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Chapter 19

Orthomyxoviruses of Fish

F.S.B. Kibenge and M.J.T. Kibenge
University of Prince Edward Island, Charlottetown, PE, Canada

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

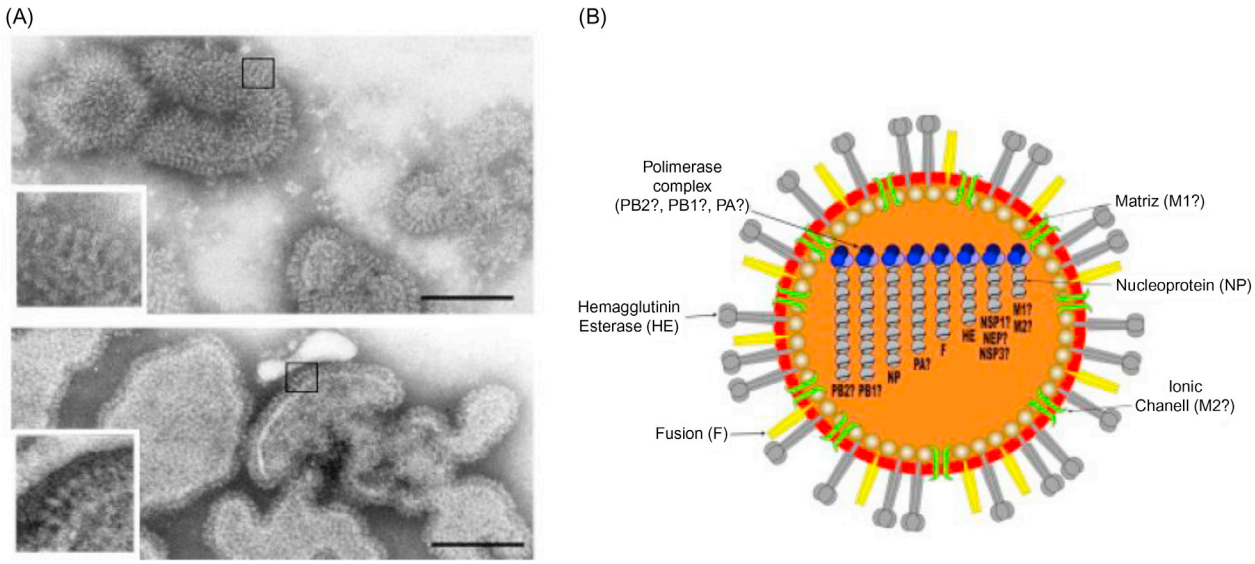

FIGURE 19.1 Influenza virus A and ISAV. (Panel A) Electron micrograph of influenza virus A/PR/8/34 (top) and ISAV (below), at a magnification of 150,000×. The squares in the photomicrographs show the amplified region that appears in the lower-left corner, at a magnification of 400,000×. Bars represent 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).*

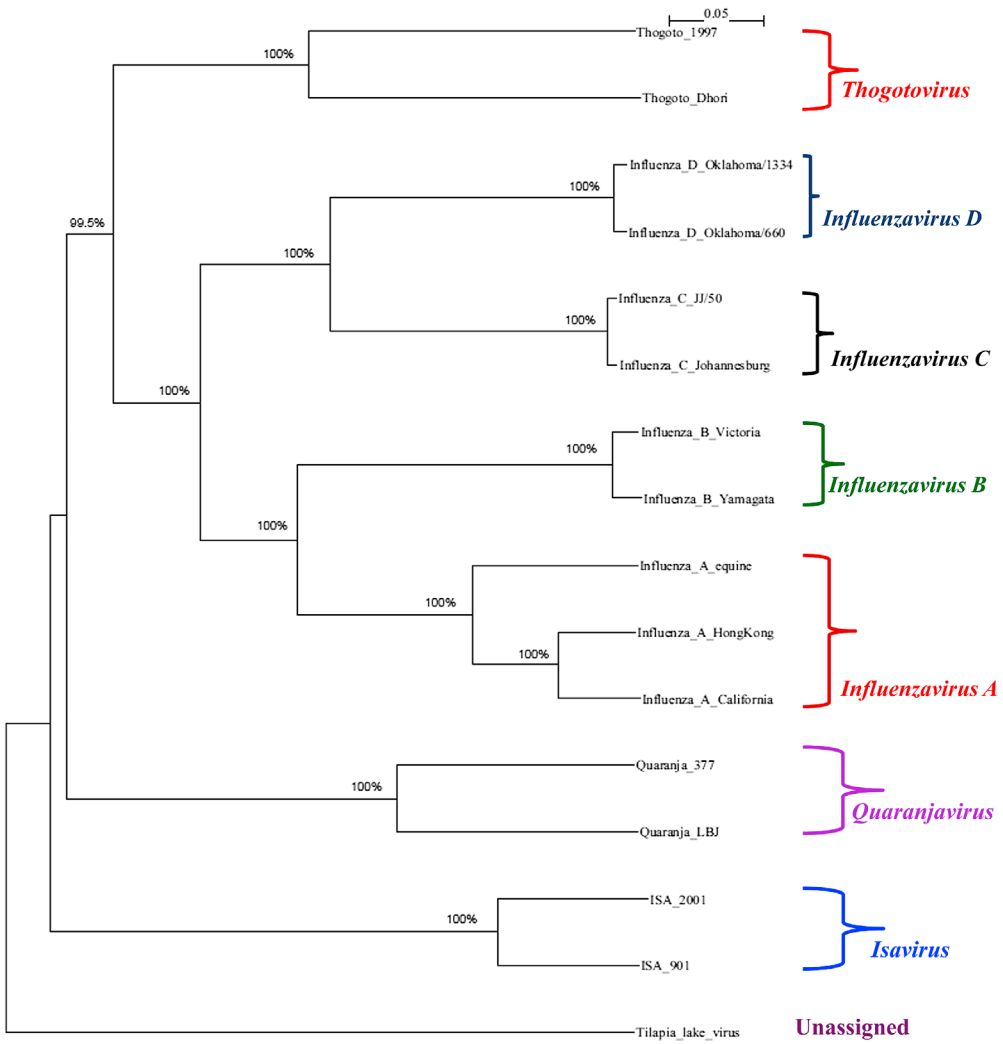

FIGURE 19.2 Phylogenetic tree showing the relationships within the family *Orthomyxoviridae*. Nucleotide sequences of polymerase basic 1 (PB1) genes of selected members in the seven genera of family *Orthomyxoviridae* were used to generate a phylogenetic tree. The GenBank accession numbers for the sequences 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

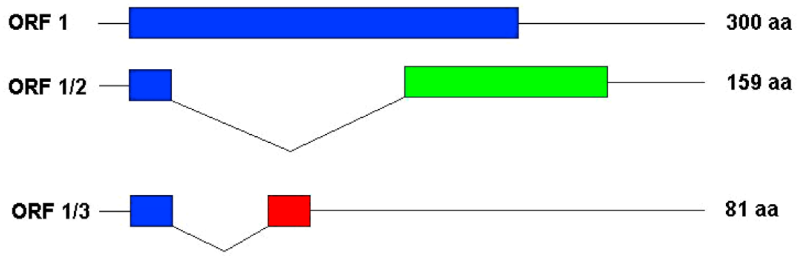

FIGURE 19.3 New gene expression model for segment 7 of ISAV of North American genotype. There are three ORFs, consisting of the primary transcript (7-ORF1) which is 300 amino acids long, and 7-ORF1/2 and 7ORF1/3, each with an intron removed from 7-ORF1 and consisting of 159 and 81 amino acids, respectively. *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

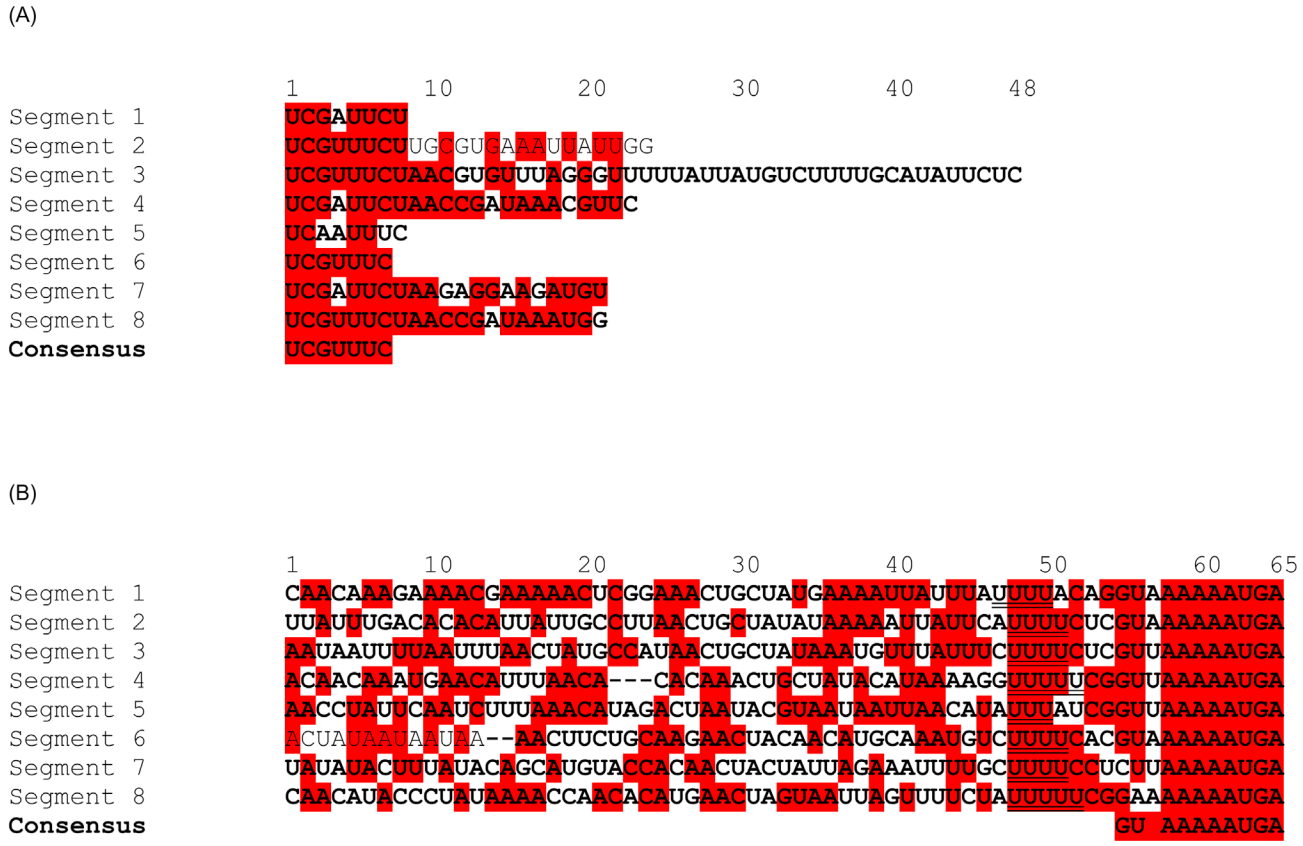

FIGURE 19.4 Alignment of the 3' and 5' noncoding regions of the eight genomic segments of the ISAV strain NBISA01 (GenBank Accession numbers HQ011270-HQ011277). Genome segment sequences were aligned manually. Sequences are in 3' to 5' orientation of vRNA. (Panel A) 3' end NCR. Only the noncoding sequences 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).

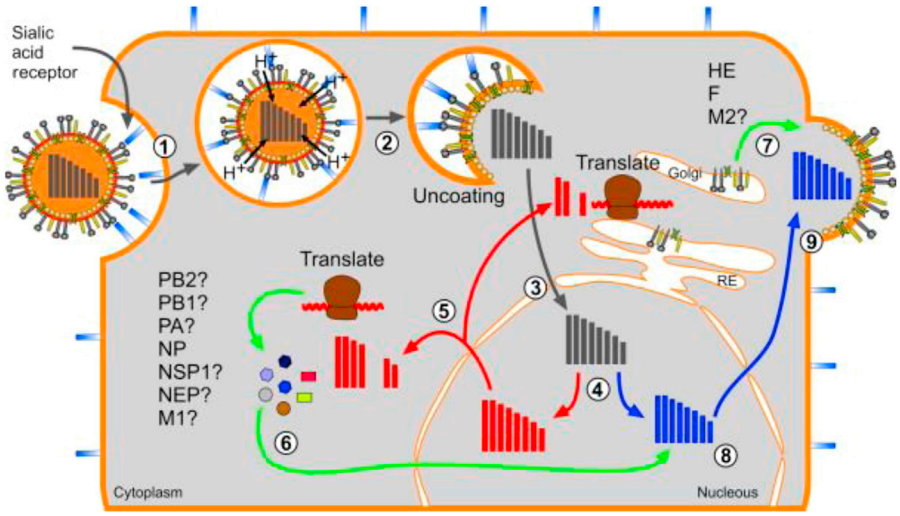

FIGURE 19.5 Schematic representation of the ISAV infection of a cell of fish. They are the most important events associated with this process and also indicate the proteins that are generated during infection. The proteins with a question mark are those in which its putative role is assumed by comparison 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).

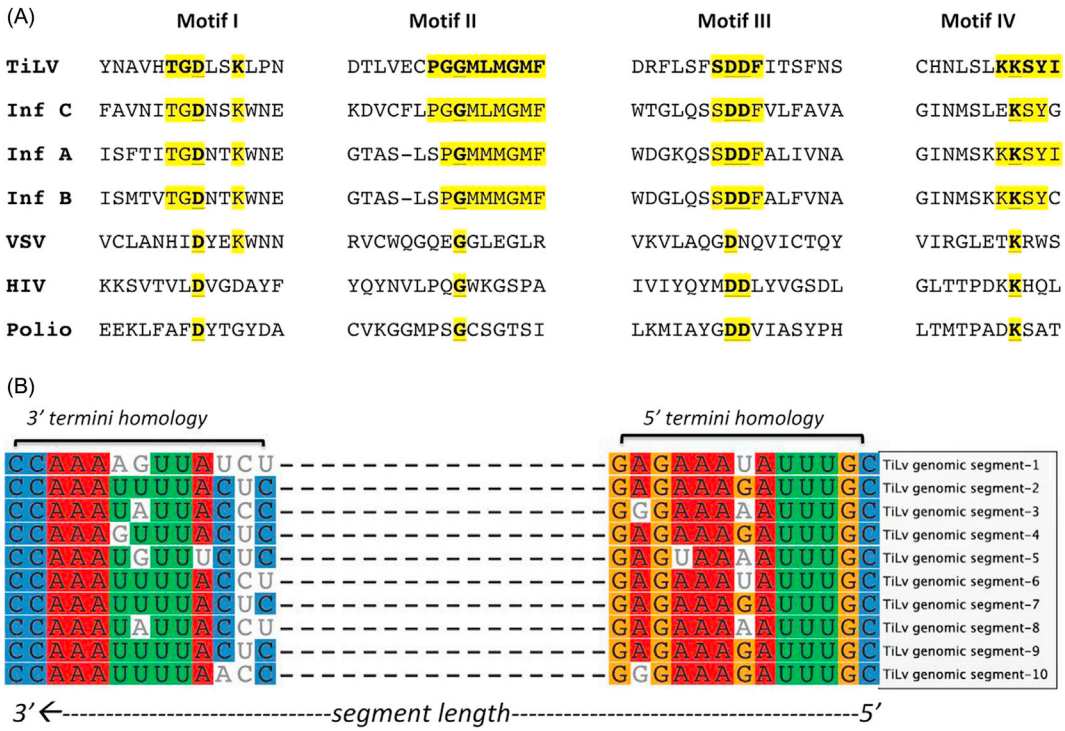

FIGURE 19.7 Genomic characterization of the segments of TiLV isolated from tilapia in Israel. (A) TiLV segment 1 putative protein shows weak homology to motifs conserved in RNA-dependent polymerases. Sequence comparison of TiLV's segment 1 predicted protein with motifs I–IV, conserved in polymerases 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/.

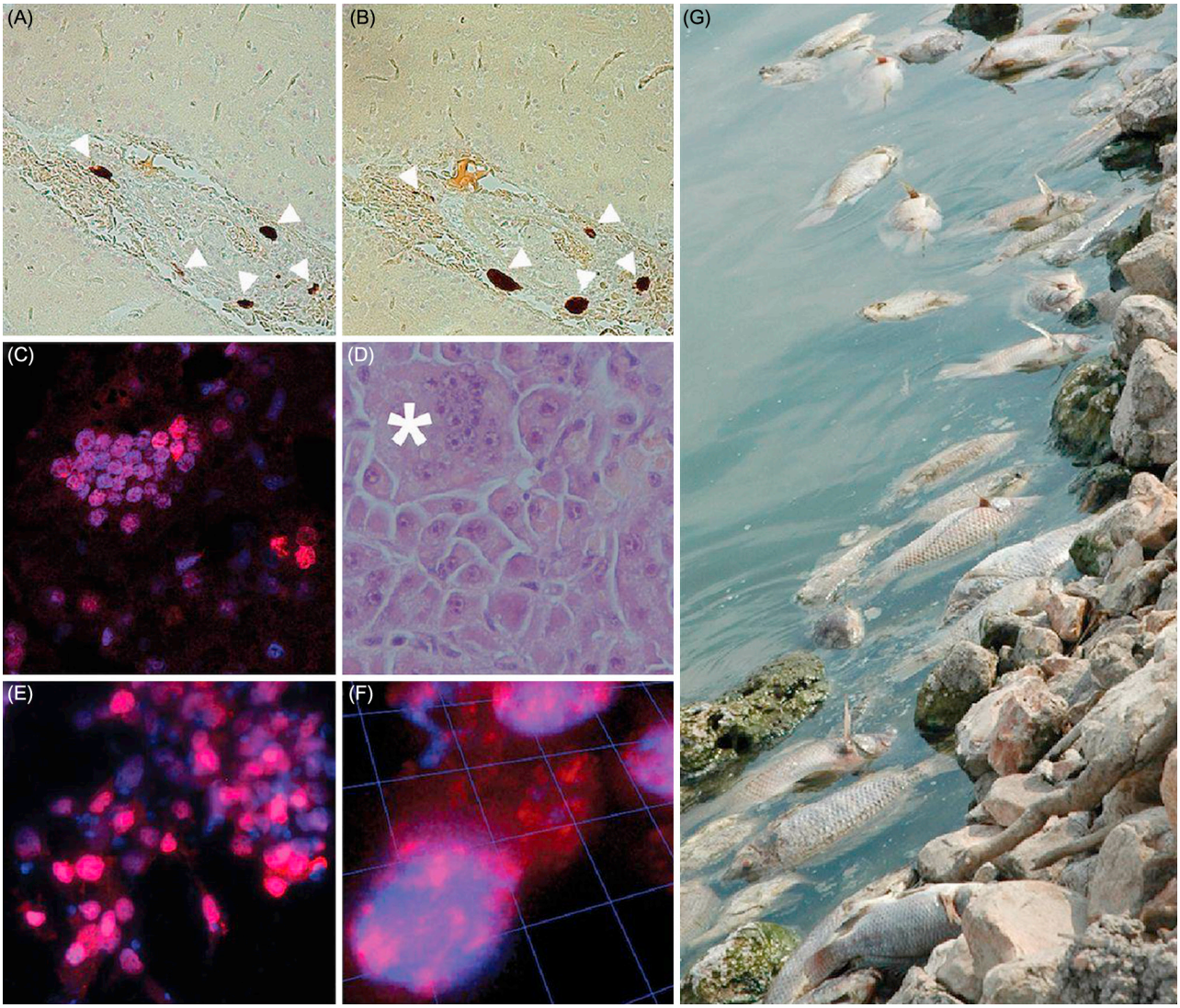

FIGURE 19.8 Detection of TiLV RNA in the brain and liver of infected tilapia and infected E-11 cells by in situ hybridization and image of dead tilapia in Israel. (A and B) Brain sections of infected Nile tilapia hybridized with Affymetrix Cy3-conjugated probes (red (light gray in print versions)) of 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

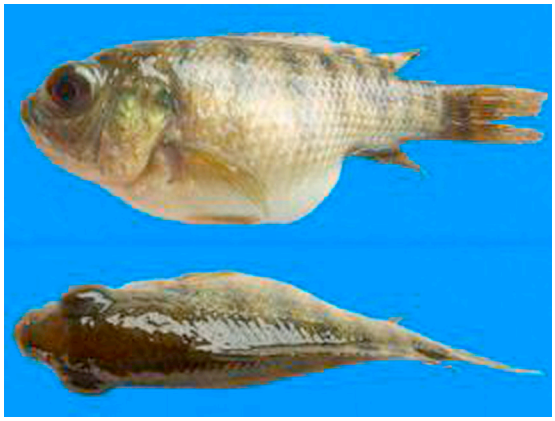

FIGURE 19.9 Two tilapia showing gross lesions of distended abdomen and exophthalmos. *From Ferguson, H.W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J.A., del Pozo, J., 2014. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. J. Fish Dis. 37, 583–589.* Figure 1 *Copyright © John Wiley & Sons Ltd (2013), with permission.*

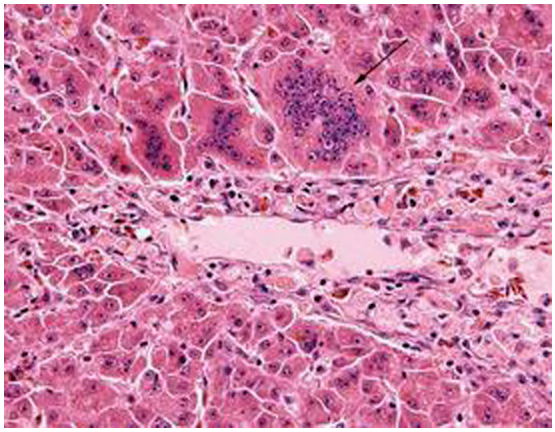

FIGURE 19.10 Liver from moribund tilapia showing syncytial giant cells (arrow), (H&E, x400). *From Ferguson, H.W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J.A., del Pozo, J., 2014. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. J. Fish Dis. 37, 583–589.* Figure 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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