and Bonami, 2007; Fig. 32.10). Negative staining of virions purified from hemolymph identified regularly spaced surface projections.

In challenge trials, injection of naïve *E. sinensis* with ultra-filtered hemolymph from a disease crab resulted in deaths accumulating rapidly from day 13 to reach 100% by day 17 (Zhang and Bonami, 2007). SD symptoms developed in approximately 30% crabs and none developed BGS suggested that BGS might be a secondary condition caused by crabs becoming moribund in pond culture environments (Zhang and Bonami, 2007). Histopathology in diseased crabs was characterized by clusters (1–5 µm dia.) of pale to deeply basophilic necrotic cells displaying hypertrophied nuclei and nuclear karyorrhesis or pyknosis, and by cytoplasmic inclusion bodies (200–800 nm diameter) filled with enveloped virions. Necrotic cells were prominent in the crab LO and in connective tissues of various organs, including the gills, hepatopancreas, heart, gut and testes. In gills, basophilic inclusion bodies were replaced by eosinophilic foci in necrotic cells and eosinophilic areas in connective tissues (Zhang and Bonami, 2007).

Due to the tissue tropism and histopathology closely resembling those manifest in shrimp infected with okaviruses, filamentous nucleocapsid precursors and enveloped rod-shaped virions sharing striking morphological similarities, except for virions appearing somewhat shorter than those described for YHV (40–50 nm × 150–200 nm, Boonyaratpalin et al., 1993; Chantanachookin et al., 1993) and GAV (34–42 nm × 183–200 nm, Spann et al., 1995, 1997), and a viral genome comprising a long (~22 kb) ssRNA, the virus was named *Es*RNV (Zhang and Bonami, 2007; Bonami and Zhang, 2011). While the properties of *Es*RNV suggest a close evolutionary relationship to okaviruses, genome sequencing is needed to determine whether it should be classified within this or a new genus of the *Roniviridae* (Cowley et al., 2012).

32.3.3 Other Crab Oka-Like Viruses

In the 1970s, viruses forming bacilliform (20–30 nm × 110–170 nm) particles together with long flexible particles up to 600 nm in length, similar in appearance to the virions and filamentous helical nucleocapsid precursors of okaviruses and bafiniviruses, were detected in the Blue crab (*Callinectes sapidus*) (Jahromi, 1977; Yudin and Clark, 1978, 1979; Johnson, 1983). *Callinectes sapidus* is indigenous to the US Atlantic coast and the Gulf of Mexico and while not an aquaculture species, supports an important wild fishery in this region. For the virus particle (20–30 nm × 110–170 nm) named tentatively as *rhabdolike-virus A* (RhVA, Jahromi, 1977; Johnson, 1983) based on morphological similarities to plant rhabdoviruses, virions were detected in Schwann cells in parallel arrays localized at ER and nuclear cistern membranes, and less frequently at plasma membranes. For the virus particle (25–30 nm × 100–150 nm) named *ecdysial gland virus* 2 (EGV-2, Yudin and Clark, 1978, 1979) due to its detection in ecdysial (Y organ or mandibular) glands of eyestalk-ablated blue crabs, virions also matured as arrays aligned perpendicularly to the outer nuclear membrane, as well as at tubular ER membranes and the plasma membrane (Yudin and Clark, 1979). EGV-2 occurred in association with a larger-diameter enveloped bacilliform particle (50–70 nm × 110–170 nm) decorated with surface projections that was named EGV-1 (Yudin and Clark, 1978). EGV-1 and EGV-2 became evident after crab eyestalk ablation, suggesting that the infections were preexisting and escalated in response to stress (Yudin and Clark, 1978). Similarities in virion morphology and morphogenesis suggest that RhVA and EGV-2 are the same virus. However, genome sequence is required to confirm their evolutionary relationships because the diameter of the bacilliform RhVA and EGV-2 virions is somewhat smaller than those described for okaviruses (Cowley et al., 2012) and bafiniviruses (de Groot et al., 2012a).

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Chapter 33

Dicistroviruses of Crustaceans

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33.1 SOME NOTES ON THIS CHAPTER

The family *Dicistroviridae* contains viruses of insects and crustaceans and is divided into two genera: *Cripavirus* and *Aparavirus*. The acronym *Dicistro* is derived from the two open reading frames (ORFs), or cistrons, encoded in the positive-sense, single-stranded RNA (ssRNA) genome whereby each ORF is translated from an independent internal ribosome entry site (IRES). The family belongs to the order *Picornavirales* (Le Gall et al., 2008; Chen et al., 2012) but differs from *Picornaviridae* in having the structural proteins at the 3' end of the genome rather than at the 5' end, and by the presence of the intergenic region (IGR) (Bonning, 2009), which serves as the IRES for ORF2. Taura syndrome virus (TSV), which infects many species of shrimp, prawn and crab (Anonymous, 2013), is in the genus *Aparavirus*, although the phylogenic tree for the 5′ non-structural polyprotein (ORF1) places it in *Cripavirus* instead of *Aparavirus* and thus may belong to a novel genus of family *Dicistroviridae* (Hertz and Thompson, 2011). Taura syndrome virus is second only to white spot syndrome disease virus (WSSDV) in terms of economic importance to the global shrimp farming industry. Recently, a new virus, *Scylla bicistronic virus–1* (mud crab dicistrovirus-l, MCDV-l) was isolated from diseased farmed Portunidae hole crab (Anonymous, 2015).

During the writing of this book, it became clear to the editors that it would not be possible to find contributors to this chapter in a timely manner because the international experts who used to work on these viruses are all retired, and there are not many "pure shrimp virologists" available, as there are no cell lines to grow shrimp viruses. So, most of the work on shrimp viral diseases is a combination of pathology and virology, and the "shrimp pathologists" believe that they are not qualified to write shrimp virology chapters. In order to help foster understanding of the historic importance and capacity of TSV to cause disease and the methods used for its detection, readers are referred to the review article by Don V. Lightner, R. M. Redman, Carlos R. Pantoja, Kathy F. J. Tang, Brenda L. Noble, Paul Schofield, L. L. Mohney, Linda M. Nunan and S. A. Navarro, "Historic emergence, impact and current status of shrimp pathogens in the Americas," from *Journal of Invertebrate Pathology* (2012), which describes the status of shrimp viruses in the Americas.

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Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00033-4
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Chapter 34

Nodaviruses of Crustaceans

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34.1 INTRODUCTION

The aquaculture industry has flourished because of the worldwide demand for seafood. Among the farmed species, the giant freshwater prawn *Macrobrachium rosenbergii* and whiteleg shrimp *Penaeus vannamei* are globally the most crucial crustacean species because they are the most farmed and are cultivated on a large scale in numerous countries. However, an infectious viral disease is a serious problem for the shrimp aquaculture industry that affects it economically. Since 1999, four virus infections have been reported to cause muscle necrosis in cultured shrimp and prawns. *Macrobrachium rosenbergii* nodavirus (*Mr*NV), which causes white tail disease (WTD) infection in freshwater prawns, was the first infection to be reported (Arcier et al., 1999; Tung et al., 1999). Another virus that infects marine whiteleg shrimp is infectious myonecrosis virus (IMNV) (Poulos et al., 2006), which causes whitish muscle necrosis. Mass mortality has been reported for both infections, ranging up to 100% and 20–60% for *Mr*NV and IMNV infections, respectively. However, these viruses belong to different virus families. *Mr*NV belongs to the *Nodaviridae* family, and IMNV belongs to the *Totiviridae* family. In 2004, another muscle necrosis-causing virus was reported, this time in *Penaeus vannamei* cultures in Belize. Infection with this virus caused a 50% reduction in production in some ponds at the affected farm. The virus was identified using cDNA synthesis, cloning and sequencing as an *Mr*NV-related nodavirus. The virus was tentatively named *Penaeus vannamei* nodavirus (*Pv*NV) (Tang et al., 2007). Since 2009, a new nodavirus named covert mortality nodavirus (CMNV), was identified and associated with covert mortality disease (CMD) in cultured *Penaeus vannamei* in China. The clinical signs and histopathological examinations of CMNV-infected shrimp revealed features similar to those of *Mr*NV, IMNV and *Pv*NV. In the CMD-infected shrimp ponds, a gradually developing mortality was observed during daily management and the cumulative mortality of *Penaeus vannamei* may range from 80% to 90%. Sequencing and alignment analyses revealed that the RdRp amino acids sequence of CMNV were 54%, 53% and 39% identical to those of insect and crustacean nodaviruses (Zhang et al., 2014). To date, *Mr*NV, *Pv*NV, respectively, and CMNV are the only three infectious nodaviruses in crustaceans (Table 34.1). This chapter will mainly examine the *Mr*NV infection because of a lack of extensive information on *Pv*NV and CMNV.

TABLE 34.1 Known Nodaviruses of Crustaceans

Virus Name	Main Disease	Host	References
Macrobrachium rosenbergii nodavirus	White tail disease (WTD)	*Macrobrachium rosenbergii*	Arcier et al. (1999)
		Penaeus vannamei	Senapin et al. (2012, 2013)
		Penaeus indicus, Penaeus monodon	Ravi et al. (2009)
Penaeus vannamei nodavirus	–	*Penaeus vannamei*	Tang et al. (2007, 2011)
Covert mortality nodavirus	Covert mortality disease (CMD)	*Penaeus vannamei, Penaeus chinensis, Penaeus japonicus*	Zhang et al. (2014)

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00034-6
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34.2 MAIN CRUSTACEAN DISEASES

34.2.1 Diseases Name

*Mr*NV and extra small virus (XSV) cause WTD, an emerging disease, leading to serious production losses in the hatchery and nursery phases of the prawn culture system (Bonami and Sri Widada, 2011). *Mr*NV is a nonenveloped, icosahedral particle measuring 26–27 nm in diameter, whereas XSV is a nonenveloped icosahedral virus, measuring 15 nm in diameter and which has a linear single-stranded RNA genome (0.9 kb) that encodes two overlapping structural proteins of 16 and 17 kDa (CP-16, 17) (Sri Widada and Bonami, 2004). XSV may be a satellite virus and *Mr*NV a helper virus because of its small size and absence of gene-encoding enzymes required for replication (Qian et al., 2003). WTD was first reported in the French West Indies (Arcier et al., 1999), then later in China (Qian et al., 2003), India (Sahul Hameed et al., 2004a; Vijayan et al., 2005; Shekhar et al., 2006), Taiwan (Tung et al., 1999; Wang et al., 2007, 2008), Thailand (Yoganandhan et al., 2006), Australia (Owens et al., 2009) and Malaysia (Saedi et al., 2012). The clinical signs of WTD-infected postlarvae (PL) prawns include lethargy and opaqueness of the abdominal muscle, beginning at the second or third abdominal segment and gradually extending from the center to the anterior and posterior muscle sections. The disease was named after the whitish muscle appearance. Since 2006, *Mr*NV has been detected in the whitish muscles of farmed *Penaeus vannamei* in Vietnam and Indonesia (Senapin et al., 2012, 2013). Furthermore, WTD has been reported in hatcheries rearing PL marine shrimp, *Penaeus monodon* and *Penaeus indicus* (Ravi et al., 2009). Therefore, *Mr*NV is a major threat to crustacean aquaculture.

Shrimp infected by similar WTD exhibit clinical signs of white, opaque lesions in their tail muscle that are similar to those exhibited by shrimp infected by IMNV. Moreover, the major histopathological characteristics observed are extremely similar to those caused by IMNV infection. However, IMNV was not detected in these affected shrimp by either reverse transcriptase-polymerase chain reaction (RT-PCR) or in situ hybridization (ISH) methods. A cDNA library was constructed from the total RNA that was extracted from the hemolymph of the infected shrimp. One clone (designated *Pv*NV-4) with a 928-bp insert was sequenced, and the sequence was similar to the capsid protein gene of *Mr*NV. Therefore, the infectious agent was tentatively named *Pv*NV (*Penaeus vannamei* nodavirus) (Tang et al., 2007).

Before 2009, shrimp farming in China was affected by a major disease, which was named CMD because most of the moribund shrimp hid in deep waters rather than swimming to the surface or in shallow waters. The CMD-infected shrimp exhibited clinical signs such as hepatopancreatic atrophy and necrosis, empty stomach and gut, soft shell and slow growth. In many cases, these clinical signs were accompanied by uneven, slightly white lesions in the abdominal segment muscles or by a slightly pale body. The onset of death began 1 month after stocking and the rates increased 60–80 days after stocking, accompanied by increased nitrite nitrogen (NO_2–N) concentration, with a cumulative mortality of up to 80%. In 2014, a report indicated that a nodavirus named CMNV caused CMD in cultured shrimp (Zhang et al., 2014).

34.2.2 Structure and Composition of Virus

Nodaviridae family viruses are small (28–37 nm), nonenveloped and isometric. These viruses characteristically package bipartite positive-sense RNA genomes comprising RNA1 (3.0–3.2 kb) and RNA2 (1.3–1.4 kb) (Schneemann et al., 2005). RNA1 encodes protein A, the RNA-dependent RNA polymerase (RdRp), and RNA2 encodes the capsid protein (CP-43) that is required for progeny virion formation. Among the nodaviruses, *Mr*NV has been isolated and characterized physicochemically. Isopycnic centrifugation was performed using 25%–40% cesium chloride gradients to obtain *Mr*NV virions, which were then separated into peaks in densities of 1.258 and 1.330 g/mL. In the two peaks, the particles were icosahedral, each with a diameter of 27 nm (Bonami et al., 2005). Nucleotide sequencing of the *Mr*NV genome indicated that RNA1 comprised 3202 nucleotides and RNA2 comprised 1175 nucleotides. In addition, a subgenomic RNA segment corresponded to the 3′ end of RNA1, but in a different reading frame. Both RNAs appeared in sense orientation, thus allowing their direct translation. The coding sequences of the following two nonstructural proteins were observed in RNA1: protein A, RdRp comprising 1034 amino acids (c. 100 kDa) and protein B, with an estimated size of 13 kDa. Protein B was encoded by at the 3′ region of RNA1 (2725–3126 nucleotides). The amino acid sequence of RdRp of *Mr*NV revealed six conserved motifs: an acidic motif at position 599–604 (motif 1), an SGT motif at position 661–665 (motif 2), a GDD motif at position 703–705 (motif 3), a basic motif at position 728 (motif 4), a basic sequence preceded by an aromatic residue at position 763 (motif 5) and an aromatic residue preceded by a basic sequence at position 830 (motif 6) (Naveenkumar et al., 2013b). Partial amino acid sequencing of the N-terminal end of CP-43 verified that this structural protein was encoded by RNA2 and its estimated size corresponded to that determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

The *Pv*NV particle exhibited a spherical shape ranging from 19 to 27 nm in diameter. The viral genome comprised two segments: RNA1 (3111 bp) and RNA2 (1183 bp). RNA1 contained two overlapped open reading frames (ORFs), ORFs A and B, encoding an RdRp and a B2 protein, respectively. RNA2 contained a single ORF encoding the viral capsid protein.

Sequence analysis revealed that the *Pv*NV genome had four RdRp characteristic motifs and two conserved domains (RNA-binding B2 protein and viral coat protein) (Tang et al., 2011).

Ultrathin hepatopancreas sections of CMNV-infected shrimp revealed the presence of spherical nonenveloped virus particles with a diameter of 24.9 nm. The purified viruses were observed using transmission electron microscopy (TEM) negative staining and the large and small virus particles measured 32 and 19 nm in diameter, respectively. The CMNV virus particle is larger than those of *Mr*NV and *Pv*NV. However, the diameter of the particle ranges from 32 to 33 nm, which is typically the size of alphanodaviruses (Zhang et al., 2014). To date, no other CMNV genome characteristics have been investigated.

34.2.3 Classification and Susceptibility of Cell Line to Virus Infection

Nonclassified nodaviruses (those belonging to neither *Alphanodavirus* nor *Betanodavirus* genera) have been reported in shrimp. The nucleotide sequences available in the GenBank database for RNA1 and RNA2 of *Mr*NV include isolates from the French West Indies (*Mr*NV-FWI; Sri Widada et al., 2003), China (*Mr*NV-CR from *M. rosenbergii* and *Mr*NV-CV from *Penaeus vannamei*; unpublished and Senapin et al., 2012, respectively), Australia (*Mr*NV-A; Hayakijkosol et al., 2012), Malaysia (*Mr*NV-M; unpublished) and India (*Mr*NV-I; Naveenkumar et al., 2013b). In addition, RNA1 and RNA2 of *Pv*NV have been sequenced from the Belize isolate (Tang et al., 2011). The deduced amino acid sequence of *Mr*NV-I RdRp was the highest homologous (99.4%) to the *Mr*NV-FWI isolate, followed by 98.6%, 97.9%, 97.8%, 95.6% and 73.1% homology in the RdRp of *Mr*NV-CV, *Mr*NV-A, *Mr*NV-CR, *Mr*NV-M and *Pv*NV respectively. The RdRp of *Mr*NV exhibited low homology with both *Alphanodavirus* and *Betanodavirus*. The *Mr*NV-I showed homology of 21.3% (Pariacoto virus) to 46.1% (Nodamura virus) with *Alphanodavirus* and 22.6% (Red spotted grouper nervous necrosis virus) to 24% (Tiger puffer nervous necrosis virus) with the RdRp of *Betanodavirus*. Deduced amino acids of the *Mr*NV-I capsid proteins exhibited the highest homology to *Mr*NV-FWI capsid proteins (98.9%), followed by homology to the capsid proteins of *Mr*NV-CV (98.4%), *Mr*NV-CR (95.7%) and *Mr*NV-A (84.3%). In addition, an identity of 44.6% with *Pv*NV capsid proteins was observed (Naveenkumar et al., 2013b). The presence of the conserved cleavage site Asn/Ala at the C-terminal region among all alphanodaviruses was observed in the *Mr*NV capsid protein. The *Mr*NV could be clearly distinguished from other nodaviruses (*Alphanodavirus* and *Betanodavirus*) by using RNA1 and RNA2 sequence analysis. *Mr*NV exhibited close homology with different geographical isolates and *Pv*NV. High variation in the homology of RdRp limits its usefulness in nodavirus classification. Phylogenetic analysis of the deduced amino acid sequences of capsid proteins suggests the existence of a new genus with two genotypes (*Mr*NV and *Pv*NV) within the *Nodaviridae* family (Naveenkumar et al., 2013b). One clone with a 1185-bp insert derived from the *RdRp* gene was established for CMNV. A blast search revealed that the amino acid sequences shared 54%, 53% and 39% identity with the Flock House virus, the black beetle virus and *Mr*NV, respectively, which belonged to insect and crustaceans nodaviruses. The resulting phylogenetic tree suggested that the deduced amino acids of the *RdRp* gene of CMNV were clustered in the genus *Alphanodavirus* (Zhang et al., 2014).

Cell culture is a basic tool for studying pathogenic infections, particularly for viruses that replicate internally. No continuous crustacean cell lines have been established for replicating these viruses. The snakehead fish cell line SSN-1, derived from the whole fry tissue of *Channa* (*Ophicephalus*) *striatus*, was used in attempted isolation of the fish nodavirus. The SNN-1 fish cell line was incubated with *Mr*NV inoculum and the cytopathic effects were recorded. The presence of de novo synthesis of viral capsid proteins was demonstrated using immunofluorescence (IF) labeling and a sandwich enzyme-linked immunosorbent assay (S-ELISA) test. Viral genomic replication was confirmed using RT-PCR and quantitative RT-PCR (qRT-PCR). Only a few empty particles were observed through electron microscopy, and attempts to isolate complete infectious particles or re-infect healthy cells were unsuccessful. Therefore, *Mr*NV could partially infect SSN-1 cells, meaning that the virus could enter cells and that its sense single-stranded genome is probably directly translated to the capsid proteins by the cell translation machinery. Nevertheless, the viral development was not complete and other elements necessary for this process were most likely lacking (Hernandez-Herrera et al., 2007). The susceptibility of the C6/36 mosquito *Aedes albopictus* cell line for *Mr*NV–XSV infection was investigated because *Mr*NV and XSV (*Mr*NV–XSV) are classified as invertebrate virus members. The replication of these viruses in C6/36 cells was confirmed using RT-PCR, acridine orange staining, infectivity study and electron microscopy. PL of prawn was challenged by immersion method using inoculum prepared from infected cells. The PL exhibited lethargy, anorexia and opaqueness of abdominal muscle, and 100% mortality was observed. The supernatant medium from the fifth passage culture initiated WTD infection in PL *M. rosenbergii*, confirming the successful propagation of *Mr*NV–XSV in C3/36 cells (Sudhakaran et al., 2007b). However, inconsistent results were obtained for the Australian isolate of *Mr*NV in C3/36 cell lines. Typical cytopathic effects, such as vacuolation and viral inclusion bodies, were observed in infected C6/36 cells, but TaqMan qRT-PCR did not confirm *Mr*NV replication in the cells for more than 14 days. Limited evidence of viral replication was observed during four serial passages (Hayakijkosol and Owens, 2013).

34.2.4 Epidemiology, Transmission and Hosts

Since 1994, episodic mortalities were reported in a hatchery in Pointe Noire, Guadeloupe in the French West Indies. The main sign of WTD was whitish coloration of the striated muscles of the cephalothorax, abdomen and tail. These signs were associated with abnormal behavior, some lethargy and anorexia. Histopathological characteristics included the presence of acute necrosis of striated muscles, characterized by severe hyaline degeneration, necrosis and muscle lysis. Oval or irregular basophilic cytoplasmic inclusion bodies measuring 1–4 μm in diameter were observed in the infected muscles and intratubular connective tissue of the hepatopancreas. No viral inclusions were observed in the epithelial cells of the hepatopancreatic tubules or in the mucosal epithelial cells of the midgut. These WTD-related mortalities varied from 5% to 90%, depending on the reproductive cycle of the broodstock. Larvae, PL and juvenile *M. rosenbergii* are highly susceptible to WTD, and WTD-related mortality is high in these three life stages. After the appearance of the first gross signs, mortality may peak after approximately 5 or 6 days. Very few WTD-infected organisms that survive the PL stage grow to the size typically intended for production. Adults are resistant to WTD, but they can act as carriers (Qian et al., 2003; Sahul Hameed et al., 2004a).

The reproductive cysts and five developmental stages (nauplii, metanauplii, juveniles, sub-adult and adult) of *Artemia* were exposed to *Mr*NV–XSV via the oral or immersion route. The cumulative mortality for the different developmental stages of the inoculated *Artemia* was not remarkably different from those of the negative controls. None of the challenged *Artemia* exhibited any signs of WTD, though they tested positive for *Mr*NV–XSV by using RT-PCR. Mortality was 100% in *M. rosenbergii* PL fed with *Artemia* exposed to *Mr*NV–XSV, and the clinical signs of WTD were observed. The results clearly indicated that *Artemia* may act as a reservoir of mechanical carrier of *Mr*NV–XSV and transmit these viruses horizontally to *M. rosenbergii* in a hatchery (Sudhakaran et al., 2006b). In addition, five species of aquatic insects from nursery ponds containing WTD-infected prawns were screened to identify potential natural carriers of *Mr*NV–XSV. RT-PCR analysis results were positive for these viruses in giant water bug (*Belostoma* sp.), dragonfly nymphs (*Aeshna* sp.), diving beetles (*Cybister* sp.) and back swimmers (*Notonecta* sp.), tested negative for water scorpion (*Nepa* sp.). The C3/36 cell line was used to determine the infectivity of the viral inoculum prepared from the aquatic insects. Electron microscopy revealed the aggregation of viral particles throughout the cytoplasm of the infected cells. Although the PCR results were positive, a lack of clarity exists regarding whether the aquatic insect species are infected with the virus or simply act as mechanical carriers. The results indicated that several aquatic insect species may pose a risk for *Mr*NV and XSV transmission to *M. rosenbergii* (Sudhakaran et al., 2008).

The adults of three marine shrimp species (*Penaeus indicus*, *Penaeus japonicus* and *Penaeus monodon*) and two freshwater prawns (*Macrobrachium rude* and *Macrobrachium malcolmsonii*) are not susceptible to *Mr*NV–XSV infection, which failed to induce mortality via the oral route or intramuscular injection (IM). RT-PCR analysis revealed the presence of *Mr*NV–XSV in organs such as the gills, abdominal muscle, stomach and intestine, and in the hemolymph, of these virus-infected shrimp. Research in this area has indicated a uniform tissue tropism pattern of *Mr*NV–XSV in freshwater prawns and marine shrimp. Reinoculation using the inoculum prepared from the *Mr*NV–XSV-infected marine shrimp or freshwater prawns caused 100% mortality in PL freshwater prawns. Moribund PL prawns tested positive for these viruses when evaluated using RT-PCR (Sudhakaran et al., 2006a; Ravi and Sahul Hameed, 2015). Subsequent studies identified WTD in hatchery-reared PL marine shrimp (*Penaeus monodon* and *Penaeus indicus*). Clinical signs observed in these shrimp were similar to those in PL *M. rosenbergii*. Infected PL shrimp tested positive for *Mr*NV and XSV when evaluated using RT-PCR. The inoculum prepared from these infected shrimp caused 100% mortality in PL freshwater prawns. Producing marine shrimp and freshwater prawn seeds in close proximity may cause the transmission of pathological organisms from the native to nonnative hosts (Ravi et al., 2009). Juvenile *Penaeus vannamei* with white muscles and high mortality rates tested negative for IMNV and *Pv*NV; however, some of these juveniles tested using RT-PCR were positive for *Mr*NV. Sequences of the two single-stranded RNA fragments in the *Mr*NV genome from *Penaeus vannamei* specimens were found to share 97% homology with the *Mr*NV sequences deposited at GenBank (NC_005094 and NC_005095). XSV was detected in some, but not all, samples. Infectivity tests were performed by feeding *Penaeus vannamei* with minced tissue from *Mr*NV-infected *M. rosenbergii* at low salinity of 2 ppt and at two water temperatures. Shrimp exhibited higher infectivity and mortality when their rearing water had a lower temperature and salinity levels (Senapin et al., 2012). The redclaw crayfish (*Cherax quadricarinatus*) was used as an experimental animal for studying *Mr*NV infections because no alternative animal experimental model for *Mr*NV has been identified in Australia. Mortality was 35% and 0% in resulting *Mr*NV and control groups, respectively. Necrosis and muscle degeneration with hemocytic infiltration were observed, and TaqMan qRT-PCR confirmed *Mr*NV infection in the infected crayfish. Although *Cherax quadricarinatus* may not be the ideal experimental animal model, it can be used for experiments because of its clinical signs, gross lesions, histopathological changes and presence of qRT-PCR titers (Hayakijkosol et al., 2011).

The clinical signs and histopathological features identified in shrimp samples collected in Belize from 2004 to 2006 were similar to those in shrimp infected by IMNV. In *Penaeus vannamei*, *Pv*NV was identified as the causative agent of muscle necrosis. In a study investigating potential vectors, *Pv*NV was detected in mosquitofish, seabird feces, barnacles and zooplankton by using RT-PCR. *Pv*NV have been suggested as potentially spreading through these carriers (Tang et al., 2011).

In China, RT-PCR results revealed CMNV infection in *Penaeus chinensis*, *Penaeus vannamei* and *Penaeus japonicus.* The host ranges of *Mr*NV, *Pv*NV and CMNV appeared to vary. *Mr*NV was isolated originally from *M. rosenbergii*, and several species of marine penaeid shrimp were identified as the reservoir host. *Pv*NV appears to have a limited host range. The results of histopathological examination and nested RT-PCR (nRT-PCR) indicated that CMNV has a wide host range in cultured shrimp species. In addition, somewhat similarly to *Pv*NV, CMNV-related mortality may be sporadic in ponds, but is aggravated by environmental stresses, such as NO_2–N concentration and high temperatures. When the air temperature exceeds 30°C and water temperature exceeds 28°C, the survival rate of CMNV-infected shrimp in ponds decreases sharply. High mortality was observed 68–80 days poststocking during a summer crop (Zhang et al., 2014).

34.2.5 Pathology and Immunity

The causative agents of WTD were identified as *Mr*NV and XSV. When transmitted experimentally to healthy *M. rosenbergii*, these viruses caused 100% mortality in PL prawn but failed to cause mortality in adult prawns. An RT-PCR assay revealed the presence of both viruses in moribund PL prawns and in the gills, head muscles, stomach, intestine, heart, hemolymph, pleopods, ovaries and tail muscle, but not eyestalks or hepatopancreas, of experimentally infected adult prawns. Pleopods are appropriate for detecting these viruses by using the RT-PCR technique in brooders. The presence of these viruses in ovarian tissues indicated the possibility of vertical transmission (Sahul Hameed et al., 2004b). Experiments were performed to determine the possibility of vertical transmission for *Mr*NV–XSV. Prawn brooders inoculated with *Mr*NV–XSV through the oral or IM route survived without mortality or any clinical sign of WTD. The brooders spawned 5–7 days after inoculation and the eggs hatched. The survival rate of larvae gradually decreased, and 100% mortality was observed at the PL stage. Typical signs of WTD were observed in the advanced larval developmental stage. The ovarian tissue and fertilized eggs tested positive for *Mr*NV–XSV when evaluated using RT-PCR, whereas the larval stages tested positive when evaluated using nRT-PCR (Sudhakaran et al., 2007a).

Macrobrachium rosenbergii prawns were intramuscularly injected with *Mr*NV–XSV, and various organ samples, such as of the gills, head muscles, pleopods and intestines, were collected 3-, 5-, 10-, 15-, 25-, 50-, 75- and 100-day postinfection (d p.i.) to study viral clearance. The results revealed that these two viruses failed to cause mortality or clinical signs of disease in adult prawns during the 100-day experimental period. Single-step RT-PCR revealed that all the samples were positive for both viruses for 3, 5 and 10 d p.i. Furthermore, the organs tested positive for viruses 15 and 20 d p.i. by using nRT-PCR, and all the organs tested negative from 25 d p.i. onward. The viral inoculum prepared from the tissues of *Mr*NV–XSV-infected *M. rosenbergii* at 3, 5, 10, 15 and 20 d p.i. caused 100% mortality in PL prawns, whereas the inoculum prepared at 25, 50 and 100 d p.i. failed to cause remarkable mortality in these prawns. Immunological parameters such as proPO activity and superoxide anion concentration increased considerably, whereas the superoxide dismutase (SOD) activity decreased remarkably in the early days p.i. and became insignificant in the later days p.i. The total hemocyte count (THC) decreased on only 1 and 3 d p.i. and no such change in the hemocyanin levels of *Mr*NV–XSV-infected and normal prawns were observed thereafter. An increase in the THC is considered a consequence of the rapid proliferation of hematopoietic cells in the host as a response against foreign pathogens. The proPO enzyme is a well-known innate immunity-related enzyme of the prophenol oxidase system, which catalyzes melanization and production of bactericidal superoxide radicals. High proPO levels enhance resistance against viral pathogens in *M. rosenbergii*. In addition, the release of superoxide anion and H_2O_2 plays a crucial role in antimicrobial activity. Increased superoxide anion levels are considered beneficial because they enhance immunity. The SOD pathway is one of the main antioxidant defense pathways in response to oxidative stress. These factors suggest that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, responsible for the release of superoxide anions, increases with decreasing SOD activity, which is responsible for scavenging superoxide anions. Increases in singlet oxygen and hydroxyl radical levels were reported to inactivate SOD and reduce enzyme activity. The activated hemocytes could produce extra antimicrobial substances such as H_2O_2 and superoxide anions, which may enhance disease resistance and viral pathogen clearance. In conclusion, increased proPO and superoxide anion levels in the virus injected prawns provide some evidence for the resistance against *Mr*NV–XSV (Ravi et al., 2010).

*Pv*NV viral inclusions were found to be closely associated with mitochondria, suggesting that the replication complexes may be located in the inner or outer mitochondrial membranes, as reported for other nodaviruses. Both *Penaeus monodon* and *Penaeus vannamei* were susceptible to *Pv*NV infection through laboratory injection and oral routes. After injection,

50% of the *Penaeus vannamei* had white tails by 11 d p.i., but no mortalities were observed throughout a 4-week bioassay. White tails or muscle necrosis was absent in *Penaeus monodon*. An RT-PCR assay was used to detect *Pv*NV in both species. Only three of five sampled shrimp from each species tested positive when orally challenged. Although *Pv*NV did not cause mortalities in the laboratory bioassay, it affected survival in grow-out ponds. *Pv*NV-related mortality is sporadic and often associated with environmental stress such as crowding and high temperatures. When shrimp are stocked at a high density ($>50\,m^{-2}$) or the temperature exceeds 32°C, the survival rate in *Pv*NV-infected shrimp ponds decreases and the food conversion ratio increases (Tang et al., 2007).

Histological examination revealed that muscle fibers containing whitish muscle lesions had muscle fragmentation tending toward coagulative necrosis, muscular lysis and myonecrosis. Multifocal myonecrosis in the striated muscle was accompanied by hemocytic infiltration and hemocytes karyopyknosis. Vacuolation in the cytoplasm of hepatopancreocytes and eosinophilic inclusions were observed within the tubular epithelium of the hepatopancreas. Inclusions and nuclear pyknosis were observed in the lymphoid spheroids. However, little evidence exists that *Mr*NV, *Pv*NV or IMNV infection can cause hepatopancreatic atrophy and necrosis. Fluorescence in situ hybridization (FISH) of CMNV-infected shrimp confirmed that the CMNV infects the tubular epithelium of the hepatopancreas. The results of viral infection through different routes revealed that the cumulative mortalities of shrimp in 0.22 μm filtered injection, unfiltered injection and oral infection groups were 100%, 100% and 84.85%, respectively, during the 10-day infection period. These results fulfill River's postulates for supporting a viral etiology for CMD. In addition, high CMNV-related mortality indicates that the virulence of CMNV is higher than that of *Pv*NV and IMNV (Zhang et al., 2014).

34.2.6 Diagnostic Methods

Conventional diagnostic methods such as gross signs, histopathology and TEM were used to detect *Mr*NV, *Pv*NV and CMNV. The gross signs and histopathological characteristics caused by *Mr*NV, *Pv*NV and CMNV infections in shrimp are not easily distinguishable. In addition, TEM is a time-consuming procedure and requires expensive equipment and excellently handling skills, rendering it unsuitable for use in field tests. Therefore, these three diagnostic methods are unsuitable for detecting *Mr*NV, *Pv*NV and CMNV. However, some specific and sensitive detection methods exist for *Mr*NV, *Pv*NV and CMNV diagnosis. Several diagnostic methods have been developed for detecting *Mr*NV, such as immunological and genome-based methods. Several antigen-based detection methods, such as sandwich ELISA (S-ELISA), triple antibody ELISA (TAS-ELISA), Western blotting, IF, immunohistochemistry (IHC) and immune dot–blot assay, were developed for detecting *Mr*NV pathogens. The genome-based detection methods include RT-PCR, nRT-PCR, multiplex RT-PCR (mRT-PCR), probe-free multiplex RT-PCR with high-resolution melt (HRM mRT-PCR), ISH, qRT-PCR, dot–blot, reverse transcription loop-mediated isothermal amplification (RT-LAMP) and RT-LAMP lateral flow dipstick (RT-LAMP-LFD). Furthermore, for detecting *Pv*NV and CMNV, RT-PCR, nRT-PCR, ISH, FISH and RT-LAMP combined with colorimetric gold nanoparticle (AuNP) probe (RT-LAMP-AuNP) were developed. These detection methods for *Mr*NV, *Pv*NV and CMNV are described in Table 34.2.

34.2.6.1 Immunological Detection Methods for MrNV

Polyclonal antibodies were produced by infecting BALB/c mice by using a purified suspension of *Mr*NV, and IgG was purified from ascitic fluid. The S-ELISA method was successfully developed. Tissue extracts from *M. rosenbergii* infected with *Mr*NV, or purified viral extracts, were successfully identified in an individual ELISA, thus confirming the validity of the method. The anti-*Mr*NV IgG did not cross-react with the healthy *M. rosenbergii* tissue, or both fish and insert nodavirus (ie, fish encephalitis virus and nodamura virus). The sensitivity of this diagnostic method was 10 μg/mL of the total protein of infected prawn homogenates (Romestand and Bonami, 2003). The IF method was successfully developed and uses anti-*Mr*NV IgG for detecting *Mr*NV proteins in SSN-1 fish cells lines (Hernandez-Herrera et al., 2007). Furthermore, the results of Western blotting revealed that 12 hybridomas strongly secreting monoclonal antibodies (Mabs) against *Mr*NV were shown to be specific and reacted with the *Mr*NV 42 kDa coat protein. TAS-ELISA was developed and proven to be a useful diagnostic tool for detecting *Mr*NV infections. No cross-reaction was reported for the TAS-ELISA procedure when these Mabs reacted with the white-spot syndrome virus (WSSV), Taura syndrome virus (TSV), fish nodavirus or bacterial infections in crustaceans in the normal PL stage. When TAS-ELISA was used for *Mr*NV detection in diseased crustaceans exhibiting clinical signs of WTD during the PL stage, the minimum detection was 1.2 μg/mL total proteins of PL homogenates. TAS-ELISA was proven to be a highly effective method for virus detection, particularly for prawn hatcheries and local aquaculture laboratories because of its low cost, rapid processing of many samples and easy interpretation of results with the naked eye (Qian et al., 2006). The antisera raised against the CP-43 protein of *Mr*NV and CP-17 protein of XSV in mice

TABLE 34.2 The Different Detection Methods Currently Used for Diagnosis of Crustacean Nodavirus Infections

Virus Names	Detection Methods	Sensitivity	References
*Mr*NV	Histopathology	–	Arcier et al. (1999), Tung et al. (1999)
	Transmission electron microscopy (TEM)	–	Arcier et al. (1999), Tung et al. (1999)
	Immunofluorescence test (IF)	–	Hernandez-Herrera et al. (2007)
	Immunohistochemistry (IHC)	–	Wangman et al. (2012)
		–	Senapin et al. (2013)
	S-ELISA	10 μg/mL total tissue protein	Romestand and Bonami (2003)
	TAS-ELISA	1.2 μg/mL total tissue protein	Qian et al. (2006)
	Dot–blot	8 pg of viral RNA	Sri Widada et al. (2003)
		600 pg/μL of GST-*Mr*NV	Wangman et al. (2012)
		100 pg of total tissue protein	Farook et al. (2014a)
	ISH	–	Sri Widada et al. (2003)
		–	Hsieh et al. (2006)
	RT-PCR	35 fg of viral RNA	Sri Widada et al. (2003)
		0.25 fg of viral RNA	Sahul Hameed et al. (2004a)
		24 copy numbers of plasmid construct	Naveenkumar et al. (2013b)
	nRT-PCR	–	Sudhakaran et al. (2006b)
		100 copies viral RNA	Senapin et al. (2010)
	mRT-PCR	25 fg of total RNA	Yoganandhan et al. (2005)
		1 fg of total RNA	Tripathy et al. (2006)
	HRM mRT-PCR	10 copies viral RNA	Senapin et al. (2010)
	qRT-PCR	<10 copies viral RNA	Zhang et al. (2006)
		–	Hayakijkosol et al. (2011)
	RT-LAMP	–	Pillai et al. (2006)
		0.3 pg of total RNA	Haridas et al. (2010)
	RT-LAMP-LFD	200 copies of in vitro transcript RNA	Puthawibool et al. (2010)
*Pv*NV	Histopathology	–	Tang et al. (2007)
	RT-PCR	–	
	ISH	–	
	TEM	–	Tang et al. (2011)
	RT-LAMP-AuNP	2 copies of in vitro transcript RNA	Suebsing et al. (2013)
CMNV	Histopathology	–	Zhang et al. (2014)
	nRT-PCR	–	
	FISH	–	

were used for developing the Western blotting and ELISA methods for detecting *Mr*NV and XSV. A time-course infectivity experiment was conducted to assess the performance of the RT-PCR, nRT-PCR, Western blotting and ELISA techniques. The results revealed that the ability to detect *Mr*NV–XSV at the PL stage was 3 h p.i. using nRT-PCR and 24 h p.i. using RT-PCR, Western blotting and ELISA, and the gross sign of the whitish muscle was 7 d p.i. (Sahul Hameed et al., 2011).

Pure forms of the viral capsid protein may be obtained using recombinant DNA technology. This technology may also be used to produce antisera or hybridomas for developing immunology-based diagnostic tools for practical applications. The gene encoding the capsid protein of *Mr*NV was cloned into an expression vector and the fusion protein was produced. The recombinant protein was obtained and then used for immunization for Mabs production. Capsid protein-specific Mabs were selected and could detect natural *Mr*NV infections in *M. rosenbergii* when used for Western blotting, dot–blot assay and IHC. No cross-reaction was observed with uninfected prawn tissues or other shrimp infected with IMNV, WSSV, TSV, yellow head virus (YHV), PmDNA, PstDNA or PemoDNA. The detection sensitivity of the Mabs was 600 pg/μL of the GST-*Mr*NV, which was approximately 200-fold lower than that of single-step RT-PCR. IHC analysis using these Mabs with infected prawn tissues demonstrated that the positive reactions for *Mr*NV were similar to those in previous reports (Wangman et al., 2012). Furthermore, Mabs were used to confirm *Mr*NV infection in *Penaeus vannamei* by using the IHC method (Senapin et al., 2013). The recombinant capsid protein of *Mr*NV (rMCP43) was used as an immunogenic antigen for producing antiserum in rabbits. A dot–blot assay using an anti-rMCP43 antibody successfully detected *Mr*NV in WTD-infected PL crustaceans as early as 24 h p.i. The sensitivity of the antiserum increased against rMCP43 was 100 pg total proteins of PL tissues. Western blotting, dot–blot assay and ELISA detected all *Mr*NV-positive coded samples similar to RT-PCR (Farook et al., 2014a). The polyclonal and Mabs against viral capsid proteins of XSV have been developed to detect XSV using similar methods. IHC analysis of *M. rosenbergii* tissue sections using the anti-XSV antibodies revealed that XSV infection colocalizes with *Mr*NV infection at varied loads (Longyant et al., 2012; Neethi et al., 2012).

34.2.6.2 Genome-Based Detection Methods for *Mr*NV

Based on the nucleotide sequence of *Mr*NV, several diagnostic methods were developed. Among these methods, the RT-PCR technique was used routinely for detecting *Mr*NV–XSV in hatcheries. Using viral RNA as a template, quantities as low as 35 fg could be amplified using conventional RT-PCR. In routine analysis, RNA content of 0.15–1.50 mg of tissue is sufficient for RT-PCR. However, inhibition was observed when RNA extracts prepared from more than 2 mg of tissue were used as templates. RT-PCR enabled the detection of *Mr*NV in samples showing no or only slight clinical signs (Sri Widada et al., 2003). In India, different primer sets were designed for *Mr*NV. Using these primer sets, sensitivity improved to 0.25 fg of total RNA when evaluated using RT-PCR. In addition, the results revealed that some WTD samples were positive for only XSV (Sahul Hameed et al., 2004a). In Taiwan, RT-PCR and ISH assays detected *Mr*NV infections but not XSV infections in some WDT-infected prawns (Hsieh et al., 2006). Similar results were obtained from field samples in Thailand (Yoganandhan et al., 2006). Techniques for detecting XSV, such as RT-PCR and dot–blot hybridization, were developed (Sri Widada et al., 2004). However, it is unclear whether XSV and *Mr*NV cause WTD or whether *Mr*NV alone does. Additional epidemiological surveys are required to further elucidate the relationship between *Mr*NV and XSV in WTD pathogenesis. In addition, the clinical signs of whitish coloration in the tail muscle were not specific to *Mr*NV infection. A new RT-PCR assay has been developed that uses a primer set designed according to the nucleotide sequence of known crustacean nodavirus to amplify the conserved regions of all reported *Mr*NV isolates and *Pv*NV (Naveenkumar et al., 2013b).

The nRT-PCR was developed in India for detecting *Mr*NV and XSV. According to the results of nRT-PCR, *Artemia* was considered a possible vector for *Mr*NV–XSV (Sudhakaran et al., 2006b). In addition, several aquatic insect species may pose a risk for *Mr*NV–XSV transmission to *M. rosenbergii* (Sudhakaran et al., 2008). A single tube, single-step mRT-PCR method was developed to detect *Mr*NV–XSV infections simultaneously in naturally and experimentally infected prawns. Experimentally infected adult prawns exhibited infection in all the organs, except hepatopancreas and eyestalk. The sensitivity test revealed that the simultaneous assay could detect the two viruses for 25 fg of total RNA. The mRT-PCR assay allowed simultaneous detection and eliminated the time and cost required to perform RT-PCR separately (Yoganandhan et al., 2005). In addition, the mRT-PCR method was developed using alternative primer sets in India. Primers were designed targeting the capsid protein genes of *Mr*NV and XSV, and the sensitivity was determined using mRT-PCR at a level of 1 fg of total RNA. This method will be useful in studying intervirus interactions in WTD (Tripathy et al., 2006).

HRM analysis is based on changes in fluorescence resulting from the melting of the PCR products in response to a 0.05°C increase in temperature. The melting temperature (Tm) and the characteristic shape of the melting curve of amplified products are highly dependent on the nucleotide sequence. A single base substitution can change the Tm of the amplified product, and this change can be detected using instruments capable of real-time fluorescence monitoring and temperature change. A probe-free multiplex RT-PCR was combined with HRM analysis (HRM mRT-PCR) to simultaneously detect

*Mr*NV–XSV. Comparing HRM mRT-PCR analysis results with those obtained using traditional nRT-PCR on the same samples and gel electrophoresis revealed that HRM mRT-PCR was more sensitive than was the traditional nRT-PCR. Among 19 freshwater prawn field samples, the HRM profiles revealed that 14 were positive for *Mr*NV–XSV, 2 were positive for *Mr*NV only and 3 were negative for both. No sample was positive for XSV alone. In addition, sequence variation in XSV could be observed from the HRM peaks and was confirmed using sequence analysis. HRM mRT-PCR technique enables the rapid and sensitive detection of *Mr*NV–XSV and has the potential to be adapted to similarly detect other mixed viral infections in shrimp (Senapin et al., 2010).

qPCR is appropriate for use in large research studies because it is a rapid test, enables quantifying a viral load and is more sensitive than conventional PCR. This assay also requires less time, has a low risk of contamination and is an attractive alternative because only one round of amplification is performed and analysis through gel electrophoresis is unnecessary. A two-step TaqMan qRT-PCR was developed for *Mr*NV–XSV to quantify the viral copy numbers in infected PL *M. rosenbergii*. The results showed that genomic copies of both viruses were remarkably high when prawns were infected with combinations containing relatively high proportions of *Mr*NV and low proportions of XSV. Signs of WTD were observed in the infected PL prawns, indicating that *Mr*NV plays a key role in WTD infection of *M. rosenbergii* (Zhang et al., 2006). The SYBR green qRT-PCR was successfully developed and applied for *Mr*NV analysis in an in vitro viral development study by using an SSN-1 fish cell line. The results revealed that the SSN-1 fish cell line was partially susceptible to *Mr*NV infections. No virus subculture or virus isolation was obtained from infected cells. However, the cDNA copies of the viral genome increased during the course of infection, as determined using qRT-PCR analysis, which suggested that the genome replication occurred within the cell (Hernandez-Herrera et al., 2007). Conversely, TaqMan qRT-PCR did not confirm the replication of the Australian *Mr*NV isolate in a mosquito C6/36 cell line over 14 days (Hayakijkosol and Owens, 2013). In addition, qRT-PCR results confirmed *Mr*NV infection in infected redclaw crayfish (*Cherax quadricarinatus*). Redclaw crayfish exhibited low susceptibility and was demonstrated to have limited capacity to carry WTD. Based on the qRT-PCR results, *Cherax quadricarinatus* was suggested to be a poor model, but one that can be used as an experimental animal model for studying *Mr*NV infections (Haykijkosol et al., 2011).

Dot–blot hybridization is a method currently used for pathogen detection. The probe derived from the RNA1 of *Mr*NV was labeled with digoxigenin (DIG) using PCR method. The sensitivity of the method was demonstrated using successive 10-fold dilutions of viral RNA extracted from purified *Mr*NV particles. The detection limit was 8 pg of viral RNA (Sri Widada et al., 2003). The application on field samples showed that dot–blot hybridization can be performed on the RNA content of the 14–140 µg of tissue of diseased animals (Bonami and Sri Widada, 2011). The methodology can be recommended for routine health monitoring in hatcheries because of its ease of use, sensitivity and ability to simultaneously process multiple samples. The ISH method requires greater technical skill and expensive equipment, but it can investigate the virus localization in the tissue using the labeled probes hybridize to the targeted nucleic acids. The ISH results revealed that *Mr*NV was found to be confined to the striated muscle and connective tissue of the abdomen, cephalothorax and appendages. Precipitation was observed in the cytoplasm. No labeling was found in the gills, hepatopancreas, or digestive epithelia or epidermis. The *Mr*NV infection was not homogenously distributed in muscular tissue. Virus particles appeared to be restricted to a certain area, whereas adjacent muscular tissue appeared free from infections (Sri Widada et al., 2003). Different DIG-labeled DNA probes were constructed, and high-intensity hybridization signals were detected in the muscles and intertubular fixed phagocytes of the hepatopancreas of *M. rosenbergii* cultured in Taiwan (Hsieh et al., 2006).

RT-PCR is currently used to detect WTD. However, PCR-based methods are time-consuming and require expensive equipment and reagents, rendering them unsuitable for use in field tests. LAMP, a novel nucleic acid amplification method, was developed and employed a *Bst* DNA polymerase with strand displacement activity and four primers that recognize six distinct sequences on the target DNA. In the LAMP method, the target DNA can be amplified from a few copies to 10^9 copies within 1 h under isothermal conditions (60–65°C) by using a simple incubator, such as a water bath or thermostat block. At the end of the reaction, the presence or absence of the target DNA is judged visually according to the appearance of a white precipitate of magnesium pyrophosphate. The presence of multiple bands of varying sizes during agarose gel electrophoresis of the LAMP-reaction products indicates that they comprise a mixture of stem-loop DNAs with various stem sizes and cauliflower-like structures containing multiple loops induced by annealing between alternatively inverted repeats of the target sequence on the same strand. When RNA is used as a template, a prior reverse transcription step is added using AMV reverse transcriptase, which remains active at 63°C. An RT-LAMP procedure was established to detect the genomic RNA molecule of *Mr*NV–XSV targeting the capsid protein gene. The RT-LAMP method was 10 times more sensitive than the RT-PCR method. When an accelerated RT-LAMP assay was performed for *Mr*NV detection by using two additional loop primers, the sensitivity was measured as being at least 10,000 times greater than that of a conventional RT-PCR. However, in some cases, for a reason not yet determined, using loop primers caused self-priming and amplification of primers, thereby causing false-positive response (Pillai et al., 2006). The standardization and optimization of a

single-tube RT-LAMP procedure was investigated for rapid WTD diagnosis. The results suggested that efficient amplification of the template RNA could be obtained within 15–45 min at 63°C and 65°C. With the LAMP reaction, no inhibition of amplification was caused by increasing the amount of the templates used. This is an advantage of RT-LAMP over RT-PCR because the increase in template use beyond the optimal level could actually inhibit detection by RT-PCR. The cost of various ingredients used in RT-LAMP was compared with those used in RT-PCR for a single reaction. The results revealed that the RT-LAMP technique for one reaction is four times less expensive than that for RT-PCR. Although RT-LAMP has numerous advantages over RT-PCR, the result indicated that RT-LAMP requires a highly purified template RNA for assay. Another disadvantage of the LAMP assay is that the high sensitivity and production of large amounts of amplified DNA makes the method susceptible to false-positive reactions because of cross-contamination. However, overall, the advantages of RT-LAMP make it highly appropriate for routine health controls in aquaculture, particularly in the field diagnosis of WTD (Haridas et al., 2010). LAMP can be combined with a chromatographic lateral flow dipstick (LFD) for much more efficient, field-friendly detection of *Mr*NV. In this research, RT-LAMP was performed at 65°C for 40 min, followed by 5 min for hybridization with an FITC-labeled DNA probe, and 5 min for LFD resulted in visualization of DNA amplicons trapped at the LFD test line. Total assay time, including 10 min for rapid RNA extraction, was approximately 60 min. In addition to a short assay time, the confirmation of amplicon identity by hybridization and elimination of electrophoresis with carcinogenic ethidium bromide, the RT-LAMP-LFD was more sensitive than an existing RT-PCR method in the detecting *Mr*NV. The method gave negative test results with nucleic acid extracts from normal shrimp and from shrimp infected with other viruses namely, DNA viruses (IHHNV, MBV, HPV, WSSV) and RNA viruses (TSV, IMNV, YHV/GAV) (Puthawibool et al., 2010).

34.2.6.3 *Pv*NV Detection Methods

For *Pv*NV, several detection methods were developed, namely, histopathology, TEM, ISH, RT-PCR, nRT-PCR and RT-LAMP-AuNP (Tang et al., 2007, 2011; Suebsing et al., 2013). Histopathological examination revealed multifocal necrosis in the skeletal muscles accompanied by hemocytic congestion, fibrocystic inflammation and phagocytosis. Basophilic cytoplasmic inclusions were found within the muscle fibers and in the lymphoid organ. In addition, lymphoid organ spheroids were observed. Under TEM, the infected cells exhibited increased the numbers of various organelles such as mitochondria, Golgi bodies and rough endoplasmic reticulum. Cytoplasmic inclusions containing paracrystalline arrays of virions were visualized. The viral particle was spherical in shape and measured 19–27 nm in diameter. The fragment of the viral genome was labeled with DIG and used for detecting *Pv*NV in the infected *Penaeus vannamei*. The probe appeared to be highly specific. No reaction was observed in any of the tissues prepared from healthy, uninfected shrimp or from those infected with IMNV, WSSV, IHHNV, TSV or YHV. The tissues reacting to the probe included the connective tissues, lymphoid organ, heart, gills and skeletal muscle in the tail. The nRT-PCR assay was developed from the viral genome sequence. The single-step RT-PCR amplified a 339 bp fragment from the viral RNA, and a two-step PCR amplified a 246 bp fragment. The nRT-PCR assay appeared to be specific for *Pv*NV because it did not cross-react with IMNV, YHV or TSV. In infected shrimp, *Pv*NV was detected in the RNA extracted from various tissues, such as pleopods, head soft tissues and hemolymph. The application of the RT-LAMP assay combined with colorimetric AuNP probes to detect the whether *Pv*NV infection in shrimp can offer a simple, rapid and sensitive technique that does not require sophisticated equipment and is applicable for small or field laboratories. The results revealed that RT-LAMP reaction successfully detected *Pv*NV at 63°C for 45 and 5 min for hybridization of the LAMP amplified product, followed by salt-induced AuNP-labeled ssDNA probe aggregation for visual color development. The method was 10 times more sensitive than conventional nRT-PCR. Cross-amplification with other shrimp pathogens (PemoNPV, PmDNA, WSSV, *Mr*NV, IMNV, TSV, YHV, IHHNV, *Vibrio harveyi* and *Pseudomonas aeruginosa*) revealed negative results, indicating that the RT-LAMP-AuNP assay was specific to *Pv*NV (Suebsing et al., 2013).

34.2.6.4 *CMNV* Detection Methods

The FISH and nRT-PCR assays were developed to enable achieving high-sensitivity CMNV detection. The results of these two detecting assays revealed that some specimens of *Penaeus chinensis*, *Penaeus vannamei*, and *Penaeus japonicus* with gross signs of atrophy hepatopancreas, soft shell, slow growth or whitish abdominal muscle were CMNV-positive. Specific analysis against the extracted RNA from healthy shrimp and nuclei acid samples from shrimp infected with YHV, *Mr*NV, WSSV and TSV indicated that nRT-PCR was specific for CMNV detection. The FISH results revealed fluorescent signals in the hepatopancreas, lymphoid organ and striated muscles of infected shrimp. The probe reacted most intensely with inclusions in the tubular epithelium and myoepithelium cells surrounding the hepatopancreatic tubules. For striated

muscles, a few inclusion-like bodies elicited an extremely strong fluorescent signal. Diffusely distributed fluorescence was observed within the muscle and lymphoid organ cells. No reaction was observed in tissues from uninfected shrimp (Zhang et al., 2014).

34.2.7 Prevention and Control

In aquaculture farms, no research has been performed into the controlling or preventing nodavirus infections in crustaceans. Preventive measures are the most efficient option to contain disease outbreaks because specific antiviral drugs are unavailable. Appropriate preventive measures such as the screening of broodstock and PL shrimp and good management practices may facilitate the prevention of nodavirus infections in shrimp aquaculture. In addition, the use of prophylactic measures such as immunostimulants and RNA interference (RNAi) (dsRNA) must be investigated to determine their effects in controlling nodavirus infections (Sahul Hameed and Bonami, 2012). The recombinant capsid (r-MCP) and RdRp proteins (RPs) of *Mr*NV are presumed to be immunostimulants but not subunit vaccines because invertebrates lack a true adaptive immune response. Adult prawns that received r-MCP exhibited a more favorable immune response than did those that received PBS (controls). Although no mortality was observed in the experimental adult prawns, the hematological and immunological parameters varied remarkably different with those of the control group. The PL *M. rosenbergii* were exposed to r-MCP by immersion route for 24 h and then infected with *Mr*NV–XSV. The qRT-PCR results revealed that the immune-related and heat-shock protein (HSP) genes enhanced mRNA expression in the r-MCP injected PL prawns. In addition, r-MCP stimulated the immune genes irrespective of the age of the prawn. Conversely, the mRNA expression of all immune and HSP genes was downregulated more in the *Mr*NV–XSV-infected PL prawns than in the PL prawns immersed in PBS. The survival of r-MCP pretreated *Mr*NV–XSV-infected PL prawns was estimated to be 76.03%, and that of untreated *Mr*NV–XSV-infected PL prawns was 0%. The results showed that r-MCP-enabled stimulation of the immune system of prawns facilitates controlling WTD (Farook et al., 2014b). In addition, adult prawns injected with 10 µg of recombinant RP of *Mr*NV exhibited significantly higher THC after 14 d p.i. The immune-related genes of PL prawns (β-glucan binding proteins, α-2 macroglobulin, cytochrome oxidase and manganese superoxidase dismutase) were remarkably upregulated 24 h and 7 d p.i. treatment, with 1 and 10 µg RP of *Mr*NV, respectively. Most of the tested samples were positive for *Mr*NV at 48 h post-immersion, as evaluated using RT-PCR analysis. After 14 days, *Mr*NV was detected in only the control prawns, and both the RP-injected groups tested negative for *Mr*NV infection. The results elucidated the potential role of RP in viral load reduction in *Mr*NV-infected prawns (Sahoo et al., 2012). The effect of the dietary brewer's yeast and nucleotides on immune responses and resistance to *Mr*NV–XSV infection was evaluated in freshwater prawns. Brewer's yeast (*Saccharomyces cerevisiae*) is a type of fungus that contains various immunostimulating compounds such as β-glucans, nucleic acids and mannan oligosaccharides, and has been used as a diet additive for various animals. Glucans and chitins are capable of enhancing innate immune responses. The cumulative survival rate was high in *M. rosenbergii* fed with 1% brewer's yeast-supplemented diet when they were infected with *Mr*NV–XSV. Immunological parameters, such as proPO activity and superoxide anion production, were considerably high in prawns fed with brewer's yeast. The THC increased remarkably in the treatment groups. The results revealed that using brewer's yeast as a dietary supplement is effective in enhancing immune responses and controlling *Mr*NV–XSV infections in *M. rosenbergii* (Parmar et al., 2012). Similar results were obtained for including nucleotide in the basal diet, which enhanced the immune responses and improved the resistance to *Mr*NV–XSV infections in freshwater prawns (Shankar et al., 2012).

Recognizing the intracellular dsRNA triggers RNAi, an innate immune response that can inhibit viral RNA in a sequence-specific manner in cells. The RNAi has been investigated to remedy a wide range of virus infections and is considered a potential antiviral tool. To test its activity against B2 and *Mr*NV viral proteins, an experimental model was developed in which RNAi were inoculated into redclaw crayfish. Mortality was 10% for redclaw crayfish exposed to stealth RNAi and *Mr*NV and 60% for those exposed to control stealth RNAi and *Mr*NV. Furthermore, the length and weight of redclaw crayfish with control stealth RNAi and *Mr*NV were remarkably smaller and lighter, respectively, than those of redclaw crayfish with stealth RNAi and *Mr*NV. According to qRT-PCR results, 2 of 10 redclaw crayfish with the stealth RNAi and *Mr*NV and 8 of 10 redclaw crayfish with the control stealth RNA and *Mr*NV treatment tested positive for *Mr*NV. Downregulating the viral defense by targeting the B2 protein gene with RNAi can effectively reduce mortality and limit *Mr*NV infection (Hayakijkosol and Owens, 2012). Synthetic short interfering RNA or dsRNA cannot be practically administered in shrimp farms; therefore, these compounds can be delivered only orally. Large-scale in vivo dsRNA production using RNase-deficient *Escherichia coli* strains is cheaper and more effective than chemically synthesis or using commercial in vitro transcription kit. Juvenile *M. rosenbergii* were fed with feed containing inactivated bacteria encapsulated with the dsRNA of *Mr*NV and XSV individually and in combination for 7 days and exposure to challenge with WTD-causing agents at 24 and 72 h, respectively, postfeeding. Test animals fed with a combination of dsRNA of *Mr*NV and XSV capsid protein

genes exhibited the highest relative percent survival. The results revealed that orally administering bacteria encapsulated specific dsRNA against *Mr*NV–XSV capsid protein genes is an effective, feasible and economical treatment option in *M. rosenbergii* (Naveenkumar et al., 2013a).

Nodavirus is one of the most serious disease affecting crustaceans, causing disastrous economic impacts on aquaculture. Currently, specific antiviral drugs are unavailable, and preventive measures are the only means of preventing disease outbreaks. Sensitive and specific diagnostic tools must be developed for detecting crustacean nodaviruses, particularly for *Pv*NV and CMNV. This is particularly critical because only limited information is available regarding the epidemiology, transmission and hosts of these viruses. For WTD, the interaction and pathogenicity between *Mr*NV and XSV requires further in-depth investigation. In addition, the resistance and immune responses mechanisms to these viruses of infected shrimp must be determined. Suitable receptive cell lines for the study of these crustacean viruses are lacking. Therefore, in vitro investigations using cell culture for viral detection and quantification must be developed.

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Chapter 35

Bunyaviruses of Crustaceans

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35.1 INTRODUCTION

Bunyaviruses infect a vast variety of vertebrates and plants and are mostly transmitted by arthropod vectors (Plyusnin et al., 2011; Walter and Barr, 2011). However, few have been identified to infect crustaceans (Table 35.1), and importantly none have yet been characterized in sufficient detail to be assigned formally within the *Bunyaviridae*. In addition, only two bunya-like viruses of crabs and two of penaeid shrimp have yet been identified that affect species of either wild fisheries or aquaculture significance (Corbel et al., 2003; Cowley et al., 2005a,b; Zhang, 2006; Li et al., 2015).

The *Bunyaviridae* currently comprises five genera (*Hantavirus* [type species Hantaan virus], *Nairovirus* [type species Dugbe virus], *Orthobunyavirus* [type species Bunyamwera virus], *Phlebovirus* [type species Rift Valley fever virus] and *Tospovirus* [type *species Tomato spotted wilt virus*]) and includes 19 groups of viruses and 21 ungrouped viruses for which insufficient data is available to define their taxonomic status (Plyusnin et al., 2011). Bunyavirus particles characteristically mature by budding through endoplasmic reticulum (ER) membranes associated with Golgi, and are often found clustered within cytoplasm vesicles (Elliott, 1996). Mature enveloped virions are generally spherical to ovoid in shape (~80–120 nm diameter) decorated with tightly packed diffuse surface projections that are sometimes visible on purified negatively-stained virions and virions detected in ultrathin tissue sections (Fig. 35.1). Viral nucleocapsids appear as beaded filaments or coils comprised of helical RNA segments bound to nucleocapsid (N) protein (Plyusnin et al., 2011; Elliott, 1996).

A prerequisite for classification as a bunyavirus is a genome comprised of three segments of single-stranded (ss)RNA designated as L (large), M (medium) and S (small) based on their size (Bishop, 1996; Walter and Barr, 2011). While the RNA segments are generally negative in polarity, the M RNA segment of tospoviruses and the S RNA segment of phleboviruses are ambisense and encode proteins in either orientation. The L RNA encodes a large RNA-dependent RNA polymerase (RdRp) (Walter and Barr, 2011). Depending on the genus, the M RNA encodes a polyprotein that is post-translationally processed in various manners to generate the two envelope glycoproteins (Pettersson and Melin, 1996). The S RNA encodes a nucleocapsid protein (N), and in the case of phleboviruses, also encodes a nonstructural protein (NS_S) in ambisense relative to the N coding sequence (Giorgi, 1996).

Arthropod-transmitted viruses of various rice, maize and wheat species classified within the unassigned *Tenuivirus* genus possess an RNA1 segment encoding an RdRp and a RNA2 segment encoding a 94 kDa protein that are most similar phylogenetically to the cognate proteins encoded in the L RNA and M RNA segments of phleboviruses (Falk and Tsai, 1998; Shirako et al., 2011). The genomic RNA segments of tenuiviruses also possess terminal-repeat sequences most similar to phleboviruses, with some RNA segments also encoding proteins in ambisense similarly, as an example, to the S RNA segment of phleboviruses (Falk and Tsai, 1998). Despite these clear evolutionary relationships, due to genomes of tenuiviruses comprising four to five RNA segments and them appearing to exist as filamentous nucleocapsids not reliant on virion formation for effective transmission, they fail to meet the criteria for formal classification within the *Bunyaviridae* (Shirako et al., 2011).

Interestingly, in retroviral elements integrated in the genome of the *Caenorhabditus elegans* nematode, a glycoprotein gene has been identified in the *env* gene location that is most closely related phylogenetically to the Gc glycoprotein encoded in the M RNA segment of phleboviruses (Malik et al., 2000). More recently, both phlebovirus-like and tenuivirus-like L RNA sequences encoding RdRp proteins have been detected in a soybean cyst nematode (Bekal et al., 2011), and sequences related to the L RNA segment of phleboviruses have been found integrated in the genomes of water fleas (*Daphnia pulex*, *Cladocera: Daphniidae*) (Ballinger et al., 2013) and the crustacean isopod *Armadillidium vulgare* (Thézé et al., 2014). These findings lend support to phylogenetic data suggesting that phleboviruses and tenuiviruses having descended from an extremely ancient viral lineage.

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00035-8
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TABLE 35.1 Bunyalike Viruses Detected in Crabs and Shrimp

Virus Name	Abbreviation	Hosts	References
Crab hemocytopenic virus	CHV	*Carcinus maenas*	Bang (1971, 1974, 1983), Hoover (1977), Hoover and Bang (1978)
S virus		*Liocarcinus depurator*	Bonami et al. (1975), Bonami (1980)
	*Es*BV	*Carcinus mediterraneus*	Mari (1987)
Cancer pagurus systemic bunyalike virus	*Cp*SBV	*Carcinus pagurus*	Corbel et al. (2003) Zhang (2006)
		Eriocheir sinensis	
Mourilyan virus	MoV	*Penaeus monodon*, *Penaeus japonicus*	Cowley et al. (2005a,b)
Wenzhou shrimp virus 1		*Penaeus monodon*	Li et al. (2015)
Wenzhou shrimp virus 2		*Penaeus monodon*	Li et al. (2015)
		Palaemon carinicauda	

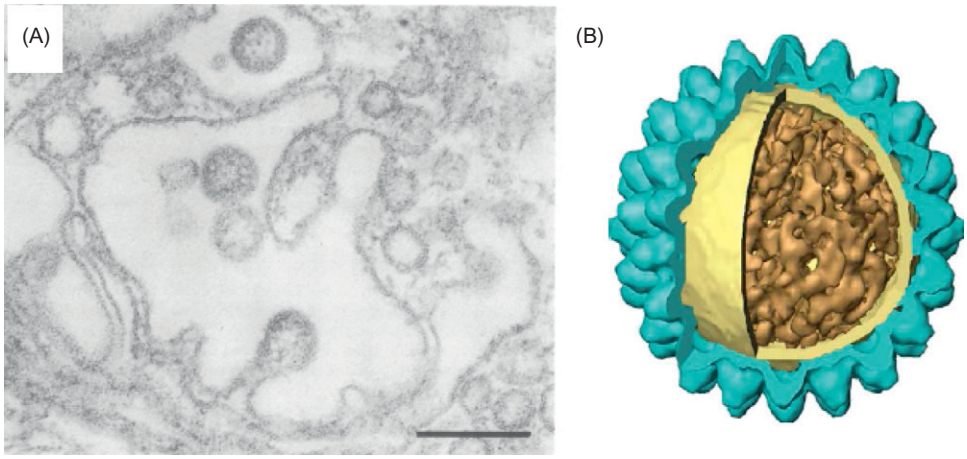

FIGURE 35.1 (A) Transmission electron micrograph of an ultrathin section of a cell infected with Uukuniemi virus (UUKV) showing mature virions within and budding into smooth-surfaced cytoplasmic vesicles, bar = 200 nm (Kuismanen et al., 1982), (B) composite model of a virion of UUKV showing the topology of the envelope glycoprotein spikes (blue (dark gray in print versions)), the viral membrane (yellow (white in print versions)) and the ribonucleoprotein-RNA segments (brown (light gray in print versions)) of the viral genome (Overby et al., 2008).

35.2 BUNYALIKE VIRUSES OF CRABS

Crustacean viruses with bunyavirus-like particle morphology and morphogenesis characteristics were first reported in the early 1970s in European shore crabs (*Carcinus maenas*) (Bang, 1971; Fig. 35.2) and Mediterranean sandy swimming crabs (*Liocarcinus depurator*) (Bonami and Vago, 1971) collected along the coast of France, and subsequently in European edible crabs (*Cancer pagurus*), which occur in abundance throughout the northeast Atlantic and are an important wild fisheries species, collected from the same region (Corbel et al., 2003; Fig. 35.3). Elongated enveloped virus particles (60–90 nm × 150–178 nm) described as *Y-organ virus* with more tenuous bunyavirus characteristics have also been detected in the Y (molting) organs of Mediterranean Green crabs (*Carcinus mediterraneus*) (Zerbib et al., 1975) and *C. maenas* (Chassard-Bouchaud and Hubert, 1975; Chassard-Bouchaud et al., 1976; see review Johnson, 1983). Except for *Cancer pagurus systemic bunyalike virus* (*Cp*SBV), which possesses a tripartite RNA genome characteristic of the *Bunyaviridae* (Corbel et al., 2003; Elliott, 1996), information on the genome composition of these viruses is limited.

Descriptions here will be restricted to Crab hemocytopenic virus (CHV) of *C. maenas*, *S virus* of Macropipus *depurator* and *Cp*SBV of *Cancer pagurus* and will be brief because their clinical features, structure and morphogenesis characteristics

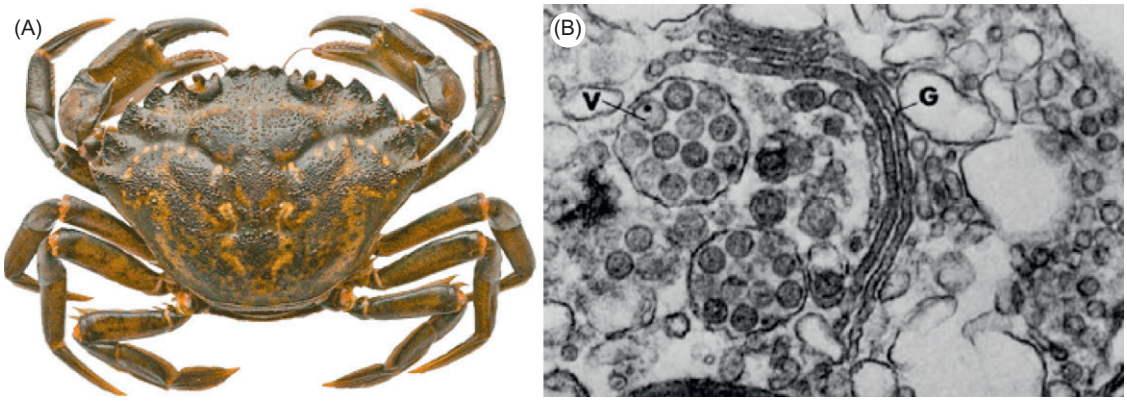

FIGURE 35.2 (A) European shore crab (*Carcinus maenas*), (B) transmission electron micrograph of an ultrathin section of a hemocyte of a *Carcinus maenas* crab showing ovoid-shaped enveloped virions of *Crab hemocytopenic virus* in close proximity to Golgi membranes and sometimes clustered within cytoplasmic vesicles, ×57,270 magnification, *V*, virion, *G*, Golgi apparatus (Hoover, 1977).

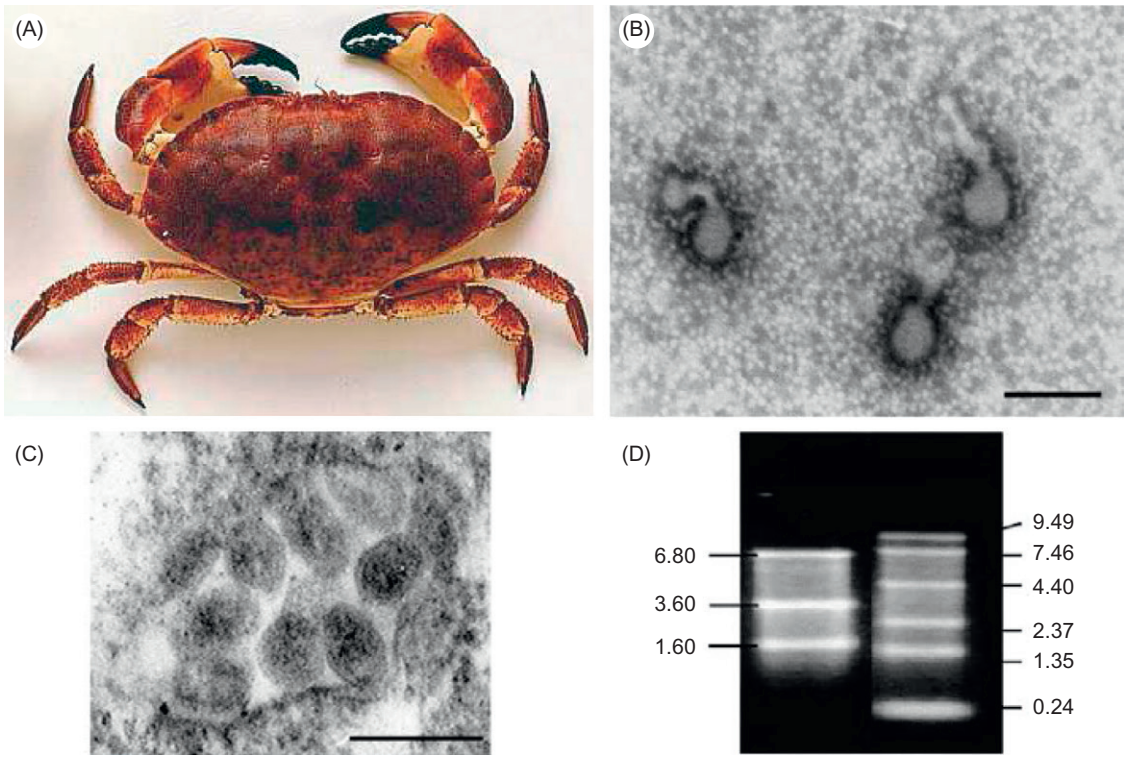

FIGURE 35.3 (A) European edible crab (*Cancer pagurus*), (B) negatively-stained virions of *Cancer pagurus systemic bunyalike virus* (*Cp*SBV) purified from hemolymph and displaying tail-like structures, bar = 100 nm (Corbel et al., 2003), (C) TEM of an ultrathin section of hepatopancreatic tissue of a *Cancer pagurus* crab showing ovoid enveloped *Cp*SBV virions clustered within a cytoplasmic vesicle, bar = 100 nm (Corbel et al., 2003), (D) L (6.8 kb), M (3.6 kb) and S (1.6 kb) genomic RNA segments of *Cp*SBV and ssRNA size markers resolved by electrophoresis in an agarose gel (Corbel et al., 2003).

have been reviewed previously (Johnson, 1983; McCladdery, 2011; Zhang, 2011), and no new information has been reported since these reviews and none of these viruses have been identified to affect crab species of aquaculture importance.

35.2.1 *CHV* or Roscoff Virus

A bunya-like virus detected in *Carcinus maenas* was named CHV based on its clinical manifestation or alternatively as *Roscoff virus* due to its discovery in one of approximately 700 crabs collected at Roscoff in France (Bang, 1971, 1974,

1983; Hoover, 1977; Johnson, 1984). Hemolymph clotting was compromised in this crab and the transfer of 0.22 µm membrane-filtered hemolymph to naïve *C. maenas* reproduced the condition in 74% crabs within 2–19 days post-challenge, with clotting function then recovering 4–6 days after onset in the 50–60% crabs that survived (Bang, 1971, 1974, 1983). When collected within the first 2 days of the clotting disorder arising, the ability of hemolymph to transmit disease reduced upon dilution. When collected at later times, however, hemolymph dilution was needed to reproduce the condition, suggesting that some auto-interference process had been activated or that host antiviral factors had accumulated (Bang, 1974; Johnson, 1983). With some crabs that recovered, the ability to transmit disease persisted for up to 29 days (Bang, 1974, 1983).

The clotting defect in CHV-infected crabs coinciding with an approximately seven-fold decrease in circulating hemocytes due to hemocytes becoming clumped within peripheral vascular channels in hepatopancreas, heart, gill and ovary connective tissues, and also with displacement of ovarian tissue and with striated muscle damage (Bang, 1971, 1983; Hoover and Bang, 1978, McCladdery, 2008). Necrotic hemocytes with distended ER structures and other cytoplasmic abnormalities were also evident. Loss of disease transmission by heating 0.22 µm membrane-filtered hemolymph at 70°C for 5 min further supported involvement of a virus.

Transmission electron microscopy (TEM) of hemocyte sections identified CHV particles typical in morphology to bunyaviruses (Bang, 1971; Hoover, 1977). The particles were spherical to ovoid in shape (95–125 nm diameter) with fuzzy surface rendering, and possessed an envelope in which the lipid membrane was sometimes evident as an electron-dense double-track structure (Fig. 35.2). Particles were commonly seen within cytoplasmic vesicles and associated with membranes in the Golgi or ER. Free CHV particles were also common in the cytoplasm, and observed at cell plasma membranes as well as in extracellular spaces as a result of endoplasmic vesicles fusing with the plasma membrane (Hoover, 1977; Johnson, 1983). CHV particles generally occurred in highest abundance in mature granulocytes and while not detected in gonad cells, virus-infected hemocytes were noted to accumulate within ovarian tissue.

CHV has not been studied further, and as yet, nothing is known about its genome composition or sequence critical to it being classified within the *Bunyaviridae*. Also nothing is known about the susceptibly of other crustacean species and, thus, what risks CHV might pose to crustaceans of significant wild fisheries or aquaculture importance.

35.2.2 S Virus

S virus was detected in captive *L. depurator* examined due to unexplained rapid mortality in the absence of disease gross signs (Bonami and Vago, 1971; Bonami et al., 1971). Hemolymph transfer to naïve crabs induced morbidity within 10 days and 70–80% mortality within 15–20 days (Bonami et al., 1971). Virions purified from hemolymph using sucrose density gradient ultracentrifugation were pleomorphic, with most ovoid in shape (80–13 nm × 190–230 nm) but others more spherical (80–150 nm diameter) or elongated (50–70 nm × 240–320 nm), and possessed a 22 nm thick electron translucent envelope decorated with distinct regularly spaced projections (Bonami and Vago, 1971; Bonami et al., 1971). Virus particles detected in the cytoplasm of cells within the heart and connective tissues in the hepatopancreas were more uniformly spherical in shape, suggesting that the more pleomorphic particles were an artifact of the virion purification process (Bonami et al., 1971, 1975).

The genome of *S virus* has been determined to comprise single-stranded RNA (Bonami et al., 1975; Bergoin et al., 1982). However, whether it is tripartite in nature is not known, and because the S virus particle morphology displays characteristics of viruses classified within both the *Paramyxoviridae* and the *Bunyaviridae*, its definitive taxonomic classification awaits more detailed genome characterization.

35.2.3 *Cancer pagurus* Systemic Bunyalike Virus (*Cp*SBV)

*Cp*SBV was detected by electron microscopy in hemolymph of wild *Cancer pagurus* collected from Brittany in France (Corbel et al., 2003; Fig. 35.2). Captive crabs were examined due to unexplained mortalities in the absence of disease gross signs. Virions purified from hemolymph and gill tissue by sucrose density gradient ultracentrifugation were enveloped and spherical to ovoid in shape (60–70 nm diameter), with many possessing tail-like appendages (25–35 nm diameter, 70–110 nm long) (Fig. 35.3). Virus particles similar in morphology, but lacking tails and displaying more obvious double-track structures characteristic of a lipid membrane, were observed congregated within cytoplasmic vesicles in connective tissue cells within various organs (Fig. 35.3; Corbel et al., 2003). A virus particle similar in morphology has been reported in moribund Chinese mitten crabs (*Eriocheir sinensis*) examined from Hubei Province in China (Zhang, 2006).

Nucleic acid extracted from purified virions was sensitive to ribonuclease, but not deoxyribonuclease digestion, and agarose gel electrophoresis identified the *Cp*SBV genome to comprise three ssRNA segments (6.8, 3.6 and 1.6 kb) consistent in length with the L, M and S RNA segments of many bunyaviruses (Corbel et al., 2003; Plyusnin et al., 2011; Elliott, 1996; Fig. 35.3).

Experimental infection of naïve *Cancer pagurus* with *Cp*SBV purified from hemolymph resulted in mortality within 7–12 days in the absence of disease gross signs (Corbel et al., 2003). Since the infected crabs originated from tanks at a commercial enterprise, it is not known whether *Cancer pagurus* is a natural host of *Cp*SBV. However, it has been speculated that *Cp*SBV infection might be a factor in post-capture mortalities that can occur during holding and transportation of this commercially fished crab species (Corbel et al., 2003; Stentiford, 2008).

As for other suspected crab bunyaviruses, taxonomic classification of *Cp*SBV within the *Bunyaviridae* awaits genome sequence information that will also relieve the current reliance on electron microscopy for virus detection.

35.3 BUNYA-LIKE VIRUSES OF SHRIMP

35.3.1 Discovery

Mourilyan virus (MoV) was discovered by serendipity in 1996 in moribund Giant Tiger shrimp (*Penaeus monodon*) collected from a farm near Mourilyan in north QLD, Australia (Cowley et al., 2005a,b). The moribund shrimp were collected from ponds experiencing mass mortality due primarily to acute infection with gill-associated virus (GAV), a crustacean nidovirus (Spann et al., 1997; Cowley et al., 2000, 2012; Cowley and Walker, 2002). To obtain GAV genome sequence information, a long (>20 kb) double-stranded (ds)RNA identified in lymphoid organ total RNA extracted from diseased *P. monodon* was gel-purified and amplified using a random RT-PCR method to generate a library of cDNA clones (Cowley et al., 2000; Cowley and Walker, 2002; Dumas et al., 1991). Among the clones sequenced, three non-GAV sequences were identified that possessed open reading frames (ORFs) most similar in amino acid sequence to the RdRp and Gn/Gc envelope glycoproteins encoded in the L and M RNA segments of Uukuniemi virus (UUKV), a tick-transmitted virus infecting birds, as well as other viruses in the *Phlebovirus* genus of the *Bunyaviridae* (Cowley et al., 2005a,b).

Recent RNA-Seq analyses of the transcriptome of 70 diverse arthropod species discovered genome sequences of 112 novel viruses (Li et al., 2015). Among these, a virus (Wenzhou shrimp virus 1) was discovered in *P. monodon* for which L, M and S RNA segments were identified that possessed sequence similarities to phleboviruses and tenuiviruses and another virus (Wenzhou shrimp virus 2) was discovered in *P. monodon* and Ridgetail shrimp (*Palaemon carinicauda*) for which L and M RNA segments were identified that had distant sequence similarities to hantaviruses (Li et al., 2015). Basic local alignment search tool (BLAST) alignments of the nucleotide- and protein-coding sequences of the MoV L, M and S1 RNA segments identified MoV and Wenzhou shrimp virus 1 to be closely related variants of the same virus (Li et al., 2015; Cowley, unpublished).

35.3.2 Genome Composition of MoV

To determine the MoV genome composition, clones were generated using a 5′-random amplification of cDNA ends (5′-RACE) method and PCR primers targeted to the putative M RNA segment clones. Sequence analysis of these clones identified the MoV M RNA segment to possess a terminus identical in sequence to the nine nucleotide (nt) inverted terminal repeats conserved at the ends of the three RNA genome segments of UUKV and other phleboviruses, and to a lesser extent, the genomic RNA segments of thrip-transmitted tenuiviruses of plants (Cowley et al., 2005a,b; Giorgi, 1996; Falk and Tsai, 1998). Amplification of random-primed cDNA using a PCR primer terminated with this sequence together with primers specific to and facing either orientation in the MoV M or L RNA sequences-generated products that completed the M RNA (2987 nt) and L RNA (6319 nt) sequences. Using this same cDNA and terminal-repeat primer in a single-primer PCR approach, three products of ~3.0, ~1.6 and ~1.4 kb in length were amplified (Cowley et al., 2005a). Sequence analysis of clones generated to the two shorter PCR products identified a S1 RNA segment, for which six clones that were sequenced all varied from 1557 to 1079 nt in length, and a 1364 nt S2 RNA segment. The S1 RNA segment length variation was due to deletions within an approximately 650 nt imperfect GA repeat sequence that extended from immediately downstream of a 246 aa ORF TAA stop codon to a 99 nt terminal sequence (Cowley et al., 2005a).

Northern blotting using lymphoid organ total RNA extracted from diseased *P. monodon* and digoxigenin (DIG)-labeled DNA probes prepared to L, M, S1 and S2 RNA segment sequences confirmed the existence and approximate length of each of the four MoV RNA segments (Fig. 35.4). The blot also showed the S1 RNA segment to resolve in heat denaturing agarose gels as a discrete ~1.6 kb band, suggesting that the shorter-length clones obtained in the single-primer PCR approach were likely artifacts of this method. Indeed, Illumina GAIIx sequence analysis of a TruSeq library generated to random-primed cDNA prepared to RNA isolated from MoV material semi-purified by ultracentrifugation from clarified shrimp hemolymph has confirmed the existence of the four MoV RNA segments, with only minor sequence and length discrepancies identified compared with the sequences determined from the various PCR-based approaches (Cowley et al.,

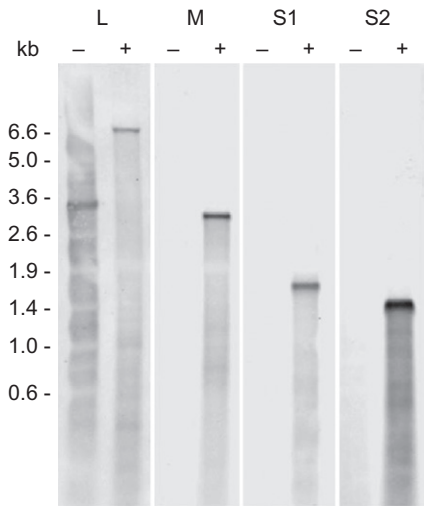

FIGURE 35.4 Northern blot of lymphoid organ tissue total RNA from naïve (−) and MoV-infected *Penaeus monodon* (+) showing the relative size of bands representing the MoV L (~6.7 kb), M (~3.0 kb), S1 (~1.8 kb) and S2 (~1.4 kb) RNA segments detected by segment-specific DNA probes (Cowley, unpublished).

TABLE 35.2 Predicted Functions, Lengths and Unmodified Masses of Proteins Encoded in the MoV L, M, S1 and S2 Genomic RNA Segments

RNA Segment	Length (nt)	UTR Lengths (nt)		Protein Name	Length (aa)	Mass (kDa)	Function
		3′	5′				
L	6319	93	57	RdRp	2051	233.0	RNA transcription/replication
M	2990	172	94	Gn/Gc	907	99.3	Precursor polyprotein
				Gn	409	45.7	Mature envelope glycoprotein
				Gc	480	51.6	Mature envelope glycoprotein
S1	1557	744	72	N	246	27.4	Nucleocapsid protein
S2	1364	99	80	NS_{S2}	394	45.6	Unknown

UTR, untranslated region; nt, nucleotide.

unpublished). The Illumina data also confirmed the sequence of one of the S1 RNA segment termini and extended terminal sequences of other MoV RNA segments not determined empirically from the PCR data.

Characteristics of the four ssRNA segments comprising the 12,227 nt MoV genome and the proteins they encode are summarized in Table 35.2. Molecular aspects of how the MoV genome is replicated and transcribed have yet to be determined, including whether mRNA transcripts acquire 5′-7-methylguanosine caps as in other bunyaviruses by the cap-snatching mechanism that involves the RdRp recognizing and utilizing short-capped "primers" cleaved from host mRNAs by a virus-encoded 3′-endonuclease (Reguera et al., 2010).

35.3.3 Proteins Encoded in the MoV RNA Segments

The MoV L RNA segment encodes a 2051 aa RdRp (233.0 kDa) containing amino acid sequences predictive of the polymerase module domains (Pre-A, A, B, C, D, E) conserved across all bunyaviruses and critical to RdRp functioning in mRNA transcription and RNA segment replication (Müller et al., 1994; Poch et al., 1990; Cowley, unpublished).

Similarly to bunyaviruses and to phleboviruses in particular (Pettersson and Melin, 1996), the MoV M RNA segment encodes a 907 aa Gn-Gc precursor polyprotein (99.3 kDa nonglycosylated mass). The MoV Gn-Gc polyprotein sequence contains four hydrophobic motifs consistent in position and structure to those functioning as either signal peptides or transmembrane-anchoring domain in the Gn-Gc polyprotein of UUKV and other phleboviruses (Pettersson and Melin, 1996; Cowley, unpublished). Cleavage at the predicted N-terminal and internal signal peptides would result in mature MoV Gn

and Gc glycoproteins 408 and 480 aa in length, respectively. Cysteine residues in the predicted Gn and Gc ectodomains are also highly conserved with those in the cognate UUKV glycoproteins. Thus as in all bunyaviruses, it is likely that the MoV Gn-Gc polyprotein precursor will be co-translationally inserted and cleavaged in ER membranes, with disulfide-linked Gn-Gc heterodimers then transported to the Golgi compartment to facilitate virion assembly (Pettersson and Melin, 1996; Walter and Barr, 2011). Like other bunyaviruses, the Gn-Gc heterodimers anchored in the envelope of MoV particles will likely have key functions in cell attachment and entry (Pettersson and Melin, 1996).

The MoV S1 RNA segment encodes a 246 aa N protein (27.4 kDa) that, in size as well as charge and structure predicted from its sequence, displays similarities to 245–293 aa N proteins of phleboviruses (Walter and Barr, 2011; Cowley, unpublished). The MoV N protein is thus expected to have primary functions in encapsidating the genomic RNA segments to form ribonucleoprotein complexes that then interact with C-terminal Gn glycoprotein sequences to facilitate virion maturation (Walter and Barr, 2011).

The MoV S2 RNA segment encodes a 394 aa nonstructural NS_{S2} protein (45.6 kDa) that is somewhat larger than the NS_S protein (248–316 aa) encoded in ambisense in the S RNA segment of phleboviruses (Giorgi, 1996). Since the sequence of the MoV NS_{S2} is unique like those of the NS_S proteins of phleboviruses, it cannot be speculated whether it might confer similar functions including the proteasomal degradation of double-stranded RNA-dependent protein kinase as found in Rift Valley fever virus and Toscana virus (Kalveram and Ikegami, 2013; Kalveram et al., 2013), or in interacting with small dsRNAs to counteract the RNA interference (RNAi) mechanism and thus disable the host antiviral response as found in the plant-infecting tospoviruses (Takeda et al., 2002; Schnettler et al., 2010). Preliminary experiments have undertaken to knockdown NS_{S2} protein expression by injecting shrimp with a S2 RNA segment-specific dsRNA prior to injection challenge with an inoculum containing GAV as well as MoV (Oanh et al., 2011). However, the data did not help resolve speculation surrounding the function of the NS_{S2} protein because this dsRNA did not reduce MoV genetic loads or enhance knockdown of GAV replication in shrimp coinjected with a dsRNA targeted to the helicase domain in the GAV ORF1b gene.

35.3.4 Phylogenetic Relationship to Bunyaviruses and Tenuiviruses

Phylogenetic analysis of the 2051 aa RdRp sequence encoded in the L RNA segment positions MoV on a branch between phleboviruses and the tenuiviruses, Gouléako virus, a potential novel bunyavirus genus found recently in various mosquito species in west Africa (Marklewitz et al., 2011) and two L RNA-encoded RdRp sequences discovered recently in a Soybean cyst nematode to be most similar to UUKV and Rice stripe tenuivirus (Bekal et al., 2011; Fig. 35.5). Phylogenetic analyses of the amino acid sequences of the MoV Gn-Gc precursor polyprotein encoded in the M RNA segment and the N protein encoded in the S1 RNA segment position MoV similarly between phleboviruses and tenuiviruses (Cowley, unpublished). No proteins with significant similarity to the 394 aa NS_{S2} protein (S2 RNA nonstructural protein) encoded in the unique S2 RNA segment of MoV currently exist in the GenBank database.

Unlike bunyaviruses, tenuiviruses exist as filamentous nucleocapsids not dependent on virion formation for replication and transmission (Falk and Tsai, 1998), but like MoV, possess genomes comprising either four or sometimes five (−) ssRNA segments rather than three, which is currently mandatory for classification in the *Bunyaviridae* (Elliott, 1996; Plyusnin et al., 2011; Walter and Barr, 2011). Based on its phylogenetic relationships, a genome comprising four discrete ssRNA segments and an ability to form virions characteristic of bunyaviruses, MoV appears to fill the evolutionary void between the phleboviruses and the unassigned plant tenuiviruses. On this premise, it might be pertinent that during a preliminary investigation into how the MoV precursor polyprotein is processed into its predicted Gn and Gc components, clones of the M RNA sequence were identified to possess stop codons interrupting the Gn/Gc coding sequence (Henquet, 2002). It is possible, therefore, that MoV carries defective M RNA segments that limit the rate at which the mature Gn and/or Gc glycoproteins can accumulate. This in turn would have consequences for the rate at which MoV particles can mature, and might explain why they are seen infrequently and only in highly necrotic cells. It is possible that the MoV genome also shares the tenuivirus characteristic of not necessarily needing to form virions to facilitate cell to cell transmission because the MoV genome comprises four RNA segments like tenuiviruses. If so, this would explain why deleterious mutations can accumulate in some M RNA segments without impacting the ability of MoV to replicate and be transmitted.

Available data on the genome composition of MoV suggest that the taxonomic criteria for virus classification within the *Bunyaviridae* might need to be relaxed to allow its inclusion as a novel genus or subfamily, which would then open the door for tenuiviruses to be included similarly.

35.3.5 Particle Morphology and Morphogenesis

Bunyaviruses typically form spherical to ovoid-shaped virions approximately 80–120 nm in diameter that possess a 5–7 nm thick lipid envelope decorated with densely packed diffuse surface projections between 5 and 10 nm in length (Murphy

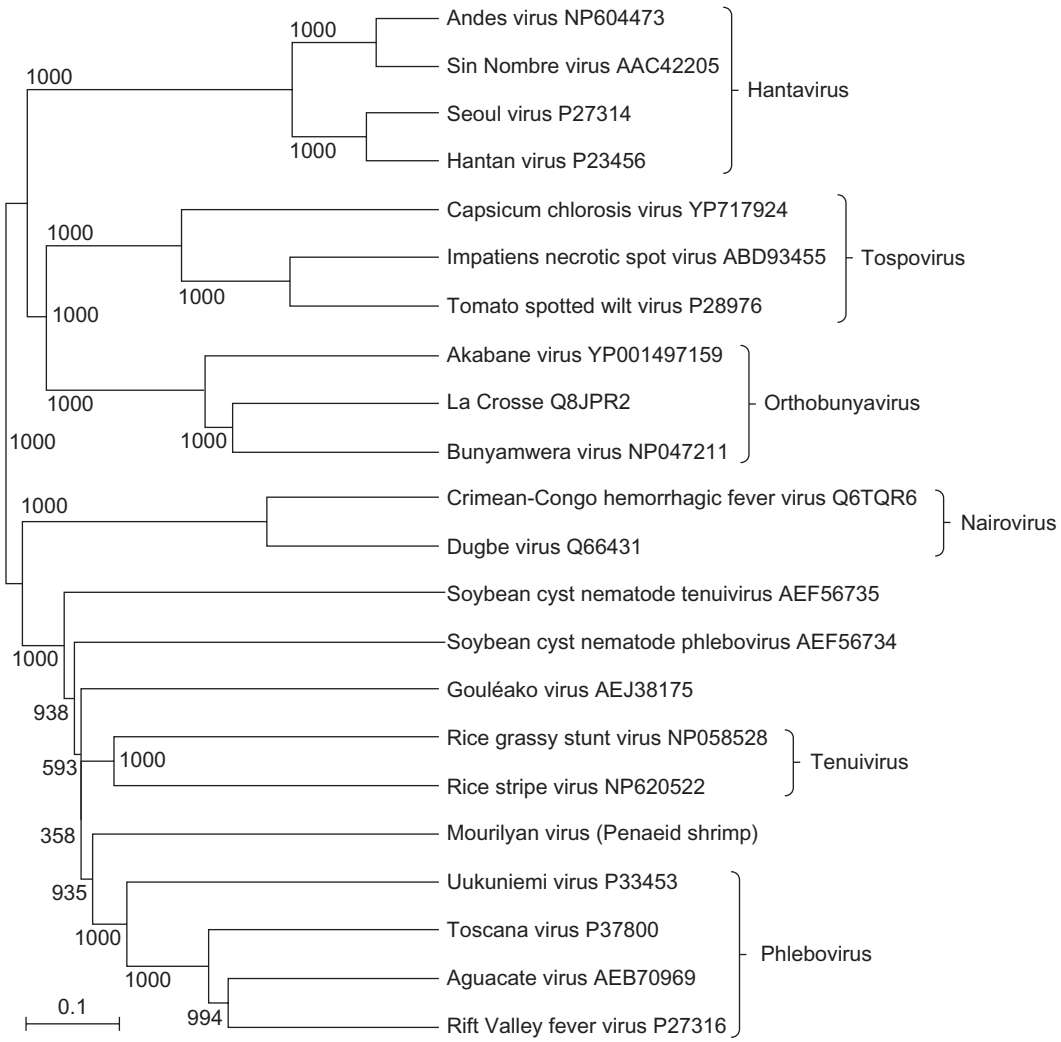

FIGURE 35.5 Neighbor joining phylogenetic tree generated from a ClustalX multiple alignment of the RNA-dependent RNA polymerase (RdRp) sequence encoded in the MoV L RNA segment with cognate RdRp sequences of representative viruses in the *Hantavirus*, *Tospovirus*, *Orthobunyavirus*, *Nairovirus* and *Phlebovirus* genus members of the *Bunyaviridae* as well as the unassigned plant *Tenuivirus* genus and selected other viruses including *Gouléako virus* isolated from West African mosquitoes (AEJ38175) and novel phlebovirus- (AEF56734) and tenuivirus-like (AEF56735) RdRp sequences discovered in a soybean cyst nematode.

et al., 1968, 1973; Bishop, 1996). The morphology of MoV particles detected by TEM in highly necrotic gill cells of *P. monodon* and in lymphoid organ cells of Kuruma shrimp (*Penaeus japonicus*) with acute MoV infections are consistent with bunyaviruses including UUKV (Figs. 35.1 and 35.6; Saikku and Von Bonsdorff, 1968; Von Bonsdorff et al., 1970; Von Bonsdorff and Pettersson, 1975) as well as the crab bunya-like viruses CHV (Fig. 35.2; Bang, 1971; Hoover and Bang, 1976) and *Cp*SBV (Fig. 35.3; Corbel et al., 2003). MoV particles are spherical (~85 nm) to ovoid (~85 nm × 100 nm) in shape with a fuzzy and sometimes dappled surface under which a double-layered electron-dense outer ring structure comprising the lipid envelope can sometimes be observed (Cowley et al., 2005a,b). Although estimated to be slightly smaller in size (50–96, 74 nm average diameter), spherical to ovoid-shaped enveloped particles similar in morphology to MoV have also been observed aggregated loosely within cytoplasmic vesicles in cells in the fasciculated zone of the eye of *P. monodon* that displayed no specific signs of disease and were examined from farms in Australia (Fig. 35.6; Smith, 2000).

In gill cuticle epithelium cells of diseased farmed *P. monodon* displaying condensed chromatin and disrupted cytoplasmic organelles indicative of advanced necrosis, MoV particles have been observed associated with the outer ring of unusual concentric "onion ring" structures composed of multiple endoplasmic membranes (Cowley et al., 2005a). The nature of these structures is not known. However, in sea urchin eggs, similar concentric ring structures of endoplasmic membranes fitting the "whorl" description can be stimulated by exposure to a hypotonic environment (Kallenbach, 1984).

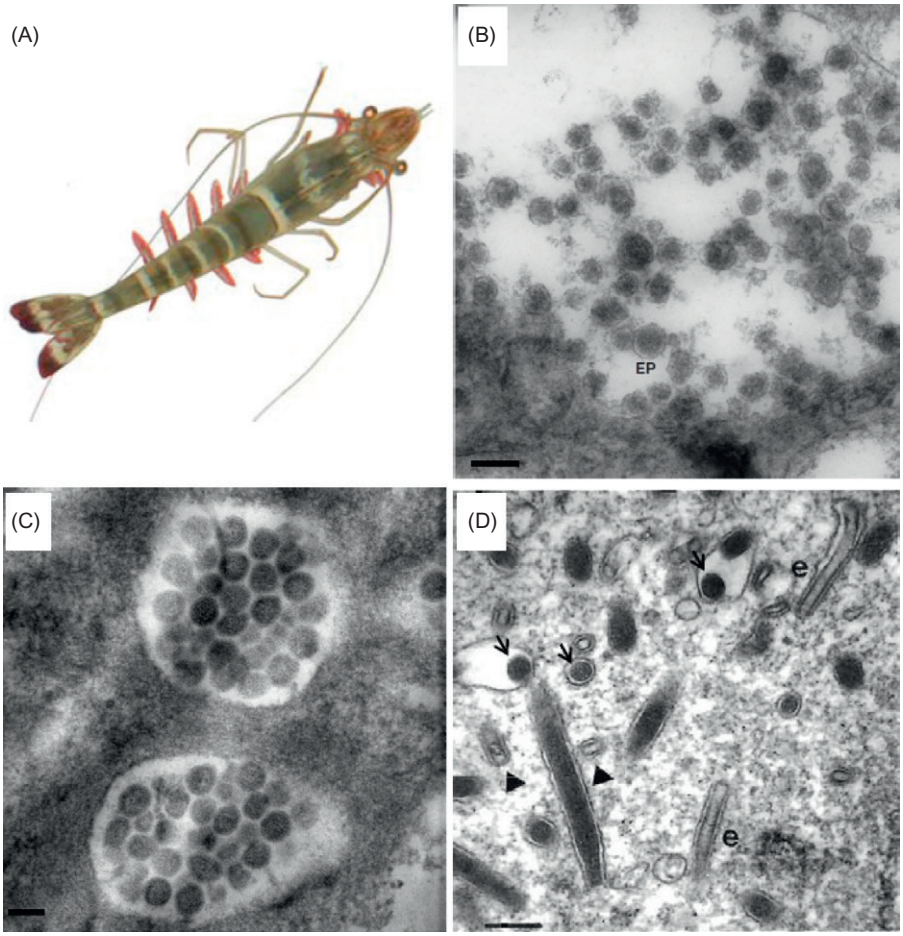

FIGURE 35.6 (A) Giant Tiger shrimp (*Penaeus monodon*), (B) TEM of enveloped particles (EP) in the proximal region of the fasciculated zone of the eye of *Penaeus monodon*, bar = 100 nm (Smith, 2000), (C) ovoid-shaped enveloped MoV particles clustered within cytoplasmic vesicle-like structures in a highly degenerated lymphoid organ cell, bar = 100 nm (Cowley et al., 2005a), (D) tubular structures described as *Penaeid hemocytic rod-shaped virus* detected in hybrids of *Penaeus monodon* and *Penaeus esculentus* (Owens, 1993). The tubular structures described as virions (119 nm × 588 nm) are visible together with narrower, less electron-dense tubules (50 nm × 496 nm) described as empty capsids (e) and enveloped structures described as capsid originators (arrowheads) in addition to ovoid-shaped enveloped virions morphologically similar to MoV (arrows) appearing to have budded into cytoplasmic vesicles, bar = 200 nm.

It possible, therefore, that these concentric membrane structures associated with MoV particles represent Golgi or ER membrane structures disrupted by osmotic changes in the cytoplasm due to the advanced necrosis and cells possibly undergoing lysis.

As for other bunyaviruses, mature MoV particles can occur in large clusters within cytoplasmic vesicles and have been observed in the vicinity of cytoplasmic tubular structures ~75–90 nm in diameter and up to 570-nm long (Cowley et al., 2005a). In interspecies hybrids of Brown tiger shrimp (*Penaeus esculentus*) and *P. monodon* examined due to gradually accumulating mortalities, similar elongated tubular structures (86 nm diameter × 542–888 nm long) suggested to be viral in origin were identified together with ovoid-shaped MoV-like particles in the cytoplasm of small-granular hemocytes present in gill tissue (Fig. 35.6; Owens et al., 1991, 1992; Owens, 1993). Tubular structures similar in dimensions have been observed in the cytoplasm of cells infected with bunyaviruses, including UUKV (Von Bonsdorff et al., 1970) and Bunyamwera virus. The tubules occur in the trans-Golgi compartment proximally to mitochondria, and have identified to contain viral proteins and cellular actin integral to their functioning as factories for virion morphogenesis (Murphy et al., 1968; Salanueva et al., 2003; Novoa et al., 2005; Fontana et al., 2008). The available data suggest that MoV particle morphogenesis occurs at Golgi membranes using processes similar to those employed by other bunyaviruses (Jäntti et al., 1997; Salanueva et al., 2003; Walter and Barr, 2011).

35.3.6 Host Range and Distribution

In eastern Australia, MoV infection occurs commonly in farmed *P. monodon* and *P. japonicus*, and PCR screening of farmed white banana prawn (*Penaeus merguiensis*) has detected it at low levels that were not presumptive of productive infection (Cowley et al., 2005a,b). MoV also occurs among *P. monodon* inhabiting Fiji (Waqairatu and Pickering, 2005), the Andaman Sea and the Gulf of Thailand in Malaysia and Thailand (Jittivadhna et al., 2003; Flegel, 2006; OIE, 2007), as well as in wild *P. monodon* inhabiting the east coast of Queensland, the Gulf of Carpentaria and Joseph Bonaparte Gulf in northern Australia (Cowley et al., 2009 and unpublished). MoV has also been detected by PCR at low prevalence and infection loads in wild *P. japonicus* captured from north Queensland (Cowley, 2005). In a 267 nt region of the Gc glycoprotein coding sequence in the M RNA segment targeted by a RT-nested PCR test (Cowley et al., 2005a), up to 5% nucleotide variation has been identified between Asian MoV strains and strains occurring in Australia (Jittivadhna et al., 2003). These data suggest the evolution of geographic variants of MoV.

35.3.7 Pathogenicity

Penaeus japonicus are farmed in small quantities in Australia and have been domesticated and selectively bred to improve growth performance (Hetzel et al., 2000; Preston et al., 1999, 2004). Despite being domesticated easily, attempts to rear *P. japonicus* broodstock in commercial ponds have been frustrated repeatedly by idiopathic mortalities accumulating progressively 4–6 weeks after shrimp transfer to heated tanks for rearing through winter (Sellars et al., unpublished). MoV infection loads can become elevated during pond rearing of *P. japonicus* (Cowley et al., 2005a), and the species is highly susceptible to experimental MoV infection via muscle infection (Rajendran et al., 2006). To specifically associate elevated MoV infection with idiopathic mortalities in *P. japonicus*, five sibling batches reared either in tanks or commercial ponds for 6 months to subadults were transferred to identical tanks systems and monitored for 56 days (Keys et al., 2004; Sellars et al., 2006). Mortalities among pond-reared shrimp began to accumulate within 4–6 days, and mean survival at the end of the 56-day period was 11% compared with 76% for the tank-reared shrimp. MoV was detected by RT-PCR in only 2 of 30 tank-reared shrimp, but in 28 of 30 pond-reared shrimp at generally higher infection loads (Sellars et al., 2006). In support of these data indicating that acute MoV infection can promote elevated mortalities in *P. japonicus*, similar gradually accumulating deaths have been observed among both pond-reared and tank-reared juvenile *Penaeus japonicus* challenged by muscle injection of a 0.22 µm membrane-filtered cephalothorax tissue homogenate of *P. monodon* infected at high loads with MoV in the absence of GAV (Cowley et al., unpublished).

As well as in moribund *P. monodon* infected acutely with GAV (Owens, 1993; Spann et al., 1997; Cowley et al., 2005a,b), extremely high-level MoV infections can occur in overtly healthy *P. monodon* either in the absence or presence of low-grade GAV infection (Cowley et al., unpublished). While no direct challenge experiments have been reported for *P. monodon*, available data suggest that MoV possesses low pathogenicity and does not contribute significantly to disease and morbidity in this species. In support of this, in *P. monodon* challenged with an inoculum containing high infectious loads of both MoV and GAV, the progress of morbidity and mortality was unaltered in shrimp in which MoV replication was inhibited completely by RNAi activated by muscle injection of a MoV L RNA segment-specific dsRNA (Oanh et al., 2011). Acute MoV infection does not thus appear to exacerbate mortality rates in *P. monodon* resulting from acute GAV infection.

35.3.8 Tissue Distribution and Pathology

In *P. japonicus* with subclinical MoV infections, lymphoid organ spheroids comprising partitioned aggregates of cells with hypertrophied nuclei and a variably vacuolated cytoplasm are often the only histopathology suggestive of viral infection (Cowley et al., 2005a,b). While in situ hybridization (ISH) of histological sections can localize MoV to cells within spheroids in infected *P. japonicus*, spheroids can occur in this species without evidence of infection by MoV or other pathogens. In subclinical *P. monodon*, vacuolization of cells within lymphoid organ spheroids is less pronounced, which might be due to shrimp in Australia commonly being infected with GAV. In such *P. monodon*, MoV ISH signal can be intense within spheroids, but also be evident throughout the stromal matrix of normal nonoccluded tubules, and particularly in cells at the tubule lumen and outer periphery (Fig. 35.7; Cowley et al., 2005a). In some *P. monodon* with subclinical MoV infections, ISH signal has also been observed variably within the cuticular epithelial lining of the stomach and the cephalothorax exoskeleton, and within the antennal gland and connective tissue surrounding tegmental glands (Cowley et al., 2005a).

Among *P. japonicus* dying as a result of acute MoV infection, the lymphoid organ can become grossly enlarged due to the accumulation of spheroids (Cowley et al., 2005b). As MoV infection severity increases, more spheroids progress from

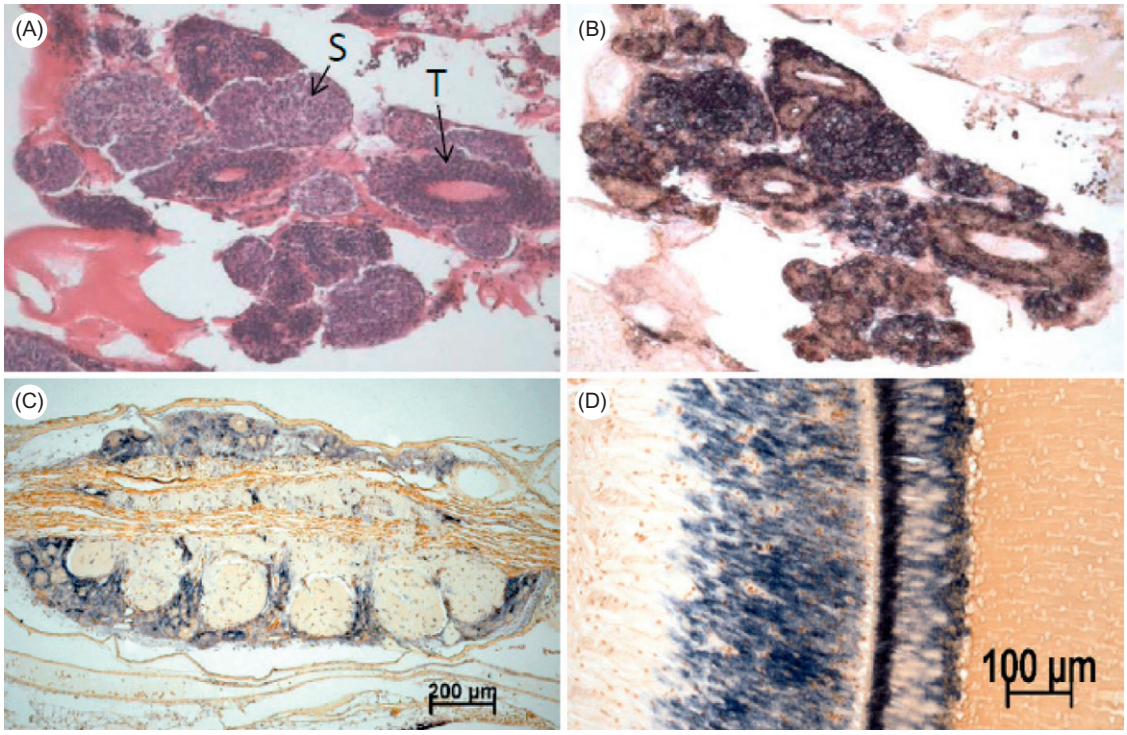

FIGURE 35.7 Examples of tissues in *Penaeus monodon* infected by MoV. (A) H&E-stained lymphoid organ (LO) section showing spheroids (S) and normal tubules (T) and in situ hybridization signal (dark blue (dark gray in print versions) color) showing the presence of MoV infection in (B) spheroids and particularly in cells at the periphery and lumen of normal tubules in a consecutive LO section, (C) segmental nerve ganglia (Cowley et al., 2005a) and (D) the fasciculated zone of the eye.

being composed of cells with a hypertrophied nuclei and variably vacuolated cytoplasm to cells also displaying necrosis typified by pyknosis (ie, condensed nuclear chromatin) and karyorrhexis (ie, nuclear fragmentation). Based on ISH signal intensities, the tissue tropism of MoV includes all cephalothorax tissues of mesodermal and ectodermal origin. Cell types supporting MoV replication include those sequestered within lymphoid organ spheroids and comprising lymphoid organ tubules, connective tissues in all organs, heart, epicardium, gills, cuticular epithelia as well as epithelia of the foregut, midgut and antennal gland, ventral and peripheral nerves, the segmental nerve ganglia and the epineurium lining of these nerves (Cowley et al., 2005b).

In *P. monodon* with high-level MoV infections, its distribution throughout mesodermal and ectodermal tissues can be as widespread as in acutely infected *P. japonicus* (Cowley et al., 2005a). Depending on the infection severity, affected tissues can include primary and secondary gill filaments, particularly in epithelial pillar cells, glial, neurosecretory and giant cells surrounding periopod segmental nerve ganglia, antennal gland, hepatopancreas connective tissues, the pericardial septum, epicardium, fixed phagocytes within the myocardium, hemocytes and hemocytic tissues, as well as antennal gland tubules, cuticular epithelium of the stomach and cephalothorax exoskeleton, and the fasciculate zone of the eye (Fig. 35.7).

When first discovered in *P. japonicus* farmed in Australia, MoV was suspected to be a possible cause of commonly observed histopathology consistent with an idiopathic disease of this species described as gut and nerve syndrome (GNS) (Lightner, 1988a,b; Lightner et al., 1984; Cowley et al., unpublished). GNS histopathology is characterized by ventral nerve cord epineurium hyperplasia and hypertrophy/thickening of the midgut basement membrane and has been linked tenuously to infection by a reo-like virus (Lightner, 1988a,b). In *P. japonicus* infected with MoV, ISH signal can associate with the multiple-layered epineurium sheaths, ventral nerve cord and segmental nerve ganglion as well as connective tissues in and muscle underlying the midgut (Cowley et al., 2005b). However, as GNS histology presents commonly in *P. japonicus* farmed in Australia that are PCR and ISH negative for MoV, and that also display no eosinophilic cytoplasmic inclusion bodies in hepatopancreas tubule or other cells indicative of reovirus infection (Lightner, 1988a,b; Cowley et al., unpublished), the cause of these pathologies remains unknown.

35.3.9 Diagnostic Methods

There are no clinical signs nor distinctive pathological features predictive of acute MoV infection. In *P. japonicus*, however, acute infection is typified by gradually accumulating idiopathic mortalities, increased mortality around molt cycles and a grossly enlarged lymphoid organ (Cowley et al., 2005b; Keys et al., 2004; Sellars et al., 2006).

35.3.9.1 Transmission Electron Microscopy (TEM)

TEM of ultrathin sections (<1 μm) of lymphoid organ and gill tissue stained with osmium tetroxide and alkaline lead citrate can be useful in detecting the characteristic enveloped spherical to ovoid-shaped MoV particles in the cytoplasm of necrotic cells (Fig. 35.6; Cowley et al., 2005a). However, as MoV particles generally become evident only in cells displaying advanced necrosis at a time when diseased shrimp are approaching death, the value of TEM as a diagnostic method is limited.

35.3.9.2 Histology and ISH

Hematoxylin and eosin-stained histology sections of cephalothorax tissues, including lymphoid organ, can aid diagnosis of MoV (Fig. 35.7). Pathology is most evident in the lymphoid organ, where infection severity can be estimated by the prevalence of spheroids and the extensiveness of nuclear pyknosis and karyorrhexis, cytoplasmic vacuolization and necrotic cell debris within spheroids (Fig. 35.7; Cowley et al., 2005a,b). In acute MoV infections, ectopic spheroids can also accumulate in gill tissue and in connective tissues associated with various cephalothorax organs. However, as other viruses such as GAV can occur together with MoV and cause similar lymphoid organ pathology and ectopic spheroids, histology alone is inadequate for diagnosing MoV infection with certainty.

ISH using a DIG-11-dUTP-labeled DNA probe prepared to a ~0.8 kb sequence in Gc glycoprotein coding region of the MoV M RNA segment provides a sensitive and specific means of detecting MoV-infected cells and estimating infection severity based on probe intensity and the diversity of cephalothorax tissues showing evidence of infection (Cowley et al., 2005b). However, to minimize RNA cross-linking with potential to restrict DNA probe binding and thus ISH detection sensitivity, cephalothoraxes should be fixed in Davidson's fixative for no more than 2 days before being transferred to 70% ethanol for subsequent processing for histology (Hasson et al., 1997; Cowley et al., 2005b).

35.3.9.3 Polymerase Chain Reaction

Sensitive and specific RT-nested PCR and TaqMan real-time quantitative PCR tests have been developed to detect and quantify MoV RNA (Cowley et al., 2005a,b; Rajendran et al., 2006). Both tests target the same region in the Gc glycoprotein coding sequence of the M RNA segment. The PCR and nested PCR tests amplify sequences 610 and 322 bp in length respectively, and the TaqMan real-time PCR amplifies a 67 bp sequence within the nested PCR sequence. Using dilutions of an accurately quantified 849 nt synthetic RNA generated to MoV M RNA segment and cDNA prepared using random hexamer primers, test detection limits have been estimated to be in the order of 6–20 RNA copies for the PCR, and 2 RNA copies when the nested PCR is applied (Cowley et al., 2005b). Using dilutions of the same synthetic RNA, the MoV TaqMan real-time qPCR reliably detects down to 10 RNA copies, and has a log linear range covering 10^9 RNA copies (Rajendran et al., 2006). Tissues providing good sources of MoV RNA for PCR analysis include lymphoid organ, gill and hemocytes. In shrimp with subclinical MoV infections, lymphoid organ will be among the tissue types containing the highest MoV RNA amounts. However, hemocytes and gill tissue provide alternatives with possibly slightly reduced detection sensitivity when nonsacrificial tissue sampling is necessary. Depending on their size, individual postlarvae or pools of either whole postlarvae heads dissected from postlarvae also provide a tissue source suitable for PCR testing.

35.3.10 Prevention and Control

Specific methods for preventing or controlling MoV infection and mortality remain limited. However, as for most shrimp viruses, generic options exist for PCR-based screening exclusion of MoV-infected broodstock and/or postlarvae and for the use of robust biosecurity systems during hatchery and pond rearing (Cowley, 2005). Available data also highlight the need to minimize environmental stress during rearing. Compared with *P. japonicus* postlarvae reared solely in tanks, increased MoV infection prevalence and loads have been noted among siblings reared in farm ponds for 6 months prior to being transferred to tanks (Keys et al., 2004; Sellars et al., 2006). It cannot be excluded that pond-rearing exposes shrimp to exogenous sources of MoV. However, the potential impacts of stress are consistent with MoV infection prevalence/loads

and mortality rates being higher among *P. japonicus* siblings reared in tanks in which the sand substrate had become anoxic (Sellars et al., 2006).

Activation of the RNAi pathway by muscle injection of a dsRNA targeted to the MoV L RNA segment (which encodes the viral RdRp critical to mRNA transcription and genomic RNA replication) has been shown to be highly effective in inhibiting MoV infection following experimental challenge of *P. monodon* with an inoculum containing GAV in addition to MoV (Cowley et al., 2012; Oanh et al., 2011). However, as with other successes in administering virus-specific dsRNA to activate the RNAi mechanism to inhibit virus replication, disease and mortality, feed-based dsRNA delivery systems identified to show promise (Attasart et al., 2013; Sanitt et al., 2014) have yet to be commercialized.

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Part VI

DNA Viruses of Mollusks

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Chapter 36

Iridolike Viruses of Mollusks

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36.1 INTRODUCTION

Iridoviruses belong to a family of large DNA viruses, *Iridoviridae*, that are 180 nm in diameter, enveloped with an icosahedral nucleocapsid and contain a large, single, double-stranded DNA genome of approximately 150 kbp. They replicate in the cytoplasm of infected cells. Several iridoviruses have been mainly reported infecting several species of insects (Jakob et al., 2001).

36.2 SPECIFIC MAIN MOLLUSK DISEASES

The potential of aquaculture to meet the challenge of food security and to generate employment and foreign exchange is clearly demonstrated by the rapid expansion of this sector. However, high-density culture conditions mean that many aquaculture animals are under stress conditions and are generally more susceptible to infections. Major disease outbreaks result in loss of income and sometimes the complete shutdown of operations. Besides causing massive mortalities in cultured species, viral pathogens are often highly infectious and easily transmissible. Several viral diseases, including iridolike virus infections, have seriously affected the aquaculture industry.

Several iridolike viruses have been reported in bivalves in different countries around the world. Two distinctive pathological infections have been described in *Crassostrea angulata* adult oysters in France (Comps and Duthoit, 1976; Comps et al., 1976; Comps, 1978, 1980). The first one, called *Maladie des branchies* (gill necrosis) was caused by gill necrosis virus (GNV) and was interpreted as the cause of recurrent mass mortalities in Portuguese oysters, *C. angulata*, from 1966 to 1968 along French coasts. In 1970, epizootic mortality events were again reported in French *C. angulata* oysters and associated with the detection of an iridolike virus called hemocyte infection virus (HIV). A possible identical GNV was also associated with disease in *Crassostrea gigas* oyster populations (Comps and Bonami, 1977). Finally, oyster velar virus disease (OVVD) was reported from hatchery-reared larval Pacific oysters on the west coast of North America (Washington State) (Elston, 1979; Elston and Wilkinson, 1985). The virus is associated with occasional mortality of oyster pediveliger larvae.

The main objective of this chapter is to give updated information on iridolike viruses that infect bivalves and replicate in their tissues. Different findings provide a presumptive link between the detection of these iridolike viruses in different bivalve species and the diseases reported. Although the presumptive link is substantial, definitive demonstration of viral etiology of the diseases must be accomplished by experimental transmission. In addition, the exact identification of these viruses has thus not been carried out. In this context, the term *iridolike viruses* needs to be used because their exact affiliation remains to be found.

36.3 DISEASE NAMES

From 1966 to 1973, mass mortality outbreaks led to the disappearance of *C. angulata* from the Atlantic French coast (Renault, 1996) and two distinct mortality events were described. Gill disease (maladies des branchies) was identified as the cause of the first mortality episode that started around 1966 in France (Marteil, 1968). This disease was later described in other European countries, namely in Great Britain (Alderman and Gras, 1969), Portugal (Ferreira and Dias, 1973) and Spain (Comps and Masso, 1978). In some years, gill disease affected 70–80% of the population, with mortality reaching up to 40% (Marteil, 1968). Although the gill disease was also observed in *C. gigas*, this taxon proved to be more resistant to the disease than *C. angulata*. Although different microorganisms were described associated with gill disease in different bivalve species, it is now generally assumed that the disease affecting *C. angulata* and *C. gigas* was caused by an iridolike

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00036-X
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virus that was called GNV. However, GNV was not isolated and no experimental transmission studies have been carried out in order to demonstrate its pathogenicity (Comps, 1988). A second period of mass mortality events occurred between 1970 and 1973 in France. Although some oysters exhibited atrophy and weakness of the adductor muscle, no distinctive clinical signs were noticed. An iridolike virus was also associated with these mortality events and was called HIV. Although GNV and HIV are very similar in terms of morphology and morphogenesis, they differ in their pathogenic effects, as described previously. Mature particles are approximately 380 nm in diameter and are icosahedral in shape. Morphogenesis takes place in the cytoplasm of oyster cells and Feulgen-positive staining reaction suggests the presence of DNA (Comps, 1988). In 1977, a similar type of HIV infection was observed in *C. gigas* in the Bay of Arcachon (France) associated with 15% mortality (Comps and Bonami, 1977). An iridolike virus similar to HIV was observed in *C. angulata* spat in France in 1984 (Bougrier et al., 1986). It must be emphasized that the pathogenicity of GNV and HIV was not demonstrated, although these viruses have been identified as the most probable cause of recurrent mass mortalities of *C. angulata* observed in Europe since 1966.

Another iridolike virus was also described in *C. gigas* larvae reared in the United States associated with high mortality and was called oyster velar virus (OVV) (Elston and Wilkinson, 1985). OVV infections affected *C. gigas* larvae greater than 150 µm in shell height. The diseased larvae were less active. Cytoplasmic inclusions bodies were observed in ciliated velar epithelium.

36.4 STRUCTURE AND COMPOSITION OF VIRUS

Transmission electron microscopy (TEM) examination demonstrated that the globular cells reported in *C. angulata* oysters with gill necrosis were hypertrophied host cells containing intracytoplasmic viral particles (Comps et al., 1976; Comps, 1978; Comps and Duthoit, 1979). GNV particles were icosahedral in shape, approximately 300 nm in diameter. A central electron-dense core, 190 nm in diameter, was surrounded by an electron-lucent zone followed by another dense layer 45 nm in thickness, two unit membranes separated by clear area-enclosed particles. The same type of virus has also been reported in association with hemocyte disease in French *C. angulata* oysters. HIV particles appeared to be icosahedral, 350 nm in diameter with an electron-dense core 190 nm in diameter (Comps et al., 1976; Comps, 1978). A third virus was also reported infecting *C. gigas* oysters in Arcachon (Atlantic coast, France) (Comps and Bonami, 1977). The virus exhibited an icosahedral morphology with viral particles 350 nm in diameter. The electron-dense core measured 250 nm in diameter.

In the case of OVVD, TEM examination of all tissues of diseased larval oysters demonstrated viroplasmic inclusion bodies in velar, oral and epithelial tissues (Elston, 1978; Elston and Wilkinson, 1985). The viroplasms were typically granular and electron-dense and correspond to intracytoplasmic inclusions visible at the light microscopy level. On the periphery of some viroplasms, the initiation of viral particle formation was reported (Elston, 1978; Elston and Wilkinson, 1985). Particles were predominantly hexagonal in profile and averaged 228± 7 nm in diameter. Viral particles were limited by two bilayered membranous structures 8.5-nm wide and separated by a space of approximately 3.6 nm. An electron-lucent space separated the limiting membranes from the adjacent central zone of moderate electron density. Within this central zone, an electron-opaque core was observed with circular and para-rectangular profiles. The circular profile measured 160 nm in diameter, and the para-rectangular profile measured 106 nm in the shortest dimension. Cells containing aggregates of particles corresponded to cells exhibiting degenerative changes at the light microscopy level (Elston and Wilkinson, 1985). Cells containing particles were characterized by poorly defined plasma membranes and loss of the normal homogenous texture of the cytoplasm in the vicinity of the particles. OVVD particles showed a mean diameter of 228 nm, which is smaller than the diameter of GNV and HIV (Comps, 1988).

36.5 CLASSIFICATION AND VIRUS REPLICATION

The various properties of the different viruses described previously, as well as their assemblage within the cytoplasm, characterized them as members of the *Iridoviridae*. However, the exact identification of the four viruses described here cannot be made at this point. Molecular characterization of iridolike viruses infecting bivalves is pending.

36.6 EPIDEMIOLOGY AND PATHOLOGY

GNV disease is regarded as the main factor of mass mortalities occurring in the late 1960s among Portuguese oysters on the Atlantic coast of France (Comps, 1970; Comps and Duthoit, 1976). During certain years, the disease affected 70% of the oyster populations from important culture areas including the Marennes Oleron and the Arcachon areas. Maximum losses occurred in 1967 and losses subsequently declined. In 1968, survivors of previous outbreaks recovered from the disease.

Gill disease also affected the Pacific oyster, *C. gigas*, although to a lesser extent. The gill lesions were always reduced, suggesting partial resistance in this species. Pacific oysters displayed into French waters from Japan in 1968 exhibited gill lesions in 27% of individuals, while 56% of a lot obtained from Korea showed lesions. At the same time, the incidence of gill lesions in *C. gigas* imported from British Columbia was only 14% (Comps, 1970; Marteil, 1976). Pacific oysters cultivated in France appeared subsequently to have acquired complete resistance to gill disease, as no gill-diseased individuals of this species were found between 1969 and 1971, although mortalities among *C. angulata* reached 70% (Comps, 1972). The first symptoms of the gill disease were the appearance of one or several yellow spots on the gills and labial palps (Alderman and Gras, 1969). In the gills, these spots increased in size and the spot center became brown and disintegrated, resulting in larger and deeper ulcerations. In advanced stages of the disease, it might lead to degeneration of the entire gill structure. Yellow or green pustules also developed on the adductor muscle and mantle, and in the latter tissue perforations might occur as on gills. The disease is characterized by tissue necrosis and massive hemocyte infiltration around the lesions (Fig. 36.1). Some oysters recovered from the disease, showing cicatrization (Comps, 1970). In oysters infected with GNV, giant polymorphic cells (c. 30 µm in size) were observed with a hypertrophied nucleus (Comps, 1988). The nucleus of the infected cells usually had an oval shape and the chromatin disappeared (Fig. 36.2). The cytoplasm was also affected, containing large inclusion bodies (5–15 µm), numerous vacuoles and finer grains (c. 0.4 µm) (Fig. 36.2). TEM examination revealed that inclusions were viroplasms and the fine granules were viral particles with an icosahedral shape (Fig. 36.3) (Comps, 1988).

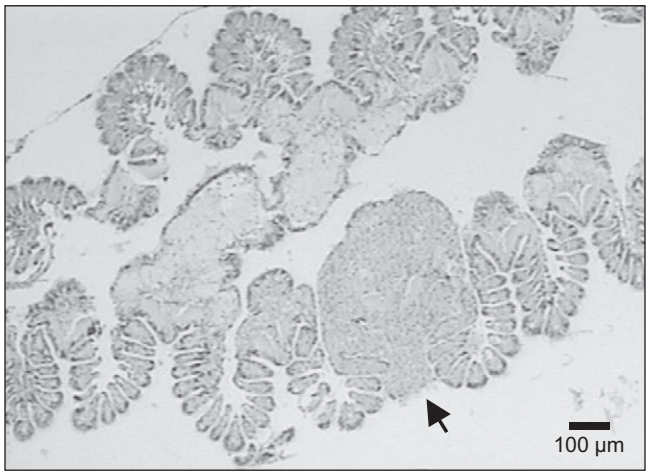

FIGURE 36.1 Histopathological changes in the gills of a *Crassostrea angulata* specimen showing a group of filaments completely destroyed and an intense cell infiltration around the lesion. Hematoxylin and eosin staining.

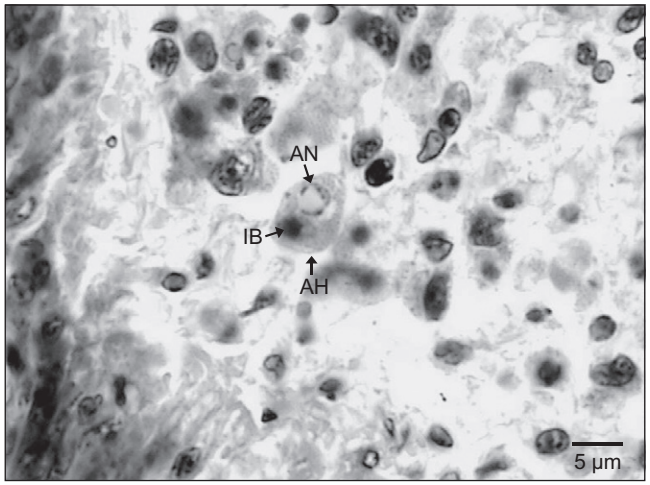

FIGURE 36.2 Atypical hemocyte (AH) of *Crassostrea angulata* infected with HIV showing an abnormal nucleus (AN) and an inclusion body (IB). Hematoxylin and eosin staining.

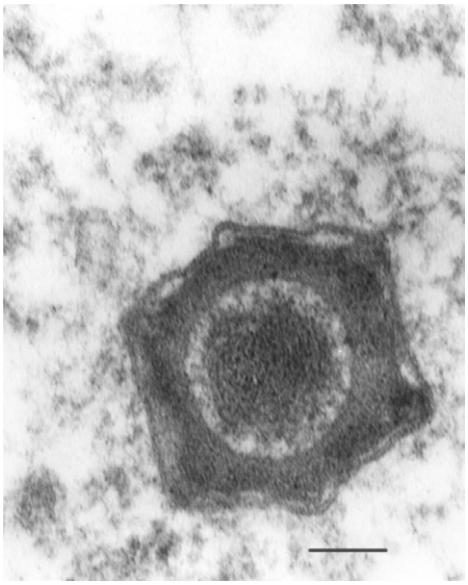

FIGURE 36.3 An iridolike virus particle from *Crassostrea angulata*. The virion is icosahedral in shape with a central electron-dense core, surrounded by an electron-lucent zone followed by another dense layer. Two unit membranes separated by a clear zone enclose the particle. Scale bar = 100 nm.

In 1970, mortality outbreaks of French *C. angulata* were again associated with the detection of an iridolike virus (HIV) infecting hemocytes. The most characteristic histological lesion of HIV infections was an acute infiltration response associated with the presence of atypical hemocytes in the connective tissue and an increase number of brown cells. These atypical hemocytes showed intracytoplasmic inclusions bodies, often with groups of fine granules. The disease affected adult oysters from 1970 to 1973, but no distinctive signs were reported (no gill lesions). Mortality was first observed in the estuary of Marennes Oleron on the French Atlantic coast and was paralleled by a similar event at Etel in Brittany (France). By 1971, the disease had also reached the Arcachon region. High mortality rates led to the almost total extinction of *C. angulata* in French Atlantic waters in 1973. Pacific oysters seemed to be resistant to the virus (HIV) and, after the disappearance of *C. angulata*, replaced the latter species in France (Marteil, 1976; Bonami, 1977). However, a similar disease associated with a morphologically similar virus was reported from Pacific oysters in France during an episode of summer mortality of that species in the Bay of Arcachon in 1977 (Comps and Bonami, 1977). In 1977 a 15% mortality was noticed in oysters kept in a purification plant in Arcachon. As was the case for *C. angulata*, affected Pacific oysters exhibited virtually no external signs of disease, except for a greyish discoloration of the visceral mass in some cases.

Pacific oyster larvae showing clinical lesions of OVVD were less active than normal animals. OVVD lesions have been reported in clinically affected individuals, but not in nonaffected larvae. Infected larvae were unable to execute normal active movements when the velum was extended. The disease resulted in the sloughing of ciliated velar epithelial cells, forming the characteristic "blisters" (Elston and Wilkinson, 1985). Infected velar epithelial cells, which were in the process of detaching from the velum, appeared as blebs along the periphery of the velum. Other cells lost cilia and the larvae became unable to move normally. Typically, the infection occurred in larvae in which the shell height is between 175 and 190 µm. Mortality of larvae greater than 170 µm in shell length typically began at about 10 days of age when cultured at 25–30°C. Detailed hatchery records from 1976 through 1984 indicated that the disease occurred each year more often from mid-March through mid-June (Elston and Wilkinson, 1985). Over the 8-year period and during the April to May period, losses of up to 50% of hatchery production were presumptively attributed to OVVD. The disease was manifested histologically by the presence of intracytoplasmic inclusion bodies and separation and detachment of infected cells from surrounding tissues. Lesions were observed in velar, oral and distal esophageal epithelia (Elston and Wilkinson, 1985). The intracytoplasmic inclusions bodies, 1.2–4 µm in diameter, were located most commonly in ciliated velar epithelium. Utilizing hematoxylin and eosin staining, these inclusions appeared basophilic, but also contained diffusely distributed eosinophilic components. The inclusion bodies exhibited a deep red, positive reaction when stained by the Feulgen and Rosenbeck reaction. Utilizing ribonuclease controls the acridine orange reaction yielded a green-staining reaction, indicating DNA presence in inclusion bodies (Elston and Wilkinson, 1985). Some epithelial cells containing inclusions appeared demarcated from surrounding cells.

36.7 DIAGNOSTIC METHODS

Several characteristics of bivalve mollusks make the diagnosis of viral diseases difficult because they do not produce antibodies and, despite several attempts, mollusk cell lines are still not available. No molecular tools are available at present time to diagnose iridolike viruses infecting different oyster species. The development of nucleic acid-based techniques can be of great importance for a rapid diagnosis of the related viral diseases, which is not possible using traditional methods such as light microscopy and TEM. Moreover, the sensitivity of these techniques, for example, polymerase chain reaction (PCR) (Martin, 1994), is high allowing the detection of virus DNA even when present in low amounts.

36.8 CONCLUSION

Viral diseases in aquaculture invertebrates are of serious concern because no specific chemotherapies and vaccines are available. Iridolike virus infections are regarded as having led to the almost total extermination of the Portuguese oyster, *C. angulata*, in French and European Atlantic waters in 1973. In this context, knowledge of viruses affecting cultured bivalves is needed in order to develop new tools for the control of viral diseases. This knowledge may give new insights into the management and control of infectious diseases in aquaculture. The basic method to diagnose virus infections has long been light microscopy, although this procedure appears to be poorly adapted to viral diseases and additional techniques, such as TEM, have to be undertaken to complete the diagnosis. Despite being time consuming and somehow inadequate for epidemiological surveys, both techniques were extensively employed through lack of suitable alternative diagnostic procedures. Within this context, the development of protocols for purification of viral particles from infected bivalves appears as a way out from both methodological deadlocks. For example, extraction and sequencing of OsHV-1 DNA from purified herpesvirus particles infecting *C. gigas* larvae rendered the development of molecular tools possible (Le Deuff and Renault, 1999). In addition, DNA extraction from archival samples could allow further retrospective analyses using PCR in order to detect iridolike virus DNA. In a recent study, iridovirus DNA was successfully detected by PCR and in situ hybridization from the mollusk *Nautilus* sp. (Gregory et al., 2006). The sequence data reported by these authors as well as the experimental approach (eg, primer pairs, PCR conditions) can be useful to study oysters infected by iridolike viruses.

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Chapter 37

Malacoherpesviruses of Mollusks

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37.1 INTRODUCTION

Herpesviruses are composed of relatively large, double-stranded, linear DNA genomes encoding 100–200 genes encased within an icosahedral protein cage called the capsid, which is itself wrapped in a protein layer called the tegument containing both viral proteins and viral mRNAs and a lipid bilayer membrane called the envelope. At least five species of *Herpesviridae*—HSV-1 and HSV-2 (both of which can cause orolabial herpes and genital herpes), Varicella zoster virus (which causes chickenpox and shingles), Epstein-Barr virus (which causes mononucleosis) and Cytomegalovirus—are extremely widespread among humans. With more than 130 virus species, herpesviruses cause diseases in a very large range of animals, including humans (Davison et al., 2009).

Herpesviruses infecting marine mollusks have attracted particular research attention due to their economic and ecological impact during the past 30 years. The first description of a virus from a marine mollusk indicating membership to the order *Herpesvirales* (Davison et al., 2009) was reported in adult Eastern oysters, *Crassostrea virginica*, by Farley et al. (1972). In early 1990s, viruses interpreted as belonging to Herpesvirales were associated with mass mortality outbreaks of hatchery-reared larval *Crassostrea gigas* in France (Nicolas et al., 1992) and New Zealand (Hine et al., 1992). Sporadic mass mortality events affecting larval *C. gigas* had then been regularly observed in commercial European hatcheries, occurring in association with the detection of a herpes-like virus (Renault et al., 1994b; Renault and Arzul, 2001). Since 1993, mass mortality outbreaks have also occurred in Pacific oyster spat cultured in different French locations (Renault et al., 1994a,b). Viral particles have been purified from French *C. gigas* larvae collected in 1995 from a French hatchery (Le Deuff and Renault, 1999). The entire virus DNA was sequenced (GenBank accession number AY509253) and the virus classified under the name Ostreid herpesvirus 1 (OsHV-1) as the first known species in the family *Malacoherpesviridae* (Davison et al., 2005, 2009). Viral infections were also reported in Pacific oyster seed suffering losses in Tomales Bay (California) (Burge et al., 2007) and along the northwestern Pacific coast of Baja California (Mexico) (Vazquez-Juarez et al., 2006; Vásquez-Yeomans et al., 2010). The serious impacts from disease outbreaks in Pacific oysters caused by OsHV-1, and more recently by the virulent variant µVar in Europe (Clegg et al., 2014; Domeneghetti et al., 2014; Lynch et al., 2012; Martenot et al., 2011; Martenot et al., 2012; Peeler et al., 2012; Renault et al., 2012; Roque et al., 2012; Segarra et al., 2010) and closely related variants in Australia (Jenkins et al., 2013; Paul-Pont et al., 2013, 2014) and in New Zealand (Keeling et al., 2014; Renault et al., 2012), stress the need to further characterize this virus and better understand its biology in order to establish control and management measures. In addition, viral infections were reported in spat and larvae of the European flat oyster, *Ostrea edulis*, in France (Renault et al., 2001b). Concomitant mortalities were observed among larvae and spat of *C. gigas* and *O. edulis*, in 1994 and 1995, with the detection of herpes-like virus particles by transmission electron microscopy (TEM) (Renault et al., 2000a). Replication of herpes-like viruses was also described in *Ostrea angasi* in Australia (Hine and Thorne, 1997), in *Tiostrea chilensis* in New Zealand (Hine et al., 1998) and in *Ruditapes philippinarum* and *Pecten maximus* in France (Arzul et al., 2001b; Renault et al., 2001a,b).

Highly pathogenic herpesviruses were also reported in different abalone species including *Haliotis diversicolor supertexta* (Chang et al., 2005), *Haliotis laevigta*, *Haliotis rubra* and their hybrids (Hooper et al., 2007; Tan et al., 2008) associated with substantial mortality outbreaks.

37.2 SPECIFIC MOLLUSK DISEASES

OsHV-1 is the etiological agent of a contagious viral disease of Pacific cupped oysters, *C. gigas*, affecting also other bivalve species. Abalone herpesvirus (AbHV) is the etiological agent of abalone viral ganglioneuritis (AVG), a contagious disease of abalone species in Australia (Ellard et al., 2009; Hooper et al., 2007) and possibly of species in other countries (Chang et al., 2005; Wang et al., 2004).

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00037-1
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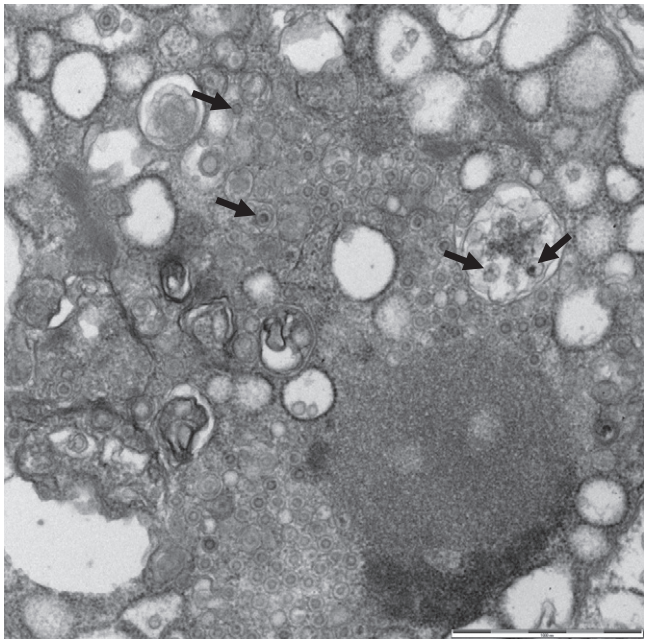

FIGURE 37.1 OsHV-1 infected Pacific oyster spat: nucleocapsids presenting different features (*arrows*). Scale bar = 1000 nm.

37.2.1 Structure and Composition of Virus

In infected Pacific oysters, OsHV-1 capsids are circular or polygonal in shape, 70–80 nm in diameter (Renault et al., 2001a,b). Some particles appear empty and are interpreted as being capsids, others contain an electron-dense toroidal or brick-shaped core and are presumed to be nucleocapsids (Fig. 37.1). Capsids and nucleocapsids are scattered throughout the nucleus in infected cells. Enveloped single virions or groups of viruses are observed in cytoplasmic vesicles (Fig. 37.2). These intracytoplasmic particles possess an envelope formed by a trilaminar unit-membrane. Core and capsid are separated by an electron-lucent gap of approximately 5 nm with fine fibrils spanning the lucent-space from core to capsid. Naked capsids are also observed free in the cytoplasm of infected cells. Extracellular viruses are usually enveloped and measure 100–180 nm in diameter (Renault et al., 2001a,b). No tegument, or a reduced tegument, is observed between the outer membrane and the capsid shell of enveloped particles. Nucleocapsids also occur in myocytes and cardiomyocytes.

The genome structure and sequence, and the capsid morphology (Davison et al., 2005) have been studied in order to assess OsHV-1 phylogenetic status in relation to vertebrate herpesviruses. Purified OsHV-1 capsids (Davison et al., 2005) appear structurally similar to those of other herpesviruses. Tri-dimensional reconstructions of OsHV-1 capsids derived by cryoelectron microscopy revealed an icosahedral structure with a triangulation number of $T = 16$ (Davison et al., 2005), a structure observed only with herpesviruses. OsHV-1 genome consists of a linear genomic dsDNA of 207 kbp (GenBank accession number AY509253) (Davison et al., 2005). The overall genome structure is: TR_L—U_L—IR_L—X—IR_S—U_S—TR_S with a 207,439 bp total genome size. TR_L and IR_L (7584 bp) are inverted repeats flanking a unique region (U_L, 167,843 bp). TR_S and IR_S (9774 bp) are inverted repeats flanking a unique region (U_S, 3370 bp), and X (1510 bp) is located between IR_L and IR_S. This overall genome structure is similar to that of mammalian herpesviruses (eg, herpes simplex virus and human cytomegalovirus). Southern blot hybridization experiments using polymerase chain reaction (PCR)-generated probes from the ends of U_L and U_S showed that the two orientations of U_L and U_S are present in approximately equimolar amounts in viral DNA, giving rise to four genome isomers. This is also a feature of the vertebrate herpesvirus genomes with similar structures. A detailed analysis of the coding potential of OsHV-1 genome sequence indicated the presence of 124 unique protein-coding genes without indication of splicing. Thirty-eight genes are identified belonging to 12 families of related genes. These include two families whose products are predicted to be secreted, three families predicted to encode membrane-associated proteins, one family whose products contain motifs of helicases, one family whose products are related to inhibitors of apoptosis, one family derived from a deoxyuridine triphosphatase gene, two families of RING-finger genes and two other families. Gene families are also present in all sequenced vertebrate herpesviruses. Database searches also provide functional information on 25 genes that are not members of families. These include genes encoding a tentative

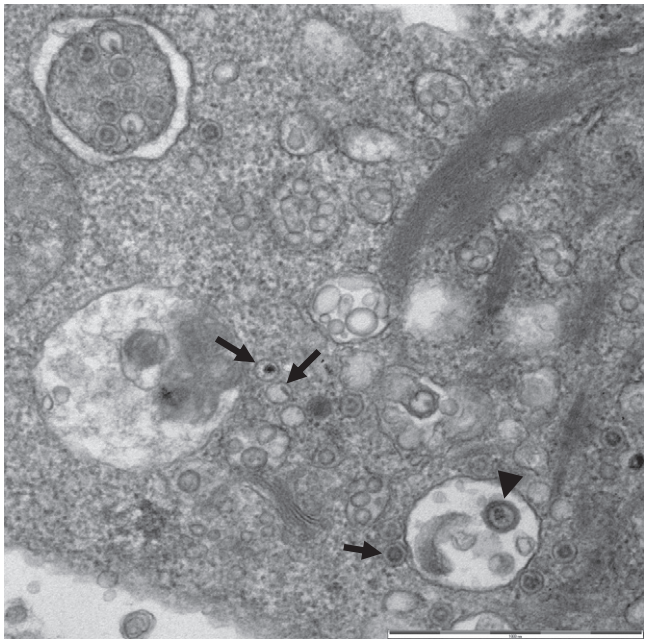

FIGURE 37.2 OsHV-1 infected Pacific oyster larva: nucleocapsids presenting different features (*arrows*) and an enveloped particle (*arrowhead*). Scale bar = 1000 nm.

primase, two subunits of ribonucleotide reductase, a helicase, the catalytic subunit of DNA polymerase, the ATPase subunit of terminase and two RING-like proteins. The ORF30 protein is related in an N-terminal cysteine-rich domain to a protein of unknown function in two subfamilies (*Beta-* and *Gammaherpesvirinae*) of the higher vertebrate herpesviruses. A total of 15 genes encode proteins that have predicted signal or transmembrane sequences and therefore may be associated with membranes.

Purified viral particles from Australian abalone (Tan et al., 2008) observed by TEM are icosahedral with electron-dense cores and 100–110 nm in diameter. The intranuclear location of AbHV particles, their size and ultrastructure are characteristic of members of the Herpesvirales order. Isopycnic gradient centrifugation (in potassium tartrate and cesium chloride density gradients) indicated a virus particle buoyant density of 1.17–1.18 g/mL (Tan et al., 2008). Based on the assembled DNA sequences of the abalone virus, several protein coding sequences show varying distant homology to herpesvirus proteins, most notably those of OsHV-1 (Savin et al., 2010). None of the coding sequences identified appear to be split by introns. Savin et al. (2010) proposed to refer to the virus infecting abalone as abalone herpesvirus type 1 or AbHV-1.

37.2.2 Classification and Virus Replication

The virus infecting Pacific oysters was classified under the name ostreid herpesvirus 1 (OsHV-1) as the first known species in the family *Malacoherpesviridae* (Davison et al., 2009). The sequence data demonstrate that OsHV-1 is not closely related to herpesviruses with vertebrate hosts (including fish). Several OsHV-1 proteins have homologues that are distributed widely in nature (eg, DNA polymerase), but these are no more closely related to homologues in other herpesviruses than to homologues in other organisms. However, a genetic indication of a common origin between OsHV-1 and vertebrate herpesviruses resides with the ATPase subunit of the terminase. Homologous genes are present in all herpesviruses, and the only nonherpesvirus counterparts are specified by T4 and related bacteriophages. Morphological features, the capsid structure and the presence of a putative terminase gene support assignment of OsHV-1 as a member of Herpesvirales despite its having very few other genetic relationships with herpesviruses found in mammals, birds, amphibians, reptiles and fish. The available data support the view that herpesviruses of mammals and birds, herpesviruses of fish and amphibians and herpesviruses of invertebrates form three major lineages of the herpesviruses (Davison et al., 2005, 2009). OsHV-1 is the first identified member of a third major domain of the herpesviruses.

OsHV-1 capsids and nucleocapsids are observed in the nucleus of infected cells. Viral particles then pass through the nuclear membrane into the cytoplasm and enveloped particles are released at the cell surface. Intranuclear and cytoplasmic

capsids present a variety of morphological types including electron lucent capsids, toroidal core-containing capsids and brick-shaped core-containing capsid.

Sequence analyses of the genome of Australian specimens of AbHV (Savin et al., 2010) have identified homologies amongst gene open reading frames (ORFs) with OsHV-1, and it has been assigned tentatively as a second member of the *Malacoherpesviridae* family.

37.2.3 Epidemiology

OsHV-1 causes an acute disease related to mass mortality outbreaks in larvae and juveniles of several bivalve species. *Crassostrea gigas*, *O. edulis, R. philippinarum*, *R. decussatus* and *P. maximus* are naturally infected (Arzul et al., 2001a; Renault et al., 2000a, 2001a). Mortality rates reported vary considerably between sites and countries and depend on the age of affected stocks. The virus can be found in adult bivalves most often in absence of mortality. Clinical signs may be dead or gaping juvenile bivalves, but these signs are not specific to OsHV-1 infection. Infected hosts may also be slow to close their valves when disturbed. Death usually occurs one week after infection (Garcia et al., 2011; Renault et al., 1994b). Infected larvae show a reduction in feeding and swimming activities and mortality can reach 100% in a few days (Hine et al., 1992; Nicolas et al., 1992). Moribund larvae swim weakly in circles with their velum noticeably less extended than healthy larvae. They exhibit velar and mantle lesions. Free pieces of velum may be observed in tank water. Shortly before death, infected larvae settle at the bottom of the tanks. For spat, sudden and high mortalities are observed in a short period (less than 1 week) often during summer time (Renault et al., 1994a,b).

Garcia et al. (2011) reported the detection of OsHV-1 DNA in samples collected during mortality outbreaks affecting French Pacific oyster spat. In the field, mortality occurred in spring and summer, suggesting an influence of the seawater temperature on OsHV-1 detection (Garcia et al., 2011). The temperature influence on OsHV-1 detection and virus expression was also reported for *C. gigas* larvae (Le Deuff et al., 1996) and spat (Burge et al., 2006; Friedman et al., 2005; Renault et al., 1995, 2014a; Sauvage et al., 2009; Petton et al., 2013). Moreover, stressful conditions including rearing practices seem to favor OsHV-1 infection (Pernet et al., 2012, 2014). Oyster transfers may facilitate OsHV-1 transmission. Moreover, spat mortality outbreaks associated with OsHV-1 detection generally presented a patchy distribution in the field (Garcia et al., 2011). This pattern can be partly explained by the nature of the virus. OsHV-1 is enveloped and assumed to present a weak resistance in the marine environment. High temperature, chemicals or sunlight (UV) may destroy its lipid-containing envelope. The lack of cell cultures for OsHV-1 has meant that in vitro studies on the virus stability with regard to infectivity have not been carried out. As an alternative, extracted viral DNA was seeded into seawater and 10 pg/μL of viral DNA were detected for 16, 9 and 1 day at 4°C, 11°C and 20°C, respectively. The longest time for DNA detection in OsHV-1 released from macerated larvae and seeded into seawater was 22 days at 4°C and 12 days at 20°C (Vigneron et al., 2004). However, the relationship between detection of viral DNA and virus infectivity is unknown. OsHV-1 DNA has been detected by real-time PCR in the water around diseased Pacific oysters (Sauvage et al., 2009) and the disease can be experimentally transmitted via the water route (Schikorski et al., 2011a). The copy numbers of virus DNA in the water in the first 48 h after injecting spat with virus reached 1×10^5 mL^{-1} of viral DNA copies, and reached a maximum of 1×10^6 mL^{-1} following infection of cohabiting oysters (Schikorski et al., 2011a,b).

OsHV-1 life cycle appears as direct from host to host (Le Deuff et al., 1994; Schikorski et al., 2011a,b). Le Deuff et al. (1994) described rapid transmission of the virus from an extract of diseased larvae to axenic larvae of *C. gigas*. In addition, experimental infection has been induced at larval stage in Pacific oyster, *C. gigas*, Portuguese oyster, *Crassostrea angulata*, suminoe oyster, *C. ariakensis*, European flat oyster, *O. edulis*, Manila clam, *R. philippinarum*, carpet shell clam, *R. decussatus* and scallops, *P. maximus* (Arzul et al., 2001a,b). Inter-species transmission from infected larvae of *C. gigas* to axenic larvae of *C. ariakensis* and *O. edulis* under experimental conditions was also demonstrated (Arzul et al., 2001b). Experimental transmission of OsHV-1 (variant μVar) has been described in Pacific oyster spat by Schikorski et al. (2011a). The viral infection can be transmitted to spat at 22°C, following intramuscular injection of an extract of naturally infected oysters, and also by cohabiting injected oysters with healthy ones. Using real-time PCR, the virus was suspected to enter the digestive gland and hemolymphatic system, following which OsHV-1 could be disseminated to other organs.

Healthy Pacific oysters including adults have been shown to be PCR-positive for OsHV-1 (Arzul et al., 2002; Moss et al., 2007; Sauvage et al., 2009). Pepin et al. (2008) showed that DNA copy numbers/mg were high (up to 10^7) in Pacific oysters from populations with abnormal mortalities and low (lowest number detected 10^1) in populations without abnormal mortalities. The presence of OsHV-1 was showed in asymptomatic *C. gigas* adults using molecular and immunological techniques (Arzul et al., 2002). Like other herpesviruses, OsHV-1 could be shown to persist in its hosts. Adult oysters may play the role of carriers and reservoirs of the virus, promoting virus transmission from adults to larvae (Le Deuff et al., 1996; Arzul et al., 2002). In a study, three successive generations of *C. gigas* were screened for OsHV-1 by PCR

(Barbosa-Solomieu et al., 2005). Viral DNA was detected in 2-day old larvae, indicating that infection may take place at early stages. The detection of viral DNA in parental oysters did not systematically correspond to a productive infection or result in a successful transmission to the progeny (Barbosa-Solomieu et al., 2004). However, the infective status of the parents appeared to have an influence on both the infection and the survival rates of the progeny (Barbosa-Solomieu et al., 2005). Crosses involving an infected male and a noninfected female resulted in hatching and larval survival rates statistically lower than those observed in the other types of cross (Barbosa-Solomieu et al., 2005). These results suggest that infected females may transmit some kind of protection or resistance against viral infection to their offspring.

A variant of OsHV-1 (var) has been identified in France (Arzul et al., 2001a,b) in *C. gigas*, *R. philippinarum* and *P. maximus*. Friedman et al. (2005) and Moss et al. (2007) also described differences in the sequences of OsHV-1 from California and Asia, respectively. Moss et al. (2007) suggested that there are at least two genotypes in Japan, one in South Korea and two in China. One of the genotype that occurred in China and South Korea was similar in sequence to the virus type from California described by Friedman et al. (2005), and the other genotype from China was similar to specimens from France. More recently PCR analysis using the primer sets IA1-IA2 and C2–C6 enabled the detection of a variant (µVar) in association with mass mortality events reported in Europe since 2008 (Segarra et al. 2010). Several differences in two genome areas (ORF4 and ORFs 42/43) including a deletion of 12 bp in a microsatellite located upstream of the ORF4 characterize this variant when compared with the reference OsHV-1 genome (GenBank accession n° AY509253) (Segarra et al., 2010). The variant µVar has been reported initially in France (Segarra et al., 2010; Martenot et al., 2010, 2011; Renault et al., 2012) and Ireland (Peeler et al., 2012) in 2008–09, and subsequently in Great Britain, in particular in the Kent area in England in 2010 (EFSA, 2010; Lynch et al., 2012). In Spain, recent studies demonstrated the presence of the variant µVar in association with mortality events (Roque et al., 2012). In Italy, simultaneous detection of the reference type and the variant µVar in the absence of any pathological sign was achieved in oysters originating from France (Dundon et al., 2011). Renault et al. (2012) analyzed 69 OsHV-1 specimens collected mainly in France from 1993 to 2010 and also in Ireland, the United States, China, Japan and New Zealand. Three areas of the virus genome (ORF4, ORFs 35/36/37/38 and ORFs 42/43) were selected for direct sequencing of PCR products and exploring the range of virus diversity. The ORF4 with 82 positions of a 460 nucleotide sequence showing a mutation appeared as the most variable. Two mutations were reported for the ORFs 42/43 and mainly a large deletion (605 bp) for the ORFs 35/36/37/38 area. The mutations present in ORFs 42/43 appear of interest because they allowed definition of two major groups among the analyzed samples: a first group containing virus specimens presenting 100% identities with the reference type (GenBank accession no AY509253) and a second one containing samples presenting 100% identities with the variant µVar (GenBank accession no HQ842610). Samples collected in New Zealand in 2010 grouped together and presented a sequence pattern intermediate between the reference type and the variant µVar. The analysis of the sequences of the particular regions (ORF4, ORFs35-36-37-38 and ORFs42-43), both independently and as concatemerized units, make it possible to define different sub-groups within known genotypes. Shimahara et al. (2012) detected variable types of OsHV-1 in main oyster-producing areas of Japan, using PCR targeting a C2/C6 fragment and sequencing PCR products. The etiological agent of OsHV-1 infections is represented by all genotypes of ostreid herpesvirus 1 (Arzul et al., 2001b; Davison et al., 2005; Segarra et al., 2010).

Currently, species known to be susceptible to AVG in Australia are the greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and hybrids of these two species. Clinical signs consistent with AVG have not been reported in other mollusk species in areas where AVG is suspected to be enzootic. In Chinese Taipei, ganglioneuritis associated with a herpesviral infection and mass mortality outbreaks in the *H. diversicolor supertexta* abalone species have been reported. The disease was reported only in *H. diversicolor supertexta*, while cohabitating Japanese black abalone *Haliotis discus* remained normal (Chang et al., 2005). Outbreaks of AVG in both farmed and wild abalone populations in Australia are associated with the rapid onset of high mortality rates (up to 90%) in all age classes (Corbeil et al., 2010). Similarly, in Chinese Taipei, during the epizootic in cultured abalone (the water temperature was 16–19°C), both adult and juvenile abalone suffered from the disease, with cumulative mortalities of 70–80%. It was reported that death of all of the abalone in a pond could occur within 3 days of the onset of clinical signs (Chang et al., 2005). A similar disease pattern occurred with experimental infections (Chang et al., 2005; Crane et al., 2009). Horizontal transmission (Chang et al., 2005; Crane et al., 2009) has been demonstrated experimentally by: (1) exposing healthy abalone to water containing diseased abalone in the same tank without direct contact between the diseased and healthy abalone; (2) placing healthy abalone in water that was previously inhabited by diseased abalone; and (3) intramuscular injection of healthy abalone with a filtered tissue homogenate from diseased abalone. In all cases, 100% mortality was observed with a preclinical period of 1–2 days following exposure and then mortality commenced until 100% mortality occurred within 2–5 days postinfection. In Australia, the initial outbreak of AVG occurred on a farm during summer 2005/06 and subsequently appeared to spread to wild populations, which experienced mortality throughout the following year (ie, during all seasons). All experimental infections to date have been carried out in the temperature range 15–18°C. In Chinese Taipei, during the reported epizootic, the water temperature was

16–19°C, and experimental infections were carried out at 17–20°C. How temperature affects viral replication and onset of disease have yet to be determined. It is not known whether the Australian virus is the same as, or different from, the virus found in Chinese Taipei. Recent genome sequence analyses have indicated that a number of genotypic variants are present in Australia (Cowley et al., 2011).

37.2.4 Pathology and Immunity

OsHV-1 infection-associated lesions are mainly observed in connective tissues of all organs (mantle, gills, digestive gland, labial palps, etc.) in which fibroblastic-like cells exhibit enlarged nuclei with perinuclear chromatin (Arzul et al., 2002; Lipart and Renault, 2002; Renault et al., 1995; Schikorski et al., 2011a). Histologically, lesions are confined to connective tissues in infected larvae (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 2001a,b). Fibroblastic-like cells exhibit an abnormal cytoplasmic basophilia and enlarged nuclei with marginated chromatin. Other cells, interpreted as hemocytes, show extensive chromatin condensation. The main histological change in spat is the presence of abnormal nuclei throughout connective tissues, especially in mantle, labial palps, gills and digestive gland (Renault et al., 1994a,b, 2000b). Abnormalities consist of enlarged nuclei associated with margined chromatin in fibroblastic-like cells and highly condensed nuclei in ovoid cells interpreted as hemocytes. Some nuclei show peculiar patterns of chromatin with a ring-shape or crescent-shape characteristic of apoptosis. Cellular abnormalities are not associated with a massive inflammatory reaction. Through TEM examination, abnormal accumulations of granular endoplasmic reticulum are often observed in affected tissues. The occasional occurrence of nuclear tubular structures 45–55 nm in diameter with a helical substructure has been reported (Renault et al., 2001a,b). Transverse sections of tubular structures show a tube within a tube arrangement similar in appearance to capsids with lucent cores (Renault et al., 2001a,b).

Renault et al. (2011) are the first authors to report data on genes related to antiviral immunity in the Pacific oyster. These authors studied virus-induced genes in Pacific oyster hemocytes challenged by OsHV-1 through suppressive subtraction hybridization. Different genes already known as immune-related genes were thus identified. Investigation of oyster gene expression by real-time RT PCR showed a significant increase of MyD88 gene transcripts in experimentally infected oysters. Segarra et al. (2014b) reported similar results and suggested that the overexpression of MyD88 could be interpreted more as a marker of the infectious process and viral replication than a marker of an effective antiviral response. Recently, Green et al. (2014) carried out a study to determine how Pacific oyster ontogeny interacts with water temperature to influence the antiviral response against OsHV-1 infection. For this purpose, they measured the effect of temperature (12°C vs 22°C) on oyster immune response after poly I:C injection. They reported that the expression of genes related to immunity was influenced by temperature and oyster age (Green et al., 2014).

AVG is an acute disease. Infected animals are likely to die within 1–2 days of demonstrating gross signs of the disease. In Victoria, Australia, AVG outbreaks in both farmed and wild abalone were associated with high mortality rates (up to 90% on farm). Clinically, abalone may demonstrate one or more of the following signs: irregular peripheral concave elevation of the foot; swollen and protruding mouth parts; eversion of the radula; minimal movement of the pedal muscle; excessive mucus production; absence of the marked extension of the foot shown in the righting reflex when healthy abalone are turned onto their backs; reduced pedal adhesion to the substrate. In Tasmania, abalone affected by AVG in processing plants exhibited "hard foot" or tetany, excessive mucus production, abnormal spawning and "bloating" (Ellard et al., 2009). Similar signs have been reported in Chinese Taipei (Chang et al., 2005). The major histopathological lesion identified in abalone affected with AVG is ganglioneuritis: inflammation confined to neural tissue. The cerebral, pleuropedal and buccal ganglia can be affected as well as the cerebral commissure and associated peripheral nerves (Hooper et al., 2007).

37.2.5 Diagnostic Methods

For diagnosis of OsHV-1 and AbHV infections, several methods are available including histology, TEM, PCR, real time PCR, DNA in situ hybridization (ISH) and nucleic acid sequence analysis. However, to date, attempts to culture viruses in both vertebrate and invertebrate cell lines and in primary mollusk cell cultures have been unsuccessful. Histology and TEM have been primarily used for viral infection diagnosis based on detection of cell lesions and direct visualization, respectively. However, PCR, real-time PCR, and nucleic acid sequence analysis have replaced histology, the traditional test used for marine mollusk diseases.

Histological examination of the animal on its own is not sufficient to identify a herpesvirus infection. While Cowdry type A inclusions (eosinophilic intranuclear inclusions with perinuclear chromatin) are typical of many herpesvirus infections, this type of inclusions has never been reported following histological examination of OsHV-1 infected Pacific oysters in France (Renault et al., 1994a,b). Moreover, intranuclear inclusion bodies were not observed, although there was other

cellular/nuclear pathology, in association with OsHV-1 infections. Sections of tissue that include mantle, digestive gland, gills and adductor muscle should be sampled and fixed (using 10% formaldehyde and processed using standard procedures, and stained with hematoxylin and eosin) for histological examination. A positive result consists of the occurrence of cell abnormalities on tissue sections: fibroblastic-like cells exhibiting enlarged nuclei with perinuclear chromatin. Condensed nuclei are also reported in other cells interpreted as hemocytes. These abnormal cells are not associated with mass hemocyte infiltration. Should perinuclear chromatin be observed by histology, TEM at the least should be undertaken to identify any virus particles present and demonstrate their location within cells. The presence of viral particles can be detected by TEM in OsHV-1 infected animals. OsHV-1 replication mainly takes place in fibroblastic-like cells especially in mantle, labial palps, gills and digestive gland (Renault et al., 1994b, 1995). Viruses observed under the TEM should be described as herpesvirus-like until further investigations are done to provide further evidence of the identity of the virus. A virus should only be described as OsHV-1 if it had been shown to have identity with the latter virus using OsHV-1 specific primers or probes with molecular tests because different herpesviruses are morphologically similar.

AbHV-infected animals demonstrate on histological examination ganglioneuritis typified by an inflammatory change with increased cellularity involving mainly hemocytes and glial cells, and cell necrosis in the affected nerves (Mohammad et al., 2011). Neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) is the prime target. TEM can be used to confirm the presence of viral particles in infected ganglia. AbHV particles are icosahedral with electron-dense cores and a diameter of 100–110 nm. The intranuclear location of the particles and their ultrastructure are characteristic of members of the Herpesvirales (Tan et al., 2008).

Although OsHV-1 specific antibodies have been developed (Arzul et al., 2002), they are not currently available for diagnostic purpose.

A breakthrough was achieved in the development of a protocol based on sucrose gradient centrifugation, for purifying OsHV-1 particles from fresh infected larval C. *gigas* (Le Deuff and Renault, 1999). This advance has served as an appropriate platform for developing molecular diagnosis reagents and techniques including conventional PCR, real-time PCR, loop-mediated isothermal amplification and DNA/RNA ISH assays (Barbosa-Solomieu et al., 2004; Burge and Friedman, 2012; Lipart and Renault, 2002; Oden et al., 2011; Pepin et al., 2008; Ren et al., 2010; Renault and Lipart, 1998; Renault et al., 2000b; Segarra et al., 2014a,b). This rise in molecular technologies has allowed improvements in diagnostic, epidemiological and research capabilities of aquatic animal health experts worldwide (Barbosa-Solomieu et al., 2004; Garcia et al., 2011; Lynch et al., 2012; Roque et al., 2012; Segarra et al., 2010; Webb et al., 2007). A real-time reverse transcriptase PCR was recently developed to measure the expression of 39 virus genes in Pacific oyster tissues (Segarra et al., 2014a). In order to detect and localize viral RNA on histological sections, a specific ISH assay was also developed (Corbeil et al., 2014). This technique provides information on the expression of three OsHV-1 genes in Pacific oyster tissues in relation to changes detected by histology and virus DNA detection by ISH and real-time PCR.

Several PCR methods are available for the detection of OsHV-1 (Batista et al., 2007). This includes conventional PCRs using the primer sets C2/C4 and C2/C6 (Batista et al., 2007) as well as real-time methods (Martenot et al., 2010; Pepin et al., 2008). Differences in sensitivity have been reported between different laboratories and different methods (Martenot et al., 2010). A Sybr Green-based real-time PCR (Pepin et al., 2008) is currently used for screening bivalve samples. Three sets of primers have been used targeting three regions of viral DNA: ORF4, ORF88 and ORF99. Primer pairs B4/B3 (Arzul et al., 2001a; ORF99 encoding a BIR protein) and C9/C10 (Barbosa-Solomieu et al., 2004; ORF4) were previously designed for single PCR, whereas the Gp4/Gp7 primer pair (ORF88 encoding a class I membrane protein) was assessed for real-time PCR. The primer pairs B4/B3, C9/C10 and Gp4/Gp7 yield PCR products of 207, 197 and 85 bp, respectively:

B4: 5′-ACT-GGG-ATC-CGA-CTG-ACA-AC-3′
B3: 5′-GTG-GAG-GTG-GCT-GTT-GAA-AT-3′
C9: 5′-GAG-GGA-AAT-TTG-CGA-GAG-AA-3′
C10: 5′-ATC-ACC-GGC-AGA-CGT-AGG-3′
Gp4: 5′-GGC-GTC-CAA-ACT-CGA-TTA-AA-3′
Gp7: 5′-TTA-CAC-CTT-TGC-CGG-TGA-AT-3′

The C9/C10 primer pair yielded reliable parameters for real-time PCR with OsHV-1 DNA, as well as the B3/B4 primer pair, which showed closely similar parameters with a slightly lower *E* value (96.3%). The Gp4/Gp7 primer pair is less efficient ($E = 91.3\%$) and less sensitive (≥50 copies/μL). The primer pair C9/C10 appeared to be the most sensitive and efficient. An additional primer pair, DPFor/DPRev, can be also used producing a 197 bp product (ORF100, DNA polymerase):

DPFor: 5′-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3′
DPRev: 5′-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3′

Targeting different OsHV-1 genome areas is important in order to define more precisely viral types. Although ORF4 is an interesting candidate to describe diversity because virus polymorphism has been already reported in this area, ORF100 (DNA polymerase) appears as less polymorphic. The sensitivity of the Sybr Green-based real-time PCR (Pepin et al., 2008) was considered to detect systematically four viral DNA copies/μL. It was possible to quantify OsHV-1 DNA copy numbers at least from 10 to 5×10^6 copies/μL. The detection limit for the technique developed by Martenot et al. (2010) is 6 UG/mg of tissues.

ISH procedures based on the use of digoxigenin (DIG)-labeled DNA probes were developed to detect OsHV-1 in formalin-fixed, paraffin-embedded tissue (Arzul et al., 2002; Barbosa-Solomieu et al., 2004; Lipart and Renault, 2002). Tissues including mantle, digestive gland, gills and adductor muscle should be fixed for 24 h in Davidson's AFA or other suitable fixative and processed using standard procedures for histological examination. Specific dark brown intracellular staining is indicative of the presence of viral DNA. Thirty Pacific oysters, *C. gigas*, adults have been analyzed using three different techniques: PCR, ISH and immunochemistry, in order to detect OsHV-1 in asymptomatic individuals (Arzul et al., 2002). PCR and ISH allowed detection of oyster OsHV-1 DNA in 93.3% and 86.6%, respectively, of analyzed oysters while polyclonal antibodies allowed detection of viral proteins in 76.6% of analyzed adult oysters. DIG-labeled RNA probes and a specific ISH assay were also developed recently (Corbeil et al., 2014). A real-time reverse transcriptase PCR has been previously developed to measure the expression of 39 virus genes in Pacific oyster tissues (Segarra et al., 2014a,b).

The search for association between virus genetic markers and clinical symptoms is of great interest and the characterization of the genetic variability of OsHV-1 specimens is an area of growing interest. Characterization of OsHV-1 specimens has already been carried out through sequencing of nucleotide sequences of PCR-amplified virus DNA fragments (Renault et al., 2012). A genotyping method has also recently been developed in order to characterize clinical OsHV-1 specimens by targeting a particular microsatellite locus located in the ORF4 area (Renault et al., 2014b). The microsatellite (H10) located in a noncoding region was selected since sequences were already available for this region, demonstrating a high level of length polymorphism. The method was used to characterize 47 clinical OsHV-1 specimens by targeting this microsatellite. Sequencing and genotyping appeared to be equally useful to differentiate clinical OsHV-1 specimens. Since an in silico analysis of the OsHV-1 genome demonstrated the presence of 12 short repeat sites including 4 mononucleotide units, 5 dinucleotide repeats and 3 trinucleotide repeats, a multiplex genotyping based on analysis of several microsatellites appears of great interest. Genotyping based on virus microsatellites can offer a first level of discrimination between specimens in order to select best candidates for complete genome sequencing.

AbHV is identified using methods that specifically detect viral nucleic acid (PCR, sequencing, real-time PCR and ISH). Following validation of the real-time PCR test targeted to ORF49 (Corbeil et al., 2010), the discovery of genotypic variants in Australia not recognized by this test necessitated other real-time PCR tests to be developed based on more conserved regions of the viral genome. Real-time PCR tests targeted to ORF66 and ORF77 have been used extensively in disease investigations and, while formal validation has not been undertaken, appear to sensitively and generically detect all AbHV variants identified to date, including specimens from Chinese Taipei. The primer pair and specific probe sequences are detailed next:

AbHV ORF66F1: 5′-TCC-CGG-ACA-CCA-GTA-AGA-AC-3′
AbHV ORF66R1: 5′-CAA-GGC-TGC-TAT-GCG-TAT-GA-3′
AbHV 66Prb1: 5′-6FAM-TGG-CCG-TCG-AGA-TGT-CCA-TG-TAMRA-3′
AbHV ORF77F1: 5′-CAA-CCA-CTT-GTT-CGG-GTT-CT-3′
AbHV ORF77R1: 5′-CAG-GGT-GAT-TAA-TGC-GGA-GT-3′
AbHV 77Prb1: 5′-6FAM-TCC-GTA-CGC-GGG-ATC-TTC-GT-TAMRA-3′

Conventional PCR may also be used for detection of AbHV in tissue samples. The AbHV1617 PCR has been shown to generate amplicons of various lengths (522–588 bp) depending on the AbHV specimens. Thus, it is potentially useful for epidemiological studies and to confirm positive real-time PCR results. The primer sequences are detailed next:

AbHV-16: 5′-GGC-TCG-TTC-GGT-CGT-AGA-ATG-3′
AbHV-17: 5′-TCA-GCG-TGT-ACA-GAT-CCA-TGT-C-3′

An ISH procedure has been developed using a DIG-labeled DNA probe (AbHV_ORF66f1: 5′-TCC-CGG-ACA-CCA-GTA-AGA-AC-3′, AbHV_ORF66r2: 5′-GCC-GGT-CTT-TGA-AGG-ATC-TA-3′) to detect AbHV in formalin-fixed, paraffin-embedded tissue sections. Specific dark blue-black intracellular staining is indicative of the presence of viral DNA.

37.2.6 Prevention and Control

As mollusks lack a "true" adaptive immune system and an immune memory (Gestal et al., 2008), vaccination cannot be used to protect them against pathogens, including viruses. In addition, the use of drugs is highly limited for animals that are most often reared in the open sea. Within this context, controlling animal transfers appears to be one of the most suitable ways to combat infectious diseases in mollusks and international rules have thus been developed. Moreover, in the absence of effective antiviral treatments, implementing high levels of on-farm biosecurity is recommended.

Although the infection caused by OsHV-1 is not listed at present as a notifiable disease by the EU legislation (2006/88/EC, Annex IV) or by the OIE (Manual of Diagnostic Tests for Aquatic Animals, 2015), an EU regulation (Regulation 175/2010) was implemented in March 2010. This regulation outlines measures to be taken in the case of increased mortality in the species *C. gigas* in connection with the detection of a particular variant of OsHV-1, the variant µVar. Increased mortalities of Pacific oysters have been reported in several EU member states since 2008. They were attributed to the presence of OsHV-1, including a newly described genotype named µVar. In the case of abnormal mortality outbreaks among *C. gigas* oysters, a targeted surveillance should be carried out to detect or rule out the presence of the variant µVar. When the presence of that virus has been detected, disease control measures should be implemented including the establishment of a containment area and the restriction to the movements out of the containment areas of *C. gigas* oysters. The measures have been applied until the end of 2010 and amended as appropriate after that period, taking into account the knowledge and experience gained. Although the infection caused by AbHV is listed as a notifiable disease by the OIE (Manual of Diagnostic Tests for Aquatic Animals, 2015), it is not listed at present as a notifiable disease by the EU legislation (2006/88/EC, Annex IV).

Rapid and accurate differential diagnosis is the key to success in controlling OsHV-1 and AbHV infection outbreaks. Molecular techniques including real-time PCR, in combination with sequence analysis of amplicons enable a much more precise analysis of OsHV-1 epidemiology in the field and may help to define the most adapted measures for disease containment.

Biosecurity may be successfully applied in confined and controlled facilities such as hatcheries and nurseries in order to protect the facility and the surrounding environment from the introduction of the virus. Following an on-farm outbreak, the destruction of infected stock, disinfection of water and equipment and fallowing procedures appear to be effective at preventing reinfection. It may be assumed that OsHV-1 and AbHV are fragile because they are enveloped viruses. High temperature, chemicals or sunlight (UV) may destroy their lipid-containing envelopes. Thus, in controlled rearing conditions (mollusk hatchery/nursery), viral infection outbreaks may therefore be controlled through quarantine and hygienic measures, including virus inactivation through adapted treatments. However, it is necessary to keep in mind that reduction of virus load depends on the initial titer and the virus reduction capacity of the techniques used for inactivation. If there was an initial concentration of 1 million viruses per L and the inactivation method used allowed inactivation of 10,000 viruses per L there would still be 990,000 infective particles per L in the treated product.

Finally, marine mollusk breeding programs targeting the production of resistant animals to a particular viral disease appear as one of the most promising approaches for aquaculture development. Selective breeding of bivalve stocks appears to be one of the most promising approaches for mollusk aquaculture development and disease management. Some work has been initiated in order to estimate genetic parameters for OsHV-1 infection resistance or tolerance (Sauvage et al., 2009; Degremont et al., 2013) and the correlations with other traits of interest, such as growth rates and quality of juvenile production. Several studies support the hypothesis of a genetic basis underlying resistance to OsHV-1 infection in the Pacific oyster, *C. gigas* (Degremont et al., 2013; Sauvage et al., 2009; Segarra et al., 2014b). In addition, differences in terms of susceptibility to the viral infection appeared in a single raceway and exposed to the same risk, obtained results that strongly suggested different susceptibility to the viral infection between oysters (Sauvage et al., 2009). Segarra et al. (2014b) reported a divergent response of Pacific oyster families in terms of mortality during an experimental OsHV-1 infection. They also suggested that oysters are genetically diverse in terms of susceptibility to OsHV-1 infection. After an active phase of virus replication demonstrated by an increase of viral DNA and RNA detection, DNA and RNA amounts decreased rapidly, suggesting that the host immune defense was activated and some oysters were able to circumvent OsHV-1 infection (Segarra et al., 2014b). Genomic tools will be developed to initiate marker assisted selection or genomic selection disease tolerance/resistance.

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Chapter 38

Papillomalike Viruses and Polyomalike Viruses of Mollusks

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38.1 INTRODUCTION

Papillomaviridae is a family of nonenveloped DNA viruses. Several hundred species of papillomaviruses (PV), traditionally referred to as "types," have been identified infecting mammals, but also birds, snakes and turtles. PVs are usually considered as highly host- and tissue-tropic, and are thought to rarely be transmitted between species. Infection by most PVs is either asymptomatic or causes small benign tumors, known as papillomas or warts. Papillomas caused by some types, however, such as human PV 16 and 18, carry a risk of becoming cancerous (Muñoza et al., 2006). All PVs have similar genomic organizations, and any pair of PVs contains at least five homologous genes, although the nucleotide sequence may diverge by more than 50%. The family *Papillomaviridae* is distinct from the *Polyomaviridae,* thus eliminating the old family name *Papovaviridae* (Bernard et al., 2012).

Polyomaviruses are DNA viruses with a circular double-stranded DNA genome of ~5000 bp. They are small (40–50 nm in diameter), and icosahedral in shape, and do not have a lipoprotein envelope. The genome is composed of early and late genes, contributing to a complex transcription program. They are potentially oncogenic (tumor-causing); they often persist as latent infections in a host without causing disease, but may produce tumors. The family *Polyomaviridae* used to be one of two genera within the now obsolete family *Papovaviridae* (the other family being *Papillomaviridae*). Many polyomaviruses have been found to infect birds and mammals. Polyomaviruses have been extensively studied as tumor viruses in humans and animals, leading to fundamental insights into carcinogenesis, DNA replication and protein processing.

38.2 SPECIFIC MAIN MOLLUSK DISEASES

The main objective of this chapter is to provide updated information on papillomalike/polyomalike viruses that infect bivalves and replicate in their tissues. The exact identification of these viruses that infect marine bivalves remains to be made. In this context, the terms *papillomalike viruses* and *polyomalike viruses* need to be used as their exact affiliations have yet to be found. In addition, definitive demonstration of viral etiology of related diseases must be accomplished by experimental transmission.

Papillomalike/polyomalike viruses have been reported from the gonadal epithelia of *Crassostrea virginica* in Canada (McGladdery and Stephenson, 1994) and the United States (Farley, 1976; Meyers, 1981; Winstead and Courtney, 2003), as well as from other bivalve species (Elston, 1997). These viruses were first reported as papovalike viruses based on morphological features. Farley (1976) reported for the first time a papilloma/polyomalike virus in hypertrophied gametocytes of eastern oysters, *C. virginica*. However, no clinical effects were recognized in infected individuals. Transmission electron microscopy (TEM) examination revealed the presence of intranuclear, nonenveloped viruslike particles ranging from 50 to 55 nm in diameter with an icosahedral shape. Norton et al. (1993) reported a papilloma/polyomalike virus infection in the golden-lip pearl oyster, *Pinctada maxima*, from Australia. Infected oysters demonstrated hypertrophied epithelial cells in labial palps. These cells presented nuclei containing eosinophilic inclusion bodies. TEM examination revealed viruslike particles 60 nm in diameter, resembling PV or polyomaviruses in those inclusions. During a health survey of cultured Pacific oyster, *Crassostrea gigas*, carried out in the Tongyoung area on the southern coast of Korea, a papilloma/polyomalike virus was detected in gonadal tissues (Choi et al., 2004). Nonenveloped, icosahedral particles 40–45 nm in diameter were detected by TEM (Choi et al., 2004). Histological examination of *C. gigas* oysters in France also revealed several cases of abnormally large basophilic cells in gonadal tissues that resembled cells characteristic of ovacystis disease

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00038-3
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(viral gametic hypertrophy (VGH)) (Garcia et al., 2006) previously reported in *C. virginica* oysters from Maine and Long Island in the United States (Winstead and Courtney, 2003). TEM examination of Feulgen-positive inclusions, observed in both males and females, revealed the presence of nonenveloped free viral particles of approximately 50 nm in diameter. Similar abnormal cells have been observed occasionally in *C. gigas* in France. However, viral particles were described for the first time in 2003 in French oysters (Garcia et al., 2006). VGH was also detected during an investigation of mortalities in Pacific oyster *C. gigas* in Ireland (Cheslett et al., 2009). TEM demonstrated the presence of small, nonenveloped, icosahedral viral particles approximately 50 nm in diameter. The viral particles described by Cheslett et al. (2009) appear to be similar to those described in *C. virginica* from the United States and Canada and to those described in *C. gigas* from Korea and France.

Ruiz et al. (2011) reported nuclear basophilic inclusion bodies in razor clam *Ensis arcuatus* from Galicia (northwest Spain) during a histological survey. TEM examination revealed the presence of viral particles with icosahedral symmetry inside the nuclear inclusion bodies. Size and symmetry of the nonenveloped virus particles suggest similarities to the families *Papillomaviridae* and *Polyomaviridae*.

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Part VII

RNA Viruses of Mollusks

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Chapter 39

Picornalike Viruses of Mollusks

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39.1 INTRODUCTION

Picornaviruses are nonenveloped, positive single-stranded RNA (between 7.2 and 9.0 kb in length) viruses with an icosahedral capsid. Picornaviruses are separated into a number of genera. The diseases they cause are varied, ranging from acute "common-cold"-like illnesses, to polio, to chronic livestock infections. Contained within the family *Picornaviridae* are many organisms of importance, such as vertebrate and human pathogens (Mettenleiter and Sobrino, 2008).

39.2 SPECIFIC MAIN MOLLUSK DISEASES

Mollusks may act as a transient reservoir in absence of viral replication for vertebrate viruses upon their ingestion (Lees, 2000; Potasman et al., 2002; Nishida et al., 2003). There is thus considerable literature on the human health implications, including the detection of picornaviruses such as hepatitis A virus, in different marine bivalve species of commercial interest (Intamaso and Ketkhunthod, 2014). However, the objective of the present chapter is to give updated information on viruses that infect mollusks and replicate in their tissues. Mollusk cultivation may be endangered by the occurrence of epizootics including viral diseases, which are considered one of the main risks to production. Mass mortality outbreaks have been described among different mollusk species associated with the presence of viruses belonging to various families. Viruses described in mollusks are interpreted as members of the *Herpesviridae*, *Papillomaviridae*, *Polyomaviridae*, *Togaviridae*, *Retroviridae*, *Reoviridae*, *Birnaviridae* and *Picornaviridae* families (Farley et al., 1972, Farley, 1976, 1978; Opandry et al., 1981; Meyers, 1979; Rasmussen, 1986). The discovery of viruses interpreted as picornaviruses in mollusks is a fairly recent event. The basic method to diagnose virus infections affecting mollusks has long been light microscopy. This procedure appears to be poorly adapted to viral disease diagnosis and additional techniques, such as transmission electron microscopy, have to be undertaken. Despite being time consuming and somehow inadequate for epidemiological surveys, both techniques were extensively employed through lack of suitable alternative diagnostic procedures. The research into virus cytopathogenic effects in cell cultures is not practical due to the absence of mollusk cell lines. Finally, no genetic data have been produced for any viruses interpreted as picornaviruses infecting mollusks. Their exact identification has thus not been carried out. In this context, the term *picornalike viruses* needs to be used because their exact affiliation remains to be found.

Virus-like particles of 27 nm in diameter were described in mussels, *Mytilus edulis*, from Denmark (Rasmussen, 1986). These virus-like particles were enclosed in vesicles, arranged singly or in paracrystalline arrays. Chronic inflammatory conditions interpreted as granulocytomas were reportedly associated with detection of the virus-like particles in hemolymph spaces of the digestive gland and mantle. Although similar granulocytomas have been reported from mussels in Britain by Lowe and Moore (1978), no study was undertaken by transmission electron microscopy to explore the presence of viral particles.

Jones et al. (1996) reported the detection of virus-like particles in farmed seed and adult green-lip mussels, *Perna canaliculus*, suffering mortality outbreaks in New Zealand. Extensive hemocyte infiltration and multifocal necrosis of interstitial cells, basal cells and digestive tubule epithelial cells were reported through histology examination. Transmission electron microscope examination demonstrated modified rough endoplasmic reticulum with the presence of electron-dense uncoated viruslike particles ranging from 25 to 45 nm in diameter (Jones et al., 1996). Similar virus-like particles and cell lesions were subsequently found in *Mytilus galloprovincialis* from the same area in New Zealand. Finally, apparent replication of small viruslike particles was described from digestive and secretory cells of scallops, *Pecten novaezelandiae*,

Aquaculture Virology. DOI: http://dx.doi.org/10.1016/B978-0-12-801573-5.00039-5
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and toheroa, *Paphies ventricosum*, collected during a mass mortality outbreak in New Zealand (Hine and Wesney, 1997). Virus-like particles ranged from 22 to 30 nm in diameter and appeared in an orderly array on the surfaces of the outer nuclear membrane and along the endoplasmic reticulum.

Miyazaki et al. (1999) reported a morphologically similar nonenveloped virus, 25–33 nm in diameter, in Japanese pearl oysters, *Pinctada fucata martensii*, collected during mass mortality events. Viral particles were associated with necrosis and degeneration of muscle fibers. The virus has been called "Akoya-virus" by Miyazaki et al. (1999). Its size is similar to that of picornaviruses and it resembles the virus from the scallop, *Pecten novaezelandiae*, reported in New Zealand by Hine and Wesney (1997). Akoya-virus was considered by Miyazaki et al. (1999) to be responsible for mass mortality events of pearl oysters, which had occurred in western regions of Japan since 1994. Necrosis and degeneration of muscle fibers were reported through histology examination. No Feulgen-positive inclusions were observed, suggesting that the putative virus had RNA genome (Miyazaki et al., 1999). The sarcoplasm of necrotic muscle fibers of the adductor muscle contained inclusion bodies containing round, nonenveloped viral particles (25–33 nm in diameter). In addition, the infected muscle fibers had thinned or fragmented myofibrils, vacuoles in the sarcoplasm, mitochondria with destroyed cristae and expanded reticula (Miyazaki et al., 1999). Miyazaki et al. (1999) proposed that juvenile oysters might be healthy carriers during the winter. These infected oysters then became diseased on the return of optimum water temperatures (above 25°C) and served as a source for horizontal transmission of the infection and resulting mass mortality events. Miyazaki et al. (2000) reported that an antiviral, recombinant feline interferon-ω (rFeIFN-ω) administered by injection was efficient in preventing mortality of *Pinctada fucata* after "Akoya-virus" injection. Hemocytes of *Pinctada fucata* might possess receptors to bind the rFeIFN-ω, an interferon produced in silkworms by a recombinant baculovirus (Miyazaki et al., 2002). However, prophylactic rFeIFN-ω administration was not completely effective in preventing mortality especially when viral contamination occurred several days after rFeIFN-ω administration (Miyazaki et al., 2000).

Virus-like particles were detected in granulomas associated with focal necrosis within the adductor muscle of the pearl oyster, *Pinctada margaritifera*, from French Polynesia (Comps et al., 1999). The lesions found in the muscle of *Pinctada margaritifera* displayed some similarities to granulocytomas from the mussel *M. edulis* infected by a picornalike virus (Rasmussen, 1986). Paraspherical or polygonal shaped viruslike particles were detected by transmission electron microscopy (Comps et al., 1999). Within the altered muscle of *Pinctada margaritifera*, numerous interstitial or glial cells containing electron-dense and membrane-bound ovoid bodies appeared associated with muscle fibers and connective tissue, which could be related to the process of regeneration of muscle tissue (Comps et al., 2001). However, the exact nature and role of viruslike particles observed in *Pinctada margaritifera* are unknown because the virus has not been purified or biochemically characterized from diseased pearl oysters. In addition, it has not been demonstrated that the "Akoya-virus" infects *Pinctada margaritifera* (Comps et al., 2001).

The first description of a virus affecting the carpet-shell clam, *Ruditapes decussatus*, was reported by Novoa and Figueras (2000). Viruslike particles were detected in the cytoplasm of connective tissue cells from clams suffering mortality outbreaks in Galicia, Spain. Nonenveloped viruslike particles, icosahedral-spherical in shape, ranging from 27 to 35 nm in diameter appeared free in the cytoplasm or associated with endoplasmic reticulum membranes and cytoplasmic vesicles. Due to their size, morphology and replication in the cytoplasm in association with endoplasmic reticulum and cytoplasmic vesicles, they were suggested to belong to the *Picornaviridae* family. Novoa and Figueras (2000) suggested that viruslike particles might be the cause of clam mass mortality. Mortality outbreaks of Manila clams, *Ruditapes philippinarum*, were also reported in southern England in 2008 (Bateman et al., 2012). Samples collected from five sites across the south coast of England were subjected to histology and transmission electron microscopy examination. Unenveloped viruslike particles were thus detected within the connective tissue of the gills and surrounding the tubules in the digestive gland. The viruslike particles appeared to be free within the cytoplasm or associated with endoplasmic reticulum membranes and cytoplasmic vesicles. Particles were icosahedral in shape, with a diameter of 25–30 nm. The location, size and morphology of the viruslike particles suggest that they belong to the *Picornaviridae* family. Finally, virus-like particles associated with large foci of hemocytic infiltration were reported in cockles, *Cerastoderma edule*, from Galicia, Spain (Carballal et al., 2003). Nonenveloped viruslike particles were detected in paracrystalline arrays in the cell cytoplasm through transmission electron microscopy examination. Icosahedral capsids measured 19–21 nm in length. They were assumed to be RNA positive because histological sections of hemocyte foci stained with methyl green pyronin demonstrated red deposits. Virus-like particles were similar to those described in mussels by Rasmussen (1986) and due to their size, morphology and the formation of paracrystalline arrays, they were considered to belong to the *Picornaviridae* family.

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Appendix A*

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A.1 ALLOHERPESVIRUSES OF FISH

These are large DNA viruses that require an intact envelope for infectivity; therefore they are easily inactivated using detergents, alcohol, heat and desiccation or Ultraviolet (UV) irradiation. Oxidizers, halide, phenolic and aldehyde disinfectants are also effective. The use of 10-ppm free chorine as an indefinite treatment, or 100 ppm for 1 h, is effective for the decontamination of equipment or tanks. If desiccation is used for decontamination, the equipment must be free of organic residue, as proteins and other organic matter can protect the virion from inactivation.

Samples to be taken for diagnostic use should be fish showing disease signs. These can be placed on ice or refrigerated at 4°C for up to 48 h for transport to a diagnostic lab. Tissue samples for cell culture where appropriate needed are whole fry, viscera from small fingerlings or target tissue from larger fish. These can be suspended in serum-free cell culture medium or saline buffered to a pH of 7.6 and supplemented with penicillin (up to 800 IU/mL), streptomycin (up to 800 µg/mL) or Fungizone (amphotericin B, up to 40 IU/mL). For polymerase chain reaction (PCR) analyses of diseased fish, samples may be taken from the samples taken for cell culture or tissues can be fixed in 90% ethanol (not formalin or any other histology fixative). For evaluating carrier status samples can be fresh tissues, samples stored at −70°C, or samples processed to less than 0.5 cm^3 pieces and stored in RNA later.

A.1.1 Catfish Herpesviruses—*Ictalurid Herpesvirus* 1 (IcHV1) and *Ictalurid Herpesvirus* 2 (IcHV2)

Neither pathogen is OIE listed; however, several countries and states require catfish to be declared IcHV1 free before importation.

There are no commercially available vaccines for these viruses, reducing water temperature to below 26°C (for IcHV1) and treating secondary infections will reduce losses if an outbreak occurs. For IcHV2, the water temperature would need to be reduced to 20°C or less.

Target tissue for channel catfish virus disease (CCVD) is posterior kidney. Nonlethal sampling for latent IcHV1 testing of large fish can be done using fin biopsies. These viruses can be cultured on cell lines from North American catfish species. The most sensitive cell line for IcHV1 is the channel catfish ovary cell line (CCO, ATCC CRL-2772), but the brown bullhead cell line (BB, ATCC CCL-59) is also sensitive. A 1:100 (tissue to medium) final dilution of homogenate decontaminated by 0.45micron filtration or antibiotic/antimycotic treatment. The cells should be incubated at 28°C–30°C and monitored daily for cytopathic effects (CPEs), which rapidly progress after first detection and consist of a syncytium that contract from the culture flask surface. IcHV2 will also grow on the bluegill fry cell line (BF2, ATCC CCL91) and the fathead minnow-derived cell line, EPC (ATCC CRL-2872).

The use of specific PCR is needed to confirm a disease diagnosis. Also, PCR directly on tissue extract is the only reliable method to identify latent virus in carriers because no infectious virus is present at this time and serology can be inconsistent due variability in antibody levels in carrier fish (Thompson et al., 2005). The method of Goodwin and Marecaux (2010) can be used to evaluate the presence of IcHV2 and distinguish it from IcHV1.

A.1.2 Cyprinid Herpesviruses—CyHV1, CyHV2 and CyHV3

Cyprinid herpesvirus 3 (koi herpesvirus) is an OIE-listed pathogen.

There are no commercially available vaccines for CyHV1 (carp pox disease). Management of primary CyHV1 infection has not been studied in detail, but increasing temperature to above 25°C will likely reduce losses; in addition, this will result in regression of papillomas produced on carrier fish. A commercially available attenuated live CyHV3 vaccine KoVax has been marketed in the United States and in Israel. Also, if an outbreak of koi herpesvirus disease occurs, reducing water temperature to below 20°C or above 28°C will reduce losses but the survivors will be carriers of CyHV3.

* Contributed to by each author of the relevant chapter.

These viruses are difficult to culture, and PCR is generally considered more reliable for diagnostic purposes. Target organs are skin (papilloma) for CyHV1, kidney for CyHV2 and gill and kidney for CyHV3. The use of specific sensitive PCR on affected tissue is needed to confirm a disease diagnosis. Also, PCR directly on tissue extract is the only reliable method to identify latent virus in carriers. The TaqMan method of Goodwin et al. (2006) is specific and sensitive for CyHV2, the method of Bercovier et al. (2005) was most sensitive of the traditional PCR methods published and the method of Gilad et al. (2004) was the most sensitive TaqMan-based PCR assay.

A.1.3 Eel Herpesvirus—*Anguillid Herpesvirus* 1 (AngHV1)

This is not an OIE-listed pathogen.

There are no vaccines available for AngHV1. Disease management consists of treating secondary pathogens and if possible lowering the temperature to below 22°C.

The tissues sample for cell culture are gills, liver, posterior kidney and spleen. The virus causes syncytium formation on the eel kidney cell line EK-1 at 25°C. PCR must be used for confirmation and detection of latent virus. The qPCR method of van Beurden et al. (2015) is sensitive and specific.

A.1.4 Sturgeon Herpesviruses—*Acipenserid Herpesvirus 1* (AciHV1) and *Acipenserid Herpesvirus 2* (AciHV2)

Neither pathogen is OIE-listed.

There are no vaccines for AciHV1 or AciHV2. Management includes the avoidance of known carriers, treating secondary pathogens and reducing the water temperature to 12°C or less.

Epidermis, kidney and liver are plated on white sturgeon skin (WSSK-1) or white sturgeon gonad cells at 15°C. CPE of AciHV1 involves syncytia formation, which results in large clumps of fused cells. CPEs of AciHV2 involve the focal destruction of the monolayer with grapelike clusters of cells surrounding infective foci (3–7 days) and progress to the complete destruction of the monolayer by 2 weeks. Confirmation requires the use of serology or molecular analysis.

A.1.4.1 Salmonid Alloherpesviruses—Salmonid Herpesvirus *1, 2, 3 and 4 (SalHV1-4)*

Salmonid herpesvirus 2 was an OIE-listed pathogen until 2009. There are many states requiring that fish be certified free of SalHV2 and USFWL Title 50 restricts the import of live salmonids and eggs to be certified SalHV2 free by inspection.

A.1.4.1.1 Salmonid Herpesvirus 1

There is little active management to control SalHV1. Avoidance (eliminating carrier brood fish) has been effectively used.

The virus can be detected on Chinook salmon embryo (CHSE-214) or rainbow trout gonad (RTG-2) cells producing extensive syncytia at 10°C and not CPEs at 15°C. Positive results should be tested with SalHV1- and SalHV2-specific antisera or SaHV2-specific PCR to rule out the more virulent closely related SalHV2.

A.1.4.1.2 Salmonid Herpesvirus 2

The most in-depth studies on controlling diseases caused by salmonid herpesviruses have been on SalHV2. This management involves avoidance of carrier fish, disinfecting eggs using iodine treatments, sanitation and sterilizing incoming water and using good facility hygiene. In addition, the use of injectable formalin killed vaccine in brood stock is advocated to reduce the vertical transmission of the virus.

Diagnosis of SalHV2 infection can be done using cell culture on RTG-2 or CHSE 215 cells at 15°C. For diagnosis of acute infection, the liver, kidney and spleen are processed for cell culture. In the tumor form of the disease, it is best to cocultivate tumor tissue with RTG-2 cells (Yoshimizu, 2012). Carrier fish can be detected by screening ovarian fluid and tissues from post-spawning adults. Confirmation can be done using serum neutralization assays using SalHV2-specific antisera or by SalHV2-specific PCR (Aso et al., 2001).

A.1.4.1.3 Salmonid Herpesvirus 3

Management to reduce SalHV3-caused epizootic epitheliotropic disease has focused on the reducing stress, reducing fish density and on minimizing recirculation of hatchery water. No vaccines have been developed or studied for this virus.

Some hatcheries that experience high losses during the initial outbreaks depopulated and disinfected their facilities, and then obtain eggs from a nonendemic source. The SalHV3-specific PCR can be used to detect carrier brood fish and to allow culling of eggs and fry that may have received the infections by vertical transmission (Yoshimizu, 2009).

Diagnosis of SalHV3 infections requires molecular methods such as PCR (Kurobe et al., 2009) or qPCR (Glenney et al., 2016). SalHV4 is closely related and PCR based on the terminase gene will may result in positive a positive result for both viruses.

A.1.4.1.4 Salmonid Herpesvirus 4

There are no vaccines for SalHV4. Management methods include the use of prophylactic salt and formalin treatments on alternating weeks, minimizing stress and treating secondary infections when the papilloma regression and sloughing occurs. The use of PCR-based assays may allow screening for carriers and pathogen avoidance procedures.

A.2 REOVIRUSES OF AQUATIC ORGANISMS

A.2.1 Name of Virus: *Aquareovirus* (Genus)

Notifiable: No, but *Grass carp reovirus* (GCRV) is one of the major fish pathogens for freshwater species (catfish, carp, etc.) in Asia
Inactivation: Complete inactivation of virus was obtained with 2% formalin within 5 min and with 42.5% isopropanol within 15 min at 15°C. Chlorine and iodine are not effective at inactivating *Turbot aquareovirus* (TRV) (Rivas et al., 1996)
Decontamination: Use chemical disinfectants to destroy the virus in hatcheries, tanks, ponds, effluent water or equipment
Fish waste treatment: None known
Available vaccines: Vaccine formulations to date include inactivated and attenuated vaccines
Treatment regimens: None known
Diagnostic standards: None
Samples needed for testing: Heart, kidney, liver.

A.2.2 Name of Virus: Piscine Orthoreovirus (PRV)

Notifiable: No, but increased mortality associated with virus is notifiabled in Chile
Inactivation: pH below 3.0 and above 9.0
Decontamination: Disinfect with bleach, Virkon, 70% isopropanol
Fish waste treatment: None known
Available vaccines: No vaccine available
Treatment regimens: None known
Diagnostic standards: Histopathology and RT-PCR are used in Norway.
Samples needed for testing: Heart, kidney, liver and skeletal muscle.

A.3 PARAMYXOVIRUSES OF FISH

A.3.1 Name of Virus: Pacific Salmon Paramyxovirus; Atlantic Salmon Paramyxovirus

Notifiable: No
Inactivation: Enveloped virus inactivated by heat (37°C), chloroform and high and low pH values
Decontamination: Presumed sensitive to standard topical virucidal compounds, including chlorine and iodophors
Fish waste treatment: None known
Available vaccines: None known
Treatment regimens: None known
Diagnostic standards: Kidney/spleen, gonadal fluids and gills from spawning adult salmonids for standard cell culture on susceptible fish cell lines with confirmation by molecular methods or immunostaining.

A.4 RHABDOVIRUSES OF FISH

A.4.1 Name of Virus: Infectious Hematopoietic Necrosis Virus

Notifiable: All countries
Inactivation: Common disinfectants and drying
Decontamination: Depopulation and complete disinfection
Fish waste treatment:
Available vaccines: DNA vaccine (APEX IHN, Novartis); only approved and Canada and the United States
Treatment regimens: No treatment regimens available
Diagnostic standards: OIE diagnostic standards (OIE, 2015a)
Samples needed for testing: Samples include anterior kidney, spleen, gill and encephalon.

A.4.2 Name of Virus: Viral Hemorrhagic Septicemia Virus, Spring Viremia of Carp Virus

Notifiable: All countries
Inactivation: Common disinfectants and drying
Decontamination: Depopulation and complete disinfection
Fish waste treatment:
Available vaccines: None
Treatment regimens: No treatment regimens available
Diagnostic standards: OIE diagnostic standards (OIE, 2015b)
Samples needed for testing: samples include anterior kidney, spleen, heart and gill.

A.5 ORTHOMYXOVIRUSES OF FISH

A.5.1 Name of Virus: Infectious Salmon Anemia Virus (ISAV)

Notifiable: All countries
Inactivation: Common disinfectants and drying
Decontamination: Depopulation and complete disinfection
Fish waste treatment:
Available vaccines: Five vaccines of inactivated whole ISAV emulsified with adjuvant and administered by intraperitoneal injection and one oral vaccine (available for the Chilean market), are available commercially (Falk, 2014)
Treatment regimens: No treatment regimens available
Diagnostic standards: OIE diagnostic standards (OIE, 2014)
Samples needed for testing: samples include anterior kidney, spleen, gill and liver.

A.6 HEPEVIRUSES OF FISH

A.6.1 Name of Virus: Cutthroat Trout Virus (CTV)

Notifiable countries: None
Inactivation: Virus inactivation was only performed during initial characterization work (Hedrick et al., 1991). CTV was stable at 37°C for 15 min; however, as incubation temperature was increased, virus was inactivated. The highest temperature tested was 60°C for 15 min, which resulted in a virus concentration reduction of almost 100,000-fold. Mammalian zoonotic hepeviruses have been studied for thermal inactivation of pork products, where internal temperatures of 71°C were necessary to completely inactivate Hepatitis E virus (Feagins et al., 2008, Barnaud et al., 2012)
Decontamination: There are no reports of testing for CTV elimination by any chemical agents
Fish waste treatment: None tested
Available vaccines: None tested
Treatment regimens: Not necessary (no disease)
National diagnostic standards/samples for testing: None have been determined. The virus is primarily isolated from spawning trout ovarian fluid samples (Hedrick et al., 1991, Batts et al., 2011). If juvenile trout are exposed to the virus, they may harbor the virus in kidney and spleen tissues for up to 6 weeks. Avoidance is the only control measure to prevent infection. The only real risk from CTV seems to be trade or movement restrictions of infected fish or eggs from infected adults.

A.7 NIMAVIRUSES OF CRUSTACEANS

A.7.1 Name of Virus: White Spot Syndrome Virus (WSSV) or White Spot Virus (WSV)

Notifiable countries: OIE-notifiable disease; notifiable in all countries
Inactivation: WSSV can be inactivated at 50°C for 120 min or less than 1 min at 60°C (Nakano et al., 1998)
Decontamination: Emergency harvest of aquacultural crop and complete removal of potential host species
Crustacean waste treatment: None tested
Available vaccines: No available vaccines; the immunostimulants available provide only limited protection
Treatment regimens: Not available
Diagnostic standards and types of samples needed for testing: OIE nested PCR test confirmed with TEM is the gold standard detection method.

A.8 MALACOHERPESVIRUSES OF MOLLUSKS

A.8.1 Name of Virus: Ostreid Herpesvirus 1 (OsHV-1)

Notifiable (in which countries): None (specific rules in EU)
Inactivation: Unknown
Decontamination: Unknown
Mollusk waste treatment: Unknown
Available vaccines: None tested
Treatment regimens: None tested
Diagnostic standards and types of samples needed for testing: Yes.

A.8.2 Name of Virus: Abalone Herpesvirus (AbHV)

Notifiable (in which countries): Yes (OIE)
Inactivation: Unknown
Decontamination: Unknown
Mollusk waste treatment: Unknown
Available Vaccines: None tested
Treatment regimens: None tested
OIE diagnostic standards and types of samples needed for testing: Yes.

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Aquaculture Virology

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Despite considerable advances in animal virology in recent years coupled with the economic importance of the global aquaculture industry, knowledge of viruses of animal aquaculture is still sparse and in some cases outdated. There has been much work done in recent years on fish viruses and many new aquatic animal viruses continue to be discovered. ***Aquaculture Virology*** provides the current state of knowledge of aquatic animal viruses within the current virus classification and taxonomic context, and thereby allows the reader to draw on the principles of general virology. This book is a systematic and concise resource for anyone involved with or looking to move into aquaculture and fisheries. Clinical veterinarians, aquaculture disease practitioners, biologists, farmers, and all those in industry, government, or academia who are interested in aquatic animal virology will find this book extremely useful.

KEY FEATURES

- Provides unique comprehensive information on animal viruses for aquaculture and fisheries
- Presents high-quality illustrations of viral structure, diagrams of viral disease processes, gross pathology and histopathology lesions, and summary tables to aid in understanding
- Describes aquatic animal viruses of the three major aquatic animals—fish, crustaceans, and mollusks—within the current virus classification and taxonomic context, thereby allowing the reader to draw on the principles of general virology

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ACADEMIC PRESS

An imprint of Elsevier

elsevier.com

ISBN 978-0-12-801573-5

9 780128 015735