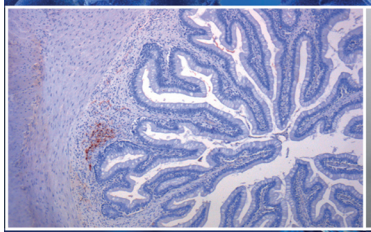
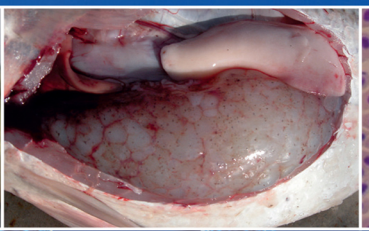
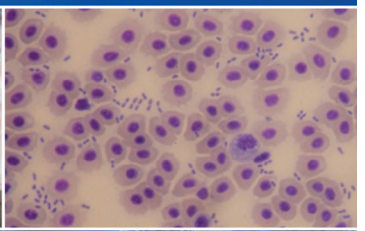
FISH VIRUSES AND BACTERIA

Pathobiology and Protection

Edited by Patrick T.K. Woo and Rocco C. Cipriano



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Preface

The main foci of *Fish Viruses and Bacteria: Pathobiology and Protection* (FVBPP) are on the pathobiology of and protective strategies against major viruses and bacteria that cause diseases and/or mortalities in economically important fish. The 25 chapters are written by scientists who have considerable expertise on the selected microbes, and the vast majority of the chapters are on notifiable microbes certified by the OIE (originally Office International des Epizooties, now the World Organisation for Animal Health). Contributors have made every effort to cite publications as recent as early 2016.

The pathogens and contributors for inclusion in FVBPP were selected by the editors. The selection of microbes/diseases was based on numerous criteria. These include the microbes/diseases that:

- were only briefly discussed in the predecessor volume published in 2011 – *Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections*, 2nd edn, eds Woo, P.T.K. and Bruno, D.W. (e.g. Koi herpesvirus, *Weissella ceti*); or
- are relatively well-studied piscine pathogens (e.g. infectious haematopoietic necrosis, *Aeromonas* spp.) that may serve as disease models for other pathogens; or
- cause considerable financial hardships to specific sectors of the aquaculture industry (e.g. viral haemorrhagic septicaemia, *Vibrio* spp.); or
- have been introduced to new geographical regions through the transportation of infected fish (e.g. Koi herpesvirus in Europe) and have subsequently become significant threats to local fish populations; or
- are pathogenic to specific groups of fish (e.g. *Oncorhynchus masou* viral disease, oncogenic viruses to salmonids in Japan); or
- are highly adaptable and not host specific, and consequently have worldwide distributions (e.g. epizootic haematopoietic necrosis, *Streptococcus* spp.).

Each chapter is arranged to provide a brief description of the selected pathogens, its host(s), transmission, geographical distribution and impact(s) on fish production. The most current information is provided on detection and diagnosis of infection with discussions on clinical signs of the disease and additional details provided for external/internal lesions (macroscopic and microscopic). The focus in each chapter is on the pathophysiology of the disease, including its effects on osmoregulation, impacts on the host endocrine system, growth and reproduction.

Finally, the most current prevention and protective control strategies are presented. These include biological, physical and legislative approaches.

Many of these pathogens have been extensively studied; however, a few are less well studied, including the newly emergent pathogens (e.g. alphaviruses, *W. ceti*). In these cases, contributors have highlighted deficiencies in our knowledge and we hope that these treatises will spur additional research in 'neglected' areas.

FVBPP is directed at research scientists in the aquaculture industry and universities, fish health consultants, managers and supervisors of fish health laboratories, and veterinary specialists in commercial aquaria. The present volume is also appropriate for the training of fish health specialists, and for senior undergraduate/graduate and veterinary students who are conducting research on diseases of fish. In addition, FVBPP may also be a useful reference book for university courses on infectious diseases, general microbiology and the impacts of diseases on the aquaculture industry. A secondary audience includes pathologists who may wish to study the combined effects of microbial/parasitic infections on fish health, and environmental toxicologists and immunologists who are studying synergistic effects of pollutants and microbial infections. We expect this secondary audience to increase as it becomes increasingly evident that fish health may also serve as an indicator of ecosystem quality.

Patrick T.K. Woo and Rocco C. Cipriano

Previous titles by Patrick T.K. Woo

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1 Infectious Pancreatic Necrosis Virus

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1.1 Introduction

Infectious pancreatic necrosis virus (IPNV), the aetiological agent of infectious pancreatic necrosis (IPN), is a double-stranded RNA (dsRNA) virus in the family *Birnaviridae* (Leong *et al*., 2000; ICTV, 2014). The four genera in this family include *Aquabirnavirus*, *Avibirnavirus*, *Blosnavirus* and *Entomobirnavirus* (Delmas *et al*., 2005), and they infect vertebrates and invertebrates. *Aquabirnavirus* infects aquatic species (fish, molluscs and crustaceans) and has three species: IPNV, *Yellowtail ascites virus* and *Tellina virus*. IPNV, which infects salmonids, is the type species.

The IPNV genome consists of two dsRNAs, segments A and B (Fig. 1.1; Leong *et al*., 2000). Segment A has ~ 3100 bp and contains two partially overlapping open reading frames (ORFs). The long ORF encodes a 106 kDa polyprotein (NH_2-pVP2-VP4-VP3-COOH) that is co-translationally cleaved by the VP4 (viral protein 4) protease (29 kDa) to generate pVP2 (62 kDa; the precursor of the major capsid protein VP2) and the 31 kDa VP3 (Petit *et al*., 2000). The short ORF encodes VP5, a 17 kDa, arginine-rich, non-structural protein that is produced early in the replication cycle.VP5 is an anti-apoptosis protein similar to the Bcl-2 family of proto-oncogenes.VP5 is not required for IPNV replication *in vivo* and its absence does not alter virulence or persistence in the host (Santi *et al*., 2005). Segment B has ~ 2900 bp and encodes the polypeptide VP1 (94 kDa) which is an RNA-dependent RNA polymerase.VP1 is found both within the mature virion as a free polypeptide with RNA-dependent RNA polymerase-associated activity and as a genome-linked protein, VPg, via guanylylation of VP1 (Fig. 1.1 and Table 1.1).

Aquabirnaviruses have broad host ranges and differ in their optimal replication temperatures. They consist of four serogroups A, B, C and D (Dixon *et al*., 2008), but most belong to serogroup A, which is divided into serotypes A1–A9.The A1 serotype contains most of the US isolates (reference strain West Buxton), serotypes A2–A5 are primarily European isolates (reference strains, Ab and Hecht) and serotypes A6–A9 include isolates from Canada (reference strains C1, C2, C3 and Jasper).

1.1.1 IPNV morphogenesis

Two types of particles (A and B) are produced during infection. After replication, dsRNA is assembled into the 66 nm diameter non-infectious particle A, in which the capsid is composed of both mature (VP2) and immature (pVP2) viral polypeptides. Proteolytic processing of the remaining pVP2 into VP2 compacts the capsid to the 60 nm diameter infectious particle, referred to as particle B (Villanueva *et al*., 2004). The VP2 protein comprises the outer capsid, while the VP3 protein forms the inner layer of the mature virion. Additionally, VP3 remains associated with VP1 and VP4, as well as with the polymerase-associated genome.

1.1.2 IPNV tertiary structure

Virions are non-enveloped with a T13 lattice icosahedral morphology 60 nm in diameter, and have a

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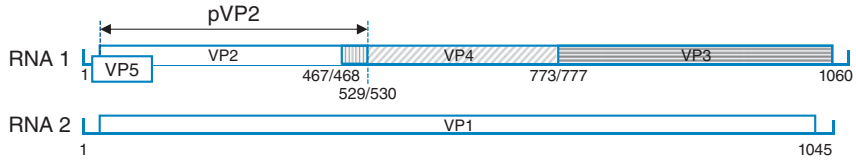

Fig. 1.1. Genome organization of infectious pancreatic necrosis virus (IPNV). The numbers at the bottom of the segments in the diagrams indicate the amino acid number(s). pVP2 is the precursor of the IPNV viral protein VP2. RNA 1 is segment A and RNA 2 is segment B of the viral dsRNA.

Table 1.1. Proteins encoded by infectious pancreatic necrosis virus (IPNV) and their functions.

Protein	Molecular mass	Function(s)	References
VP1	94 kDa	RNA-dependent-RNA polymerase; viral replication	Calvert *et al*., 1991; Leong *et al*., 2000; Graham *et al*., 2011
VP2	54 kDa	Major capsid protein, contains most antigenic determinants; has a structural function	Coulibaly *et al*., 2010
VP3	31 kDa	Minor capsid protein, interacts with the major capsid protein VP2 in capsid formation, associates with the dsRNA genome, recruits polymerase into capsids, contains some antigenic epitopes	Leong *et al*., 2000; Bahar *et al*., 2013
VP4	29 kDa	Protease involved in processing the polyprotein encoded in segment A of IPNV dsRNA	Feldman *et al*., 2006; Lee *et al*., 2007
VP5	17 kDa	Arginine-rich anti-apoptosis protein, similar to the Bcl-2 family of proto-oncogenes	Magyar and Dobos, 1994; Santi *et al*., 2005

buoyant density of 1.33 g/cm^3 in CsCl (Delmas *et al*., 2005). The viral capsid surface contains VP2 proteins, and the three-dimensional (3D) structures of these are known for IPNV and IBDV (Infectious bursal disease virus) (Fig. 1.2A). The IPNV VP2 capsid is made of 260 trimeric spikes that are projected radially and carry the antigenic domains as well as determinants for virulence and cellular adaptation. These are linked to VP3 in the interior of the virion (Fig. 1.2B). However, the spikes in IPNV are arranged differently from those in IBDV in that the amino acids controlling virulence and cell adaptation are located at the periphery in IPNV but in a central region for IBDV. The base of the spike contains an integrin-binding motif and is located in an exposed groove, which is conserved across all genera of birnaviruses (Coulibaly *et al*., 2010).

1.2 Geographical Distribution

IPN occurs worldwide among cultured and wild salmonid fishes. It was first detected in freshwater trout during the 1940s within Canada and during the 1950s within the USA (Wood et al., 1955). The virus was first isolated in 1960 (Wolf *et al*., 1960). It was subsequently reported in Europe during the early 1970s and has also been reported in many other countries (e.g. Japan, Korea, Taiwan, China, Thailand, Laos, New Zealand, Australia, Turkey) that are involved either with importing salmonids or active in aquaculture. IPN outbreaks are often traced to importations and the subsequent distribution of infected ova/fingerlings (Munro and Midtlyng, 2011).

1.3 Economic Impacts of IPN

Historically, IPN is one of the top three causes of losses in the salmonid industry. This was reflected in a survey conducted by the Shetland Salmon Farmers Association in 2001 that showed an average loss of 20–30% with a cash value of 2 million pounds due to IPN (Ruane *et al*., 2007). From 1991 to 2002, IPN had an impact on salmon post-smolt survival in Norwegian epizootiological studies of from 6.4 to 12.0% (Munro and Midtlyng, 2011). In 1998, the economic losses were estimated to exceed €12 million (Munro and Midtlyng, 2011). Even today, IPN remains an important risk for

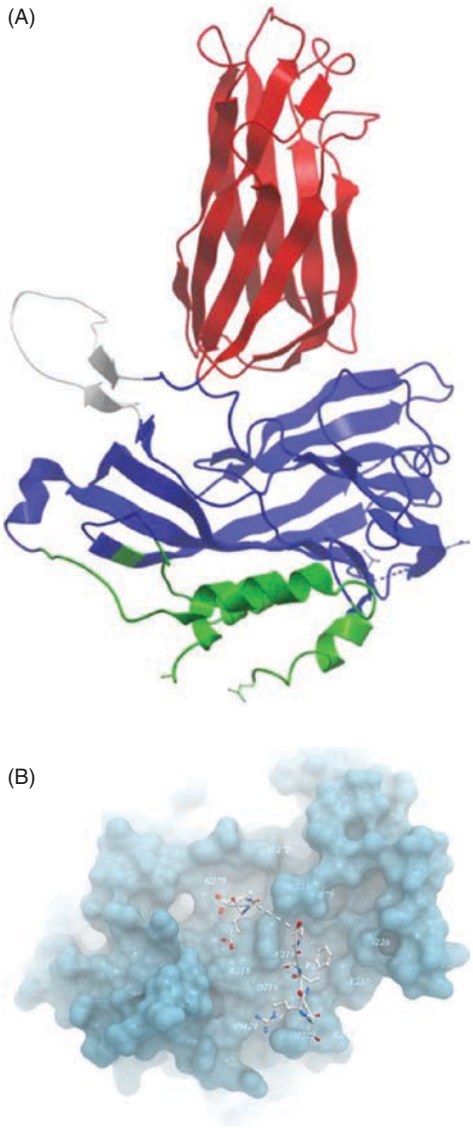

Fig. 1.2. (A) The crystal structure of infectious pancreatic necrosis virus (IPNV) viral protein VP2 showing the base domain (green), shell domain (blue) and variable P domain (red). The molecular graphic was prepared using the free ICM-Browser software (MolSoft LLC, San Diego, California, downloadable at http://www.molsoft.com/icm_browser.html). (B) The crystal structure of viral protein VP1 (blue surface with residue labels) and the VP3 protein C-terminal (white stick and ribbon). VP3 interacts with the finger domain of VP1. VP3 residues 227–231 and 236–238 are shown and missing residues that were not resolved are depicted by dashed lines in the ribbon. As in (A), the molecular graphic was prepared using the ICM-Browser software.

salmonid culture. For example, it was reported in 48 salmon farming facilities in Norway during 2014, although this was fewer than in previous years (Norwegian Veterinary Institute, 2015). IPN is most important in the first 6 months after sea transfer. The industry still reports significant losses due to mortalities and subsequent weakening of the surviving fish. A recent report on cumulative mortality in the first 6 months indicated an increase to 7.2% compared with a baseline mortality rate of 3.4%; this is more than doubling the cumulative mortality (Jensen and Kristoffersen, 2015). This same study showed that IPNV-infected cohorts challenged with other stressors showed increased levels of cumulative mortalities. For example, with pancreas disease (PD), mortality increased to 12.9%, whereas heart muscle and skeletal muscle inflammation (HSMI) increased to 16.6% when all other factors were normalized.

1.4 Diagnosis of the Infection

1.4.1 Clinical signs and viral transmission

IPNV and IPNV-like birnaviruses have been isolated from salmonids as well as from non-salmonid fishes (e.g. *Cyprinus carpio*, *Perca flavescens*, *Abramis brama* and *Esox lucius*), molluscs, crustaceans and pseudocoelomates (McAllister, 2007). External clinical signs include darkened colour, exophthalmia, abdominal distention, the presence of a mucoid pseudocast ('faecal cast') extruding from the vent, and haemorrhages on the body surface and at the bases of fins. Infected fish swim in a rotating manner along their longitudinal axis and death generally ensues within a few hours. Internal signs can include a pale liver and spleen, and an empty digestive tract filled with clean or milky mucus. Haemorrhages can occur in visceral organs (Munro and Midtlyng, 2011). IPN outbreaks characteristically consist of a sudden increase in fry and fingerling mortalities. The disease can also occur in post-smolts in the first few weeks after transfer to the sea (Jensen and Kristoffersen, 2015). Stress on the host plays a key role in enhancing viral replication, mutation and even reversion to virulence (Gadan *et al*., 2013). The survivors of outbreaks often carry IPNV for their entire lives without clinical signs. These carriers serve as reservoirs that transmit the virus either horizontally through sheddings in faeces and urine, or vertically through contaminated reproductive products (Roberts and Pearson, 2005).

1.4.2 Viral detection

Clinical signs and pathology cannot be used to distinguish IPN from other viral diseases and the absence of clinical signs does not ensure that fish are free of IPNV. The tentative diagnosis of IPN is based on prior disease history of the farm and fish population, clinical signs and findings from gross necropsy. Confirmatory diagnosis involves isolation of the virus in cell culture followed by immunological or molecular confirmation. Serological or molecular techniques are especially useful for monitoring fish with and without clinical signs. Tissues suitable for virological examinations include the kidney, liver, spleen, the ovarian fluid from brood stock at spawning or whole alevins. The isolation of IPNV in cell culture is done using blue gill fry (BF-2), Chinook salmon embryo (CHSE-214) or rainbow trout gonad (RTG-2) cell lines (OIE, 2003). Identification of the virus from cell culture is done using neutralization assay, fluorescent antibody assay, enzyme-linked immunosorbent assay (ELISA), immunohistochemical staining using IPNV-specific antibody, or reverse-transcriptase-polymerase chain reaction (RT-PCR) (OIE, 2003; USFWS and AFS-FHS, 2007).

In recent years, SYBR Green and TaqMan-based real-time RT-PCR methods have been developed to detect IPNV (Bowers *et al*., 2008; Orpetveit *et al*., 2010). Real-time based methods are 100× more sensitive than conventional PCR, and detect the virus in subclinical animals (Orpetveit *et al*., 2010). Using real-time RT-PCR, the IPNV load in pectoral fin clips was found to be as high as in the spleen and head kidney (Bowers *et al*., 2008). Therefore, non-lethal tissue sampling coupled with real-time RT-PCR could be valuable tools for surveillance and monitoring wild and farmed fish, as well as minimizing the need to sacrifice brood stock at spawn.

1.5 Pathology

IPNV infection presents a variety of pathological changes. Pancreatic tissues undergo severe necrosis characterized with pyknosis (chromatin condensation), karyorrhexis (fragmentation of the nucleus) and cytoplasmic inclusion bodies (Fig. 1.3). The pylorus, pyloric caeca and anterior intestine also undergo extensive necrosis. Intestinal epithelial cells slough and combine with mucus to form thick, whitish exudates that may discharge from the vent. Degenerative changes also occur in the kidney, liver and spleen. In persistently infected fish, IPNV is in macrophages within the haematopoietic tissue of the kidney, and can multiply in adherent leucocytes isolated from carrier fish (Johansen and Sommer,

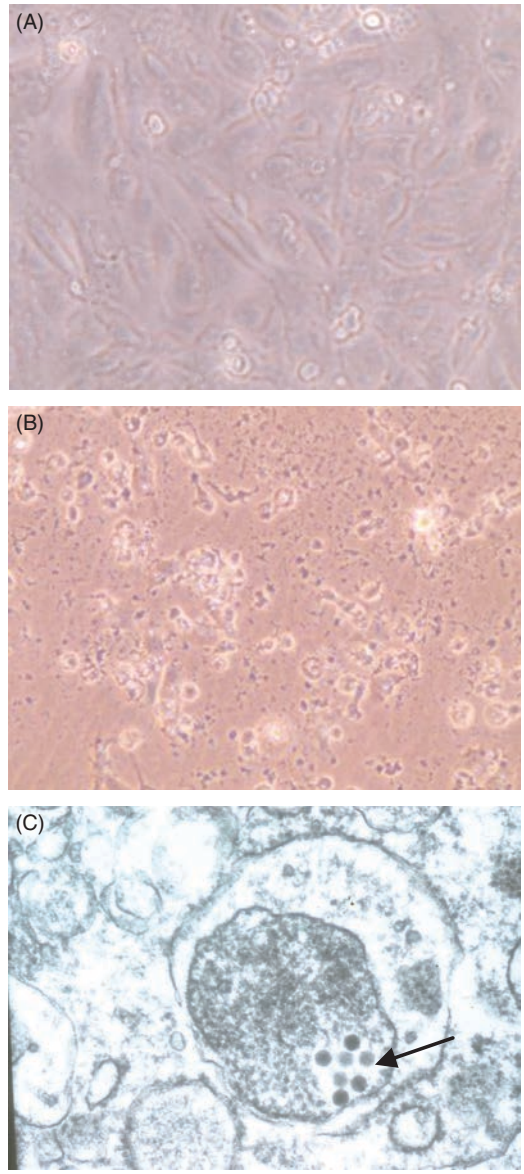

Fig. 1.3. (A) Cells of uninfected Chinook salmon embryos (CHSE-214); (B) cells infected by infectious pancreatic necrosis virus (IPNV) exhibiting a lytic type of cytopathic effect (magnification, 100×); and (C) a transmission electron microscope (TEM) image showing the IPN virus particles (indicated by an arrow, magnification, 27,500×) displaying a characteristic hexagonal profile in cytoplasmic vesicles.

1995). There are indications of reduced immune response in leucocytes isolated from carrier fish, and of increased *in vitro* viral replication after the stimulation of resting leucocytes with phytohaemagglutinin (Knott and Munro, 1986).

1.6 Pathophysiology

The susceptibility of fish to IPN infection and mortality depends on species, age or developmental stage, physiological condition of the host, virus strain, genetic background of the host, and environmental and management factors (Munro and Midtlyng, 2011). In cultured trout and salmon, infection varies from subclinical with little or no mortality to acutely virulent with high mortality. Although in trout and salmon the disease produces severe pancreatic necrosis, it also causes histological changes in the renal haematopoietic tissue, gut and liver. The liver is a key target (Ellis *et al*., 2010), while the virus is also present in the islets of Langerhans and in the corpuscles of Stannius in the kidney (Fig. 1.4), which suggests that it could also affect metabolism. McKnight and Roberts (1976) reported the clinical sign of 'mucosal damage' with a description that fits what is currently referred to as acute enteritis caused by faecal casts. They postulated that this damage might be more lethal than necrosis of the pancreas. Cell necrosis of the digestive glands and of the mucosal gut epithelium is also thought to be responsible for the shedding of infective virus with faeces. Severe necrosis of the intestinal mucosa and pancreas may also cause anorexia that exacerbates conditions such as 'pinhead' fish and 'failing smolts', which are often observed among the survivors of an epizootic (Smail *et al*., 1995). Roberts and Pearson (2005) also reported that in seawater, after a loss of 50% or more to IPN, many fish failed to grow, became chronically emaciated and were prone to sea louse infestation.

Subclinical infections may not affect the growth of Atlantic salmon (*Salmo salar*) parr or post-smolts. However, in laboratory studies, both feed intake and specific growth rates of healthy post-smolts were depressed after an immersion infection with IPNV (Damsgard *et al*., 1998). Viral titres were determined in the kidney and pyloric caeca before and after the experimental infection, and no mortality occurred in the infected or in the control groups. In the infected fish, the titres in both the kidney and pyloric caeca increased significantly. Between 16 and 44 days after infection, the titre in the pyloric caeca decreased significantly from 10^6 to 10^3–10^4 plaque-forming units (pfu)/g. From approximately 20 days after infection, feed intake and specific growth rates were significantly lower in infected fish than in uninfected fish. The results indicated that IPNV-infected fish require relatively high viral titres in the kidney and pyloric caeca before reduced feeding is detectable.

IPNV induces programmed cell death as apoptosis markers have been found in hepatic, intestinal and pancreatic tissues that correspond to viral accumulation and pathological changes (Imatoh *et al*., 2005; Santi *et al*., 2005). It was hypothesized that apoptosis might limit rather than enhance the negative consequences of an IPNV infection.

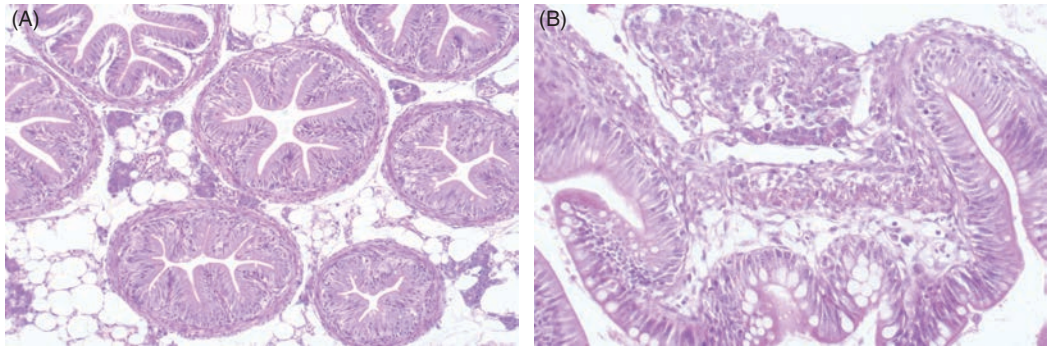

Fig. 1.4. Necrosis of pancreatic acinar cells that are located in the adipose tissue between the cylindrical pyloric caecae and sloughing of the intestinal mucosa from a fish infected with infectious pancreatic necrosis virus (IPNV) (H&E (haematoxylin and eosin) stained, magnification 400×).

Sadasiv (1995) found that viral clearance was minimal even in the presence of viral neutralizing antibodies. It was suggested that the virus may infect leucocytes, possibly persistently, and thereby subvert the neutralizing antibody response. Dual infections of rainbow trout (*Oncorhynchus mykiss*) with IPNV and IHNV (infectious haematopoietic necrosis virus) have been reported, but the potential effects of mixed infections on the immune system were not described (LaPatra *et al*., 1993). Rainbow trout pre-exposed to IPNV and later challenged with viral haemorrhagic septicaemia virus (VHSV) had significant resistance to VHSV compared with fish that had not been previously exposed to IPNV (de Kinkelin *et al*., 1992). The authors termed this phenomenon 'interference-mediated resistance' and suspected that it was due to the production of interferon.

In a similar manner, when Atlantic salmon carrying IPN were exposed to infectious salmon anaemia virus (ISAV), the mortality of non-IPNV infected post-smolts was consistently higher than that of fish that had been exposed to IPNV 3 weeks earlier (Johansen and Sommer, 2001). In contrast, when the fish were challenged with ISAV at 6 weeks post-IPNV infection, there was no difference in the mortality between IPNV carriers and non-carriers. These authors also reported a similar short-lived protection of subclinical IPNV infection against *Vibrio salmonicida* and attributed this to non-specific effects due to IPNV-induced interferon production. Additionally, no significant effects associated with intraperitoneal vaccination using a trivalent oil-adjuvanted bacterin were observed in the IPNV carrier group versus non-carrier controls. However, the IPNV carrier fish eventually had a moderate IPN outbreak with cumulative mortality in the unvaccinated carrier fish of 24% versus 7% in the bacterin-vaccinated carrier fish. In another study, no differences were seen in mortality between immunized IPNV carriers and non-carriers after experimental furunculosis or coldwater vibriosis challenge and in either group's humoral immune response to *Aeromonas salmonicida* (Johansen *et al*., 2009). In addition, when IPNV carrier and non-carrier Atlantic salmon fry (mean weight 2–4 g) were immunized against enteric redmouth disease (ERM), there was no difference in protection after experimental challenge with *Yersinia ruckeri* (Bruno and Munro, 1989). These studies indicate that IPNV infection, even in small fish, had no detrimental effect on bacterial vaccine-induced protection.

1.7 Protective and Control Strategies

Because there is no therapy for IPN disease, avoidance is the best strategy. Epizootiological studies of IPNV transmission in salmon farms have shown that viral spread is unpredictable. Since non-clinical carriers serve as a source of infection through viral shedding in faeces and sexual products, intensive monitoring and biosecurity can reduce the prevalence of the virus. It is essential to obtain stock from pathogen-free sources and maintain strong biosecurity on a pathogen-free water supply whenever new fish are introduced. UV treatment of incoming water to the hatchery is an example of a suitable control measure. Treatment with disinfectants such as formalin (3% for 5 minutes), sodium hydroxide (pH 12.5 for 10 minutes), chlorine (30 ppm for 5 minutes) and iodine compounds is also capable of inactivating the virus (OIE, 2003).

1.7.1 Selection for improved IPN-resistant fish lines

Because significant numbers of fish survive IPN epizootics, it was postulated that breeding could enhance resistance. Ozaki and colleagues reported that quantitative trait loci (QTLs) could be correlated with improved resistance to IPN. A recent review on the current status of DNA marker-assisted breeding for improved disease resistance in commercially important fish is available (Ozaki *et al*., 2012). The marker-assisted selection (MAS) of more resistant lines using genomic traits is a powerful tool for the development of IPNV-resistant salmonid lines (Moen *et al*., 2009) and is also being expanded for other diseases (Houston *et al*., 2008; Ozaki *et al*., 2012). A recent report tied the epithelial cadherin gene (*cdh1*) with resistance to IPN (Moen *et al*., 2015). Strains of IPN-resistant Atlantic salmon are marketed by companies such as Aquake, Trondheim, Norway (http://aquagen.no/en/products/salmon-eggs/product-documentation/resistance-against-ipn/); IPN resistance in these fish was linked to a single QTL that could prove useful for future efforts to develop resistant lines of fish using MAS (Moen *et al*., 2009).

1.7.2 Available biologics

A number of IPN vaccines are available (Table 1.2), but there is a need to develop more cost-effective vaccines that can be delivered to all life stages. The

Table 1.2. Approved vaccines against infectious pancreatic necrosis (IPN).

Name	Content and/or vaccine against[a]	Type	Delivery	Company	Licensed in:
Alpha Ject® 1000	Whole IPNV	Inactivated virus	IP	Pharma AS, Norway	Chile
Alpha Ject® 2.2	IPN Furunculosis		IP	Pharma AS, Norway	UK
Alpha Ject® 4-1	IPN SRS Furunculosis Vibriosis		IP	Pharma AS, Norway	Chile
Alpha Ject® 5-1	IPN ISA SRS Furunculosis Vibriosis		IP	Pharma AS, Norway	Chile
Alpha Ject® 6-2	IPN Furunculosis Coldwater vibriosis Winter sore		IP	Pharma AS, Norway	Norway Faroe Islands
Alpha Ject® IPNV-Flevo 0.025	IPNV Flavobacteriosis		IP	Pharmaq AS, Norway	Chile
Alpha Ject® micro 2	IPN SRS		IP	Pharmaq AS, Norway	Chile
Alpha Ject® micro 3	IPN SRS Vibriosis		IP	Pharmaq AS, Norway	Chile
Alpha Ject® micro 7 ILA	IPN Vibriosis Furunculosis Coldwater vibriosis Winter sore ISA		IP	Pharmaq AS, Norway	Norway Faroe Islands
AquaVac® IPN Oral	VP2 and VP3 capsid proteins	Subunit	Oral	Merck Animal Health, New Jersey, USA	Canada, USA
Birnagen Forte	Whole IPNV	Inactivated virus	IP	Aqua Health Ltd., Novartis, Canada	Canada
IPNV	Whole IPNV	Inactivated virus	IP	Centrovet, Chile	Chile
Norvax® Minova 6 Norvax® Compact 6	VP2 capsid protein	Subunit	IP	Intervet International BV, The Netherlands Merck Animal Health	
SRS/IPNV/Vibrio	VP2 protein	Subunit	IP	Microtek International, Inc., British Columbia, Canada	Canada, Chile

[a]IPNV, infectious pancreatic necrosis virus; ISA, infectious salmon anaemia; SRS, salmon rickettsial septicaemia; VP2, VP3, viral proteins 2 and 3.

use of inactivated wild type virus to induce immunity was the earliest approach to fish viral vaccines and it is still a reliable standard by which other vaccines are evaluated. The Alpha Ject® micro 1 ISA (Pharma/Novartis) and Alpha Ject® 1000 vaccines (Table 1.2) are examples of such vaccines that target infectious salmon anaemia (ISA) and IPN, respectively (http://www.pharmaq.no/products/injectable/). Inactivated viral vaccines induce strong responses because they retain surface-exposed antigens and the inactivated genomic component.

Subunit vaccine based on major viral antigen(s) is another option for producing viral vaccines. The intrinsic ability of some viral structural proteins to

self-assemble into particles that mimic the native virus in both size and processing by the host have led to the development of a class of subunit vaccines referred as virus-like particles (VLPs; Kushnir *et al*., 2012). VLPs have been expressed in bacteria, yeast, transgenic plants and cell culture. A number of human vaccines (e.g. Gardasil vaccine®9, Human Papillomavirus 9-valent Vaccine, Recombinant) have been produced using this technology (Kushnir *et al*., 2012). Recent efforts have used this approach to produce vaccines against IPNV. IPNV VLPs containing VP2 and VP3 proteins and measuring 60 nm in diameter have been produced in insect cells and *Trichoplusia ni* larvae using a baculovirus expression system. When Atlantic salmon post-smolts were intraperitoneally immunized with purified antigen and challenged via immersion, the cumulative mortalities 4 weeks post-challenge were lower (56%) than in control fish (77%) (Shivappa *et al*., 2005).

Another IPN vaccine is based on IPNV VP2 protein alone (Allnutt *et al*., 2007). The VP2-based subviral particles (SVPs) expressed in yeast were 22 nm in size compared with 60 nm for the native virus. SVPs induced a strong anti-IPNV antibody response in rainbow trout. The antigen was delivered via injection or through the diet, and the reduced IPNV load was 22- and 12-fold in injected and orally vaccinated fish, respectively (Allnutt *et al*., 2007). To further explore the possibility of using the IPNV SVP to develop multivalent vaccine, a foreign epitope (human oncogene *c-myc*) was expressed on the SVP, and the chimeric SVPs induced antibody response to both IPNV and the *c-myc* epitope (Dhar *et al*., 2010). Further research has led to the successful display of an ISAV haemagglutinin epitope on the surface of this IPNV SVP, and the chimeric SVPs, when injected into rainbow trout, induced antibody response against IPNV as well as ISAV (Dhar *et al*., unpublished data). Three other vaccines based on the IPNV VP2 capsid protein are marketed, including IPNV (licensed in Chile and from Centrovet, Chile), Norvax (Intervet-International BV, The Netherlands), and SRS/IPNV/*Vibrio* (licensed in Canada and Chile and from Microtek International Inc., British Columbia, Canada; since December 2010 fully integrated into Zoetis Veterinary Medicine Research & Development and Zoetis Canada) (Gomez-Casado *et al*., 2011). The Centrovet vaccine provides oral delivery of both an inactivated IPNV and a recombinant protein to provide flexibility in delivery (http://www.centrovet.com/index.php/products/aqua/vaccines99). The Norvax vaccine is another recombinant protein vaccine that is delivered via intraperitoneal injection and only addresses IPN. The SRS/IPNV/Vibrio vaccine is a trivalent recombinant protein vaccine that is also delivered by intraperitoneal injection and provides the user with the convenience of addressing three different pathogens.

An experimental IPNV DNA vaccine (expressing the VP2 antigen), delivered via injection, provided almost 80% relative percent survival (RPS) upon challenge by an infectious homologous virus in 1–2 g rainbow trout fry (Cuesta *et al*., 2010). Another DNA vaccine encapsulated in alginate and delivered in food pellets reduced or eliminated the IPNV titres in rainbow trout after waterborne virus challenge (Ballesteros *et al*., 2015). In this study, the VP2 gene was cloned into a DNA vector, incorporated into alginate microspheres and delivered orally to rainbow trout using a pipette to assure uniform delivery of the vaccine (Ballesteros *et al*., 2012). The alginate-bound DNA vaccine was also incorporated into food pellets to induce an immune response (Ballesteros *et al*., 2014). Both IgM and IgT increased at 15 days postvaccination but were much higher at 30 days postvaccination. A cellular immune response was also monitored by looking at the T cell markers CD4 and CD8. Both markers were elevated at day 15 but returned to background levels by day 30. The RPS was 85.9 and 78.2% when fish were challenged with IPNV at days 15 and 30 postvaccination, respectively. Recently, another study reported a fusion protein of IPNV VP2–VP3 proteins expressed in *Escherichia coli* and delivered via injection-induced IgM production against IPNV; this provided an RPS of 83% in juvenile rainbow trout (Dadar *et al*., 2015).

1.7.3 Subunit vaccines

Designer whole viral vaccines were produced using reverse genetics based on the Sp strain of IPNV (Munang'andu *et al*., 2012). Avirulent and virulent motifs were added to the Sp strain, which was then inactivated for use as a vaccine. The inactivated virus was compared with DNA, subunit and nanoparticle subunit vaccines made against IPN using a cohabitation challenge system. The inactivated whole virus vaccine provided a similar antibody titre to the other vaccines but outperformed them in survival after viral challenge with 48–58% RPS

while the VP2-fusion protein-, subunit- and DNA nanoparticle-based vaccines had values of 25.4–30.7, 22.8–34.2 and 16.7–27.2% RPS, respectively (Munang'andu *et al*., 2012).

The delivery of VP2 or VP3 antigens by a recombinant *Lactobacillus casei* was evaluated as a potential vaccine strategy (Liu *et al*., 2012). VP2 and VP3 were engineered either to be secreted by the *L. casei* or to be surface displayed. When these recombinant *L. casei* vaccines were orally delivered to rainbow trout, the VP2 secretory strain provided a much higher serum IgM titre than the other *L. casei* lines. On challenge with IPNV, the VP2-secreting *L. casei* was also more effective in reducing the viral load of the fish (~ 46-fold reduction compared with ~3 fold for the VP3-secreting strain).

Other ongoing research includes the improvement of the oral delivery of IPN antigens. For example, a recent study in Atlantic salmon showed that alginate-encapsulated IPNV antigens significantly improved the titre of IPNV-targeted antibodies and induction of immune-related genes when compared with the delivery of the same IPNV antigens in non-encapsulated form (Chen *et al*., 2014). The expense and inconvenience of injectable vaccines limit their usefulness in large-scale aquaculture beyond one vaccination cycle, so the use of oral vaccines to boost immune activation is attractive. The alginate-encapsulated vaccine was compared with the antigen alone and it was conclusively shown that protection of the antigen in the alginate was required for improved efficacy. The initial booster vaccination was done a year after the injection vaccination, when two oral doses were provided 7 weeks apart. Serum IgM was boosted after the first oral vaccination but IgT was not upregulated. In contrast, after the second booster, both IgT and IgM were upregulated (Chen *et al*., 2014).

Some examples of experimental vaccines against IPNV are shown in Table 1.3.

1.8 Conclusions and Suggestions for Future Research

Aquabirnavirus is the largest and most diverse genus within the family *Birnaviridae*. IPNV is one of the most extensively studied and widely distributed viruses infecting marine and freshwater fishes. Since the initial report of IPN-associated disease outbreaks in the 1940s, large numbers of IPNV and IPNV-like viruses have been isolated worldwide from diseased and apparently healthy salmonids and non-salmonids, as well as invertebrates (ICTV, 2014). Due to the extensive diversity of viral species belonging to the genus *Aquabirnavirus*, it has been difficult to classify the virus to species level (Crane and Hyatt, 2011). It remains unknown whether phylogeny based on whole genome sequence data and structure-based analysis of the VP2 and VP3 proteins will help to delineate the aquabirnaviruses to species level.

IPN is economically important due to its lethality for salmonid fry in freshwater production, and in post-smolts after transfer to seawater. The development of improved biosecurity protocols, targeted vaccines and resistant brood stocks has been very beneficial to the control of IPN, but despite these efforts, the disease remains a serious challenge to salmonid farming worldwide. One of the major constraints in developing effective vaccines has been the lack of a repeatable infection model to evaluate vaccine efficacy. However, recently, a

Table 1.3. Experimental vaccines against *Infectious pancreatic necrosis virus* (IPNV).

Vaccine	Description	References
IPNV virus-like particle (VLP)	Both viral proteins VP2 and VP2/VP3-based VLPs have been produced and shown to induce a strong immune response	Martinez-Alonso *et al*., 2012
Live recombinant *Lactobacillus casei*-expressing VP2	Orally delivered recombinant bacteria expressing a secreted VP2 were most effective in reducing viral load	Liu *et al*., 2012
Plasmid expression of segment A of IPNV dsRNA (whole or in parts)	Protection only with all of the large open reading frame (ORF) polyprotein	Mikalsen *et al*., 2004
Recombinant inactivated whole virus vaccine (IWV)	Reverse genetic constructs of virulent and avirulent IPNV-based nanoparticles	Munang'andu *et al*., 2012

cohabitation challenge for IPNV has been developed in Atlantic salmon (Munang'andu *et al*., 2016). Further validation of this infection model, combined with non-invasive tissue sampling to determine viral titre, may enhance IPN management.

Considering the extensive diversity of IPNV and IPNV-like viruses, the efficacy of IPNV vaccines can perhaps be improved by employing a structure-based vaccine design approach. In a rapidly evolving *Norovirus* GII.4 infecting humans, it has been shown that chimeric VLP-containing epitopes from multiple strains incorporated into a single VLP background induce a broad blocking antibody response, not only against GII.4 VLPs from GII.4-1987 to GII.4-2012, but also against those strains that were not included in the chimeric VLP (Debbink *et al*., 2014). A similar approach could be applicable to developing IPNV vaccine that provides protection against a number of prevailing strains for salmonid aquaculture.

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2 Infectious Haematopoietic Necrosis Virus

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2.1 Introduction

Infectious haematopoietic necrosis virus (IHNV, infectious hematopoietic necrosis virus) is a *Rhabdovirus* that causes significant disease in Pacific salmon (*Oncorhynchus* spp.), Atlantic salmon (*Salmo salar*), and rainbow and steelhead trout (*O. mykiss*). The disease that it causes, infectious haematopoietic necrosis (IHN), was first detected in cultured sockeye salmon (*O. nerka*) in the Pacific Northwest of North America and IHNV was first cultured in 1969 (see Bootland and Leong, 1999). IHNV is the type species and reference virus for the *Novirhabdovirus* genus of the family *Rhabdoviridae*. The viral genome is a linear, single-stranded RNA (~11,140 nucleotides in length) of negative sense with six genes that read from the 3′ end of the genome as N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein), NV (nonvirion protein) and L (RNA polymerase). The name *Novirhabdovirus* is derived from the unique *nonv*irion gene present in this genus (Kurath and Leong, 1985; Leong and Kurath, 2011).

IHNV causes necrosis of the haematopoietic tissues, and consequently it was named infectious haematopoietic necrosis by Amend *et al.* (1969). This virus is waterborne and may transmit horizontally and vertically through virus associated with seminal and ovarian fluids (see Bootland and Leong, 1999). Convalescent rainbow trout fry often clear the virus, but some fish can harbour it for 46 days, (Drolet *et al.*, 1995). The virus persists in the kidneys of some survivors for a year after infection (Drolet *et al.*, 1995; Kim *et al.*, 1999). Rainbow trout that survived the infection and were kept in virus-free water for 2 years had infectious virus in seminal and ovarian fluids at spawning (Amend, 1975). These fish are potential reservoirs. However, adult sockeye salmon collected in seawater and held to maturity in virus-free water had no detectable virus at spawn, while cohorts allowed to migrate naturally had prevalences of 90–100% (Amos *et al.*, 1989); this suggests the importance of horizontal transmission during river migration and that persistence of IHNV differs in different hosts. Recently, Müller *et al.* (2015) demonstrated the persistence of IHNV in the brains, but not in the kidneys, of sockeye salmon survivors. Despite the absence of disease and mortality among survivors, 4% of the fish had IHNV viral RNA in their brains at 9 months postexposure. This supports the hypothesis that a small percentage of infected fish become carriers. If the virus from the brain is infectious, the finding would have serious impacts on strategies for viral containment. Other potential reservoirs include virus adsorbed to sediment and virus detected, albeit rarely, in invertebrates or nonsalmonid fish hosts.

IHNV is reportable to the World Organisation of Animal Health (OIE) and countries with confirmed or suspected cases include: Austria, Belgium, Bolivia, Canada, China, Croatia, Czech Republic, France, Germany, Iran, Italy, Japan, Korea (Republic of), The Netherlands, Poland, Russia, Slovenia, Spain, Switzerland and the USA (OIE, 2015; last updated 23 July 2015; and Cefas, 2011, last updated 31 January). The virus is originally endemic to western

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North America, where it has the largest diversity of host species and the longest history of disease impacts, infects both wild and cultured fishes, and is most genetically diverse. IHNV has also been introduced into Asia and Europe, where it has become established largely in rainbow trout farms. Global phylogenetic analysis of IHNV has defined five major genetic groups (genogroups): group U in North America and Russia; groups M and L in North America; group J in Asia; and group E in Europe (Kurath, 2012a).

Economic losses from IHNV can be a direct consequence of fish mortality, or an indirect effect related to regulations that restrict the movement of IHNV-infected fish or require that infected stocks be destroyed. Disease outbreaks have devastated both commercial aquaculture (e.g. rainbow trout and Atlantic salmon) and conservation/mitigation programmes for Pacific salmon and trout in western North America. Since the disease has spread to Europe and Asia, an example of the potential economic impact of IHNV on salmon and trout fisheries/aquaculture was recently provided by Fofana and Baulcomb (2012). Although IHNV has not been isolated in the UK, it has been estimated that the direct and indirect costs of a theoretical IHN outbreak over 10 years (1998–2008) would be 16.8 million British pounds (~$25.5 million US dollars). Direct costs would be due to culling, mortality and the disposal of dead fish. Indirect costs would include lost revenue from consumer responses, impact on reduced exports and the increased expense of implementing additional surveillance strategies. The OIE maintains a database of IHN outbreaks, and in 2012, there were detections of IHNV in the western USA, Germany, Italy, Poland, China, Japan, Korea and British Columbia in Canada.

2.2 Clinical Signs of Disease and Diagnosis

2.2.1 Clinical signs

IHNV infection causes serious disease in young salmonid fishes though the virus can infect salmonids at all ages. Typically, at the start of an epizootic, moribund fish become lethargic, with periods of sporadic whirling or hyperactivity; fry may have a dark coloration, distended abdomens, exophthalmia, pale gills and mucoid, opaque faecal casts (Fig. 2.1A). Petechial haemorrhages may occur at the base of the fins and vent and occasionally in the gills, mouth, eyes, skin and muscle (Figs 2.1B,C). Some fry may have a subdermal haemorrhage immediately behind the head. Older fish have fewer external clinical signs. Sockeye salmon smolts have gill and eye haemorrhages, clubbed and fused lamellae and cutaneous lesions, while 2-year-old kokanee salmon (landlocked sockeye salmon, *O. nerka*) have erratic swimming and haemorrhages near the base of the fins. Some fish succumb to IHN disease without visible signs (see Bootland and Leong, 1999).

The liver, spleen and kidney of infected fry are pale due to anaemia, there may be ascites and the stomach is filled with a milky fluid but without food. The intestine contains a watery, yellowish fluid and there may be petechial haemorrhages in the visceral mesenteries, adipose tissue, swim bladder, peritoneum, meninges and pericardium. Older fish may have empty stomachs, intestines filled with yellowish mucus and lesions in the musculature near the kidney (see Bootland and Leong, 1999).

2.2.2 Diagnosis

Preliminary diagnosis can be based on fish with clinical signs at a site where there is history of the disease. Histologically, the observation of necrosis of the granular cells of the alimentary tract is pathognomonic (Wolf, 1988). A preliminary diagnosis must be confirmed by specific identification. The most widely accepted diagnostic method is the isolation of the virus in cell culture (presumptive diagnosis) followed by identification using a serum neutralization test or PCR-based methods. Immunological and molecular methods are described in the (online) *Manual of Diagnostic Tests for Aquatic Animals* from the OIE (2015), the Canadian *Fish Health Protection Regulations: Manual of Compliance* (Department of Fisheries and Oceans, 1984; revised 2011), and the IHNV chapter (LaPatra, 2014) in the American Fisheries Society (AFS)-Fish Health Section (FHS) *FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, 2014 edn (AFS-FHS, 2014).

Many teleost cell lines are susceptible to IHNV infection, but those specified by the OIE Manual, the Canadian Manual of Compliance and the *FHS Blue Book* are the epithelioma papulosum cyprini (EPC) and/or fathead minnow (FHM) cell lines. The *FHS Blue Book* also recommends the Chinook

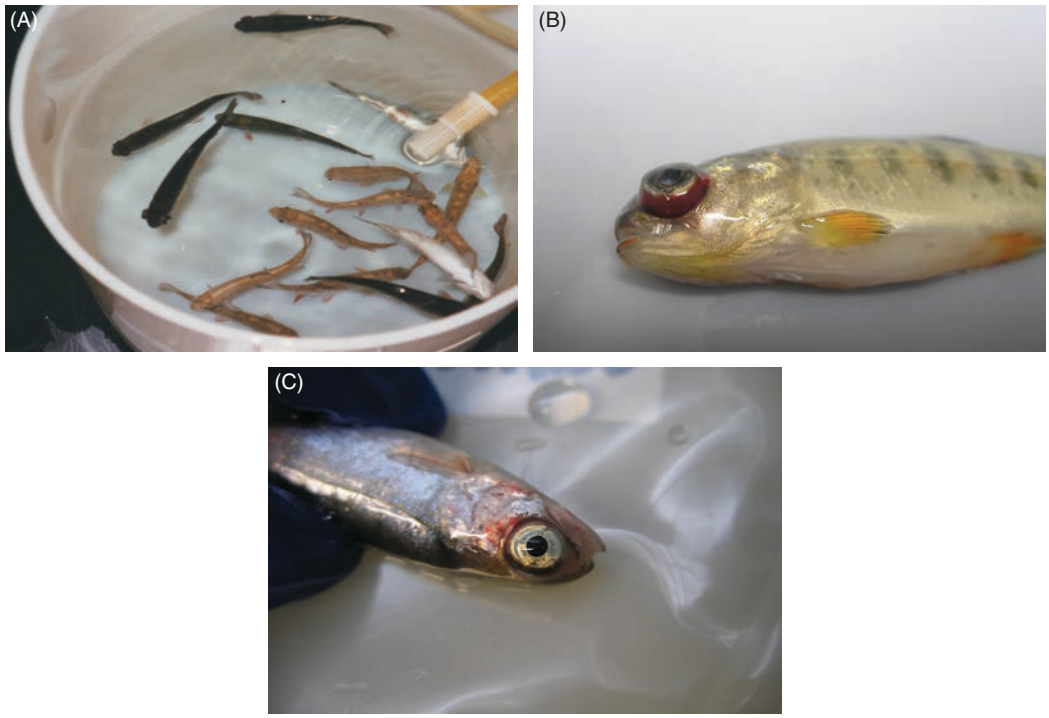

Fig. 2.1. (A) Juvenile rainbow trout at 7 days after immersion exposure to IHNV. The lighter fish show no evidence of infection and the darker fish exhibit the typical signs of infectious haematopoietic necrosis (IHN) disease with darkening coloration and pronounced exophthalmia. The dead fish to the right side of the tank exhibit petechial haemorrhages. (B) Juvenile rainbow trout with IHN disease showing pronounced exophthalmia with bleeding in the eye orbit. (C) Juvenile sockeye salmon fingerling with IHN disease showing petechial haemorrhages around the eye orbit and on the gills and fins.

salmon embryo cell line, CHSE-214. EPC cells are the most susceptible to IHNV (Lorenzen *et al*., 1999). Other fish cell lines susceptible to IHNV cytopathogenicity are discussed extensively in Bootland and Leong (1999). The optimum temperature for growth is approximately 15°C (Mulcahy *et al*., 1984); the higher range of 23–25°C does not support viral replication. IHNV is heat labile, and is inactivated within several hours at 32°C (Pietsch *et al*., 1977).

Cell culture detection of IHNV can require 14 days. End-point titrations and plaque assays are typically used. The latter are more sensitive and are the standard for quantifying IHNV. Pretreatment of cell monolayers with polyethylene glycol improves the speed and sensitivity of plaque assays and produces larger plaques. The typical cytopathic effect (CPE) consists of grape-like clusters of rounded cells, with margination of the chromatin of the nuclear membrane. Typical IHNV plaques consist of a cell sheet that retracts or piles up at the inner margins of the opening; the centre may contain granular debris. Cells should be examined for 14 days. If no CPE occurs, the supernatant may be passed on to fresh cells for a 'blind passage'. The absence of CPE indicates that the sample is virus negative (see Bootland and Leong, 1999)

The preferred tissues for isolating IHNV are the kidney and spleen; mucus and pectoral fin clippings have also been used as non-lethal samples. For testing brood stock, the ovarian fluid is preferred, because the virus is less frequently detected in the milt. The sampling of post-spawning females, storage of ovarian fluid or incubation of ovarian fluid cells enhances the sensitivity of viral detection. Milt samples should be centrifuged and, after the pellet is incubated in water, the water is assayed for virus (see Bootland and Leong, 1999, 2011).

Serological assays require either polyclonal or monoclonal antibodies and IHNV immunodetection

kits are commercially available (Bio-X Diagnostics, Rochefort, France; see http://www.biox.com). Assays for the identification of IHNV include serum neutralization, indirect fluorescent antibody testing, direct alkaline phosphatase immunocytochemistry (APIC) and enzyme-linked immunosorbent assay (ELISA), as well as the Western blot, dot blot, staphylococcal co-agglutination and electron microscopy (see Bootland and Leong, 1999). The molecular methods include reverse transcriptase-dependent polymerase chain reaction (RT-PCR), real-time (quantitative) RT-PCR (qRT-PCR; see Bootland and Leong, 2011; Purcell *et al*., 2013), multiplex RT-PCR (Liu *et al*., 2008), loop-mediated isothermal amplification (Gunimaladevi *et al*., 2005) and a molecular padlock probe (Millard *et al*., 2006). With the exception of the latter, an initial RT step must be used to create cDNA from the IHNV viral RNA (mRNA, genome and antigenome) followed by amplification using PCR. These methods and their relative sensitivities (if available) have been described by Bootland and Leong (2011) and their comparative sensitivities are given in Table 2.1. For research applications, modified qRT-PCR assays have been developed for the specific detection of positive- and negative-sense IHNV RNA (Purcell *et al*., 2006a) and for genotype-specific detection of individual IHNV strains within mixed infections (Wargo *et al*., 2010).

Salmonids infected with IHNV may mount a strong antibody response that persists for months (see Lorenzen and LaPatra, 1999). Monitoring the antibody response is not lethal and may be useful in surveillance of a population for previous exposure to IHNV (Bootland and Leong, 2011).

2.3 Pathology

2.3.1 Histopathology

Histopathological findings include degenerative necrosis in haematopoietic tissues, the posterior kidney, spleen, liver, pancreas and digestive tract. In the anterior kidney, the initial changes are small, lightly stained focal areas consisting of apparent macrophages and degenerating lymphoid cells. As the disease progresses, degenerative changes become noticeable throughout the kidney. Macrophages increase in number and may have a vacuolated cytoplasm and chromatin margination of the nuclei. Pyknotic and necrotic lymphoid cells may be present. Necrosis may be so severe that the kidney tissue consists primarily of necrotic debris. Focal areas of cells in the spleen, pancreas, liver, adrenal cortex and intestine show nuclear polymorphism and margination of the chromatin, with eventual necrosis. Extensive necrosis in all organs is accompanied by pyknosis, karyorrhexis and karyolysis. A pathognomonic feature of IHN is degeneration and necrosis of granular cells in the lamina propria, stratum compactum and stratum granulosum of the alimentary tract, and sloughing of intestinal mucosa may give rise to faecal casts. Smolts and yearlings tend to show less severe histopathology. The kidney, spleen, pancreas and liver may show necrosis, but there is only moderate sloughing of the intestinal mucosa and no faecal casts. Fish have a normocytic aplastic anaemia and the blood of those that are affected has leucopenia with degenerating leucocytes and thrombocytes, a reduced haematocrit and osmolarity, and a slightly altered biochemical profile. Cellular debris (necrobiotic bodies) in blood smears or kidney imprints is pathognomonic for IHN (Wolf, 1988; in Bootland and Leong, 1999).

2.3.2 Disease progression

In rainbow trout, the IHN virus enters transiently through the gills, skin, fin bases and oral region to the oesophagus/cardiac stomach region before spreading to internal organs. The haematopoietic tissues of the kidney and spleen of young fish are most severely affected and are the first tissues to show extensive necrosis. Typically, within a day after immersion exposure, low titres of IHNV are detectable in the gills, skin and intestine of young rainbow trout before the infection spreads to the kidney (2–4 days) and subsequently becomes widespread (Bootland and Leong, 1999, 2011).

During infection, viral prevalence and titres peak within 5–14 days (Drolet *et al*., 1994; Peñaranda *et al*., 2009; Purcell *et al*., 2009). Some rainbow trout have been shown to be still infected at 28 days but infectious virus was no longer detected after 54 days (Drolet *et al*., 1994). Drolet *et al*. (1994) proposed that the infection progressed via two major routes: from the gills into the circulatory system and from the oral region into the gastrointestinal (GI) tract and then into the circulatory system. Both routes induced systemic viraemia. These authors proposed that the initial infection of the kidney was not via the GI route but rather

Table 2.1. Sensitivity of different diagnostic methods for IHNV detection

Method	Reported sensitivity	Calculated no. of physical particles[a]	Reference
Infectivity in fish cell lines	1 infectious unit	1 $TCID_{50}$ units = ~ 500 1 PFU = 0.69 $TCID_{50}$ units = ~345	Durrin, 1980
Staphylococcal co-agglutination	1–6 × 10^4 PFU	3.45–20.7 × 10^6	Bootland and Leong, 1992
Western Blot			
Peroxidase labelled antibody	10 ng of virus[b]	7.67 × 10^7	Hsu and Leong, 1985
Iodine^{125} labelled antibody	2.5 ng of virus	1.53 × 10^7	Hsu and Leong, 1985
Alkaline Phosphatase labelled secondary antibody			
Polyclonal mouse anti IHNV N	1 PFU	345	Kim *et al*., 2001
Monoclonal mouse anti IHNV N	3 × 10^3 PFU	1.035 × 10^6	Kim *et al*., 2001
Enzyme-linked immunosorbent assay			
Polyclonal mouse anti-IHNV N	10 PFU	3450	Kim *et al*., 2001
Monoclonal mouse anti-IHNV N	10^3 PFU	3.45 × 10^6	Kim *et al*., 2001
Chicken and rabbit anti-IHNV	70 PFU	2.42 × 10^4	Medina *et al*., 1992
Immunoblot assay			
Peroxidase Labelled	0.85–4.0 ng virus	0.66–3.07 × 10^7	McAllister and Schill, 1986
Biotinylated antibody	10^2 PFU, 5.5 ng virus	3.45 × 10^4, 4.22 × 10^7	Schultz et al. 1989
RT-PCR, N gene, single round	4 × 10^2 PFU	1.38 × 10^5	Arakawa *et al*., 1990
RT-PCR, G gene, single round	2.2 × 10^3 G gene RNA copies[c]	5.9 × 10^2 viral gRNAs, 25 particles	Emmenegger *et al*., 2000, Purcell *et al*., 2013.
Immunomagnetic bead capture with monoclonal anti IHNV N, followed by RT-PCR	10^6 PFU	3.45 × 10^6	Kim *et al*., 2001
Dot blot RNA Hybridization (biotin or alkaline phosphatase label) if chemiluminescent reaction is used	20 pg viral RNA[d] 4 pg viral RNA	3.37 × 10^6 viral gRNA 4.22 × 10^4 viral gRNA	Gonzalez *et al*., 1997 Gonzalez *et al*., 1997
RT-qPCR, N gene	7 N gene RNA copies[e]	1.1 viral gRNA, 0.05 particles	Purcell *et al*., 2013

[a]The sensitivity of each method was used to determine the equivalent number of physical IHNV particles quantified using electron microscopy (Durrin, 1980).

[b]The weight of the IHNV particle was derived from a 21:1 ratio of virion protein to RNA and the estimate that the molecular weight of the viral genome is 3.57 × 10^6 (Kurath and Leong, unpublished observation). Thus, the molecular weight of a virion is 7.497 × 10^7 daltons of protein plus 3.57 × 10^6 daltons of RNA. The calculated weight of one virion is 1.30418 × 10^{-16} grams, and the calculated weight of one viral genome is 5.928 × 10^{-18} grams.

[c]The IHNV G gene conventional PCR assay detection was calibrated to viral RNA copy number based on data from Purcell *et al*., 2013 (M. Purcell and G. Kurath, unpublished), and includes both viral genomic (gRNA) and mRNA . Within this viral RNA copy number the ratio of gRNA:G mRNA was reported as 1:2.7, and the ratio of gRNA:PFU as 8000:1 (Purcell *et al*., 2006). These ratios were used to calculate the equivalent number of viral gRNA copies, PFU, and then physical particles.

[d]Gonzalez *et al*, 1997, used partially purified virus for their RNA extraction, consequently the actual virus RNA number might actually be lower.

[e]The universal IHNV N gene RT-qPCR assay detection limit was calibrated as described in footnote c, but was modified based on the reported molar ratio of IHNV G mRNA:N mRNA as approximately 1:2. Thus a ratio of gRNA: N mRNA of 1:5.4 was used to calculate the equivalent number of gRNA copies.

through the highly vascularized tissue lining the oral cavity. From there, the virus travelled through the blood to the kidney and haematopoietic tissues. Using Immunogold-labelled secondary antibody to detect the binding of an anti-N monoclonal antibody to infected cells, Helmick *et al.* (1995a,b) identified an early IHNV target area, the esophagus/cardiac stomach region (ECSR), particularly the cardiac mucus-secreting cardiac gland (MSSG). There was evidence of the attachment and internalization of IHNV in the ECSR mucosal epithelial cells in rainbow trout and coho salmon (*O. kisutch*) within 1 h postinfection. The MSSG of coho salmon showed a milder reaction, which supported previous findings that the virus replicated less efficiently in coho salmon. In Chinook and sockeye salmon fry there may be hepatic deposits of ceroid (Wood and Yasutake, 1956; Yasutake, 1970). In the final stages of the disease, necrosis is seen not only in the haematopoietic tissues of the kidney, but also in the glomeruli and kidney tubules.

Another study in rainbow trout used bioluminescence imaging in living fish to follow infection with recombinant IHNV strains expressing luciferase (Harmache *et al.*, 2006). In addition to identifying fin bases as a site of entry, there was continuing viral replication in fin tissues and internal organs. Some survivors maintained localized viral replication, mostly in the fins. More recently, the progression of infection with a high temperature-adapted IHNV strain was monitored in transparent zebrafish (*Danio rerio*) larvae (Ludwig *et al.*, 2011) infected by injection and held at 24°C. Macroscopic signs of infection slowed down and then arrested blood flow despite continuing heartbeat. This was followed by a loss of reactivity to touch and the fish were dead in 3–4 days. Using *in situ* hybridization in whole larvae, the first infected cells were detected at 6 h postinfection in the major blood vessels and the venous endothelium was a primary target of infection. This suggested that infection spread from damaged vessels to the underlying tissues. By transferring the larvae to 28°C (no viral replication), a critical threshold resulting in irreversible damage was reached in less than a day, before clinical signs appeared.

2.4 Pathophysiology

IHN is characterized by a severe depletion of the alkali reserve and an imbalance of blood electrolytes, resulting in decreased blood osmolality (Amend, 1973). These changes have been ascribed to the loss of renal function from the viral induced necrosis of kidney tissues. Packed cell volume, haemoglobin, red blood cell count and plasma bicarbonate were significantly depressed in 4 days (Amend and Smith, 1974). Plasma chloride, calcium, phosphorus, total protein and blood cell types did not change during the 9 days of study. An increase in lactate dehydrogenase B ($LDHB_4^{2'}$) isoenzyme levels was consistently associated with early development of the disease. Increased LDH was not observed in fish infected with infectious pancreatic necrosis virus (IPNV) or three bacterial pathogens.

Amend and Smith (1975) found reduced plasma bicarbonate, chloride, calcium, phosphorus, bilirubin and osmolality in moribund rainbow trout. When the fish showed signs of disease, plasma glucose and anterior kidney ascorbates were unchanged. Infected fish had reduced corpuscular counts, haemoglobin and packed cell volume, but mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentrations remained normal. The percentage of immature erythrocytes increased, but the percentage of leucocytes was unchanged. Neutrophils decreased, lymphocytes increased, but monocytes did not change. Plasma pH increased and the alpha 2 and 3 fractions of the serum proteins were altered. The alkali reserve diminished and acid:base and fluid balances were altered. Death probably resulted from severe electrolyte and fluid imbalances caused by renal failure.

2.5 Control Strategies

Control strategies rely primarily on avoidance of exposure to the virus through biosecurity policies and best practices for hygiene at culture facilities (Winton, 1991). The successful Alaskan sockeye salmon culture policy implemented in 1981 provides guidelines for IHNV control within a virus-endemic region based on three criteria: a virus-free water supply, rigorous disinfection; and the compartmentalization of incubating eggs and rearing juvenile fish (Meyers *et al.*, 2003). Thus, eggs are disinfected, typically using treatment with an iodophor (a disinfectant containing iodine complexed with a solubilizing agent), and then incubated in separate egg lots in virus-free water. Fry are also reared in virus-free water. Secure water sources (free of susceptible host fish) are used as long as possible throughout the rearing period. The recent

success of a delayed exposure rearing strategy at a large steelhead hatchery illustrates the importance of water supply in minimizing viral transmission between free-ranging and hatchery fish (Breyta *et al*., 2016). Disease outbreaks are controlled in areas where the disease is not endemic by culling, disinfection and quarantine (McDaniel *et al*., 1994). Additional precautions generally practised within British Columbia salmon farms include the maintenance of single-year class and single-species sites, reduced fish movements between pens, the culling/accelerated harvest of smaller fish when infection occurs and fallowing between restockings (Saksida, 2006). The introduction of IHNV into new geographical areas has never been associated with the movement of killed fresh fish or frozen products and the risk is considered negligible for processed rainbow trout (LaPatra *et al*., 2001a).

In cell cultures, IHNV replication is inhibited by methisoprinol (Siwicki *et al*., 2002), and chloroquine inhibits IHNV *in vivo* by reducing viral binding and cell entry (Hasobe and Saneyoshi, 1985; De las Heras *et al*., 2008). Similarly, the pretreatment of cultured cells with the antiviral agents amantadine or tributylamine also reduced IHNV binding and cell entry. When antisense phosphorodiamidate morpholino oligomers (PMOs) complementary to the 5′ end of IHNV genomic RNA were tested against IHNV in cell culture, inhibition was sequence and dose dependent. The compound was cross-linked to a membrane-penetrating peptide that enhanced entry of the MPO into cultured cells and live fish tissues (Alonso *et al*., 2005). Anti-IHNV activity has also been reported in bacteria from aquatic environments (Myouga *et al*., 1993). A peptide (46NW-64A), produced by *Pseudomonas fluorescens* biovar 1 completely inhibited the replication of IHNV (100% plaque reduction). Inclusion of the carotenoid, astaxanthin, in feed produced significant resistance to IHNV challenge in rainbow trout fry (Amar *et al*., 2012). Bovine α_2-CN casein hydrolysates and total casein hydrolysates are potent antiviral agents against IHNV on fish cell monolayers (Rodríguez Saint-Jean *et al*., 2012). When three month old brown trout (*Salmo trutta*) were fed casein at day 0 and day 3 postexposure to IHNV, the treated group showed nearly 50% protection against a virus dose that killed 93% of the control fish (Rodríguez Saint-Jean *et al*., 2013).

Since McIntyre and Amend (1978) reported 30% heritability for resistance to IHNV in sockeye salmon, efforts have continued to develop IHNV-resistant strains of trout and salmon. The resistance can be species specific and confined to a particular viral genogroup. However, resistance to IHN disease does not mean resistance to infection. In terms of disease, sockeye salmon are resistant to M group virus but sensitive to U group virus, while rainbow trout have the converse phenotypes (Garver *et al*., 2006). Rainbow trout hybrids from crosses with more resistant species, (e.g. coho salmon, or brook trout, *Salvelinus fontinalis*) are more resistant to IHNV (LaPatra *et al*., 1993), whereas coho and chinook salmon hybrids are susceptible to IHNV-induced mortalities (Hedrick *et al*., 1987).

The identification of the genes responsible for resistance has been the focus of selective breeding programmes for disease-resistant rainbow trout and Atlantic salmon. The majority of genetic studies on IHNV resistance have used rainbow trout backcrossed within families (Khoo *et al*., 2004) or with less susceptible cutthroat trout (*O. clarkia*) (Palti *et al*., 1999, 2001; Barroso *et al*., 2008) or steelhead trout (Rodriguez *et al*., 2004). Genetic linkage maps based on molecular markers (microsatellites and amplified fragment length polymorphisms (AFLPs)) and quantitative trait loci (QTLs) have been characterized, such studies but have not identified highly associated loci (Overturf *et al*., 2010) or significant associations within very large blocks of linkage disequilibrium (Rodriguez *et al*., 2004; Barroso *et al*., 2008). Genome sequencing has identified 19 single-nucleotide polymorphism (SNP) markers in rainbow trout that were associated with IHNV resistance and can be used in a marker-assisted selection (MAS) programme (Campbell *et al*., 2014). It is probable the resistance genes are related to MHC (major histocompatibility complex) haplotypes and T cell immunity, as has been reported by Yang *et al*. (2014). Interestingly, Verrier *et al*. (2012) found that cell lines derived from trout clonal lines resistant to viral haemorrhagic septicaemia virus (VHSV) were also resistant to rhabdovirus infection under *in vitro* conditions.

2.5.1 Vaccines

A DNA vaccine referred to as APEX-IHN (Novartis Animal Health Canada Inc.) was approved in 2005 by the Canadian Food Inspection Agency (CFIA) for farm-raised Atlantic salmon (CFIA, 2005). This was the first DNA vaccine approved for fish and one of only four DNA vaccines commercially available for animals. The vaccine was based on the

demonstration that an intramuscular injection of plasmid DNA encoding the IHNV G gene under the control of a constitutive viral promoter induced protection in rainbow trout fry (Anderson *et al*., 1996). Since then, numerous experimental studies in the USA have confirmed the efficacy of similar DNA vaccines and examined several practical aspects, such as vaccine dose, duration of protection, cross-species protection, routes of administration and vaccine safety (see Kurath, 2008). It was confirmed that the DNA vaccine must encode the IHNV G gene, not other IHNV genes, to induce protection and neutralizing antibodies in rainbow trout fry and in sockeye salmon. The vaccine was effective at extremely low doses (0.1–1 µg) in 1–2 g rainbow trout fry; larger fish required a higher dose. Vaccinated rainbow trout fry were protected against IHNV from different geographical locations, suggesting that it would be useful worldwide. The DNA vaccine induced rapid non-specific innate immunity (LaPatra *et al*., 2001b; Lorenzen *et al*., 2002) with the expression of Mx protein, an indicator of alpha/beta interferon induction (Kim *et al*., 2000; Purcell *et al*., 2006b), followed by long-term specific protection with relative percentage survival (RPS) > 90% for 3 months and RPS > 60% for 2 years (Kurath *et al*., 2006). The IHNV-G DNA vaccine induced protection in rainbow trout (see Kurath, 2008), Atlantic salmon (Traxler *et al*., 1999), Chinook salmon, sockeye salmon and kokanee salmon (*O. nerka*) (Garver *et al*., 2005a). DNA vaccines containing G genes from either the M or U genogroups protected fish against intra- and cross-genogroup IHNV challenges (Peñaranda *et al*., 2011).

The DNA vaccine is administered by intramuscular injection, which is labour intensive and difficult to implement with small fish. Equivalent high levels of efficacy were detected in rainbow trout fry when the vaccine was delivered by cutaneous particle bombardment using a gene gun. However, only partial protection was obtained by intraperitoneal injection, and several other routes of vaccination did not induce protection (Corbeil *et al*., 2000). Recently, an oral DNA vaccine against IHNV was developed using alginate microspheres to encapsulate the DNA vaccine; using this, significant protection was observed in rainbow trout (Ballesteros *et al*., 2015).

The IHNV DNA vaccine is safe and well tolerated by fish. No vaccine-specific pathological changes were observed in tissues for as long as 2 years after vaccination (Garver *et al*., 2005b: Kurath *et al*., 2006). The plasmid backbone of the vaccine contains the cytomegalovirus (CMV) immediate early promoter, which is derived from a human pathogen. As regulatory authorities may consider such a vaccine 'unsafe', Alonso *et al*. (2003) tested DNA vaccines containing the IHNV G gene linked to three different rainbow trout promoters. A vaccine with the interferon regulatory factor 1A promoter from rainbow trout provided protection equivalent to that of the pCMV-G vaccine (Alonso and Leong, 2012).

Extensive efforts have been made to develop more conventional vaccines against IHN, such as subunit vaccines and attenuated vaccines. Experimental IHNV vaccines reviewed in Winton (1997) and Bootland and Leong (1999) showed varying degrees of efficacy and have not resulted in a commercial product. Other vaccines, including a baculovirus-derived IHNV G protein vaccine produced in insect cells and a recombinant subunit vaccine produced on the surface of *Caulobacter crescentus* have shown some efficacy in experimental studies (Cain *et al*., 1999; Simon *et al*., 2001). A recent re-examination of killed IHNV vaccines demonstrated the critical importance of inactivating agents, and a β-propiolactone-inactivated whole virus vaccine induced both 7 day and 56 day protection (Anderson *et al*., 2008). Hence, there is still potential for effective killed vaccines.

2.6 Conclusions and Suggestions for Future Research

IHNV is a global problem for wild and domestic salmonids, and its spread to salmonid farms is increasing. The virus has adapted to new hosts and biological environments as evidenced by the evolution of the trout-adapted M, J and E genogroups in North America, Japan and Europe, respectively. Effective control strategies are needed to prevent the spread of the virus to naive fish populations and IHNV-free geographic regions. Perhaps the spread of IHNV can be contained by developing a source of salmon or trout eggs that are certified as specific pathogen free (SPF). Briefly, eggs would be obtained from IHNV-free brood stock and the resultant progeny reared on SPF water. These viral-free fish can then produce eggs and fingerlings for sale as IHNV-free stock. Certainly, faster diagnostics and regular surveillance for IHNV can assess the viral status of both wild and domestic fish.

Sensitive detection assays for the virus are now available and thorough investigation of potential vectors and reservoirs for IHNV is possible. Genetic typing is used in molecular epizootiology and fish health management (Breyta *et al*., 2016) and centralized databases of epizootiological and genetic information are publically available for virus isolated in North America or Europe (Johnstrup *et al*., 2010; Emmenegger *et al*., 2011; Kurath, 2012b). Although the first DNA vaccine has been approved for use in fish in Canada, other countries have not licensed this product. The efficacy of the vaccine is well established, but delivery by injection is often impractical, and finding an alternative method to deliver the vaccine remains a high priority (Munang'ando and Evensen, 2015).

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3 Viral Haemorrhagic Septicaemia Virus

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3.1 Introduction

Viral haemorrhagic septicaemia virus (VHSV), the aetiological agent of viral haemorrhagic septicaemia (VHS) is a member of the family *Rhabdoviridae* within the order *Mononegavirales*. These viruses have single-stranded, negative-sense (nega) RNA genomes with enveloped bullet- or cone-shaped nucleocapsids. The economically most important pathogenic rhabdoviruses are VHSV, infectious haematopoietic necrosis virus (IHNV; both in the genus *Novirhabdovirus*) and spring viraemia of carp virus (SVCV; new name *Carp sprivivirus*, genus *Sprivivirus*). Please refer to the International Committee on Taxonomy of Viruses for details (ICTV, 2015). VHSV and IHNV share the same six genes that read from the 3′ end to the 5′ end of the genome as N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), G (glycoprotein), NV (non-virion) and L (RNA polymerase) (Einer-Jensen *et al*., 2004). SVCV lacks the gene for NV and has other minor genomic differences (Hoffmann *et al*., 2002). VSHV was comprehensively reviewed by Smail and Snow (2011) and LaPatra *et al*. (2016).

Serotyping was originally used to subgroup isolates (Olesen *et al*., 1993); however, genotyping is currently done using the genes for G and N. Genotypes are broadly distributed geographically and their differentiation has proven immensely invaluable for understanding epizootiological spread and pathogenesis (Nishizawa *et al*., 2002, Snow *et al*., 2004, Brudaseth *et al*., 2008). If the genotype and infected species is not specified, generalizations on VHSV pathogenesis, host range, virulence, etc. can be misleading.

Genotypes I and IV are the most relevant to this discussion, although all four genotypes I–IV occur in the northern Hemisphere. Genotypes I–III are European, with genotype I divided into freshwater rainbow trout (*Oncorhynchus mykiss*) isolates (Ia) and marine isolates from various species of wild fish (Ib). The Ia genotype is associated with the original descriptions of VHS in rainbow trout in which mortality approached 100%. Genotype IV is found in the Pacific Ocean (IVa) and North America, including the western Atlantic Ocean (IVb; Gagne *et al*., 2007) and the Great Lakes (IVb; Lumsden *et al*., 2007). A trend that has occurred in Europe and is very likely to have also occurred in North America is the movement of marine isolates into fresh water along with the expansion of host range (Einer-Jensen *et al*., 2004; Snow *et al*., 2004; Gagne *et al*., 2007; Thompson *et al*., 2011). Genotypes have an impact on the regulatory control of VHSV and the movement of fish because a diagnosis of genotype Ia outside Europe has greater implications than a geographic expansion of IVb in North America. The detection of any VHSV must be reported to the World Organisation for Animal Health (OIE, 2015).

One of the most remarkable features of VHSV is its broad host range; 28 species of fish are infected by genotype IVb in the Great Lakes (APHIS, 2009) and about 80 species are affected by all four genotypes (OIE, 2015). The ubiquitous phosphatidylserine and fibronectin, are receptors for VHSV in fish cells (Bearzotti *et al*., 1999; see LaPatra *et al*., 2016), which may account for its broad host range, though fibronectin is also a receptor for IHNV (Bearzotti *et al*., 1999). Transmission is horizontal

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with virus excreted from the urine and reproductive fluids (Eaton *et al*., 1991) and with uptake at least by gill epithelium (Brudeseth *et al*., 2008). Even though VHSV is in the reproductive fluid and VHSV RNA is inside the gonads of fish infected with VHSV IVb (Al-Hussinee and Lumsden, 2011a), vertical transmission has not been documented.

VHS occurs at water temperatures between 4 and 15°C (McAllister, 1990), although the virus grows at higher temperatures in cell culture (Vo *et al*., 2015), which emphasizes the importance of host factors in disease. Experimental infection using IVb gave bluegill (*Lepomis macrochirus*) mortality of 90% at 10°C, but 0% above 18°C (Goodwin and Merry, 2011). Temperature ranges and optima are similar for genotypes I and IV (Goodwin and Merry, 2011; Smail and Snow, 2011), and above 20°C mortalities due solely to VHSV are unlikely (Sano *et al*., 2009). The results of surveillance performed at water temperatures above 20°C should be critically evaluated. In deep waters, thermoclines rarely reach the bottom, so the virus may still be detected into summer in demersal fish. Outside the host, the virus remains infective for variable periods. It survives for a year in filtered fresh water at 4°C (Hawley and Garver, 2008) and survival is longer at lower temperatures (Parry and Dixon, 1997). An average survival in raw seawater at 15°C is 4 days (Hawley and Garver, 2008).

3.2 Diagnosis

The clinical signs of and gross lesions in VHS-affected fish are numerous, but none are pathognomonic, and the disease should always be considered given the broad geographic area over which it is endemic. It should be borne in mind as a differential diagnosis if the temperature, geographic location and fish species are consistent with morbidity, and if the fish have haemorrhages, exophthalmia and enlarged abdomens with ascites. Infected fish may also have anaemia, a darkened colour, lethargy and abnormal swimming behaviour, and they may die rapidly (OIE, 2105). However, infected fish may also die without gross lesions; this is common for round goby (*Neogobius melanostomus*) in Lake Ontario (Groocock *et al*., 2007), thus emphasizing the importance of histopathology and ancillary diagnostics. Similarly, clinically normal fish may be positive for VHSV, so emphasizing the need for either PCR or viral isolation to confirm detection.

Because VHS is an OIE reportable disease, that organization requires specific diagnostic techniques for confirmation (OIE, 2015). Viral isolation using BF-2 (bluegill fry), EPC (epithelioma papulosum cyprini) or other cell lines is used routinely, but it is labour intensive and expensive. Many laboratories use either standard reverse transcriptase (RT)-PCR or quantitative RT-PCR (qRT-PCR; Matejusova *et al*., 2008, Hope *et al*., 2010), which can detect all four genotypes of VHSV (Garver *et al*., 2011). These techniques are more sensitive and rapid than viral isolation, and they are more easily standardized and allow high throughput (Hope *et al*., 2010). ELISA and immunofluorescence (the indirect fluorescent antibody (IFA) test (IFAT); see OIE, 2015) and reverse transcription loop-mediated isothermal amplification (Soliman *et al*., 2006) are also used.

When PCR is used, tissues should be saved to allow confirmatory testing, which includes viral isolation (OIE, 2015). Viral isolation also requires a minimum amount of tissue and/or pooling of fish to allow for a positive identification (AFS-FHS, 2014). Quantitative RT-PCR is ideal for a presumptive diagnosis or surveillance with banking of tissues in the case of a presumptive positive. A conclusive diagnosis of VHS requires viral isolation and serum neutralization followed by one of three confirmatory tests; IFAT, ELISA or RT-PCR (OIE, 2015).

3.3 Pathology

The pathology of VHSV has previously been reviewed (de Kinkelin *et al*., 1979; Wolf 1988; Evensen *et al*., 1994; Brudeseth *et al*., 2002, 2005; OIE, 2015; LaPatra *et al*., 2016). Gross lesions of many acute systemic diseases are often stereotypical, including haemorrhages, enlarged abdomen and exophthalmia, which are typical macroscopic lesions in VHSV-infected fish (OIE, 2015). Petechial haemorrhage in the dorsal musculature in rainbow trout infected with VHSV Ia, is very common (OIE, 2015). The lesions included in the present discussion are generalizations.

Mortality can occur without significant gross lesions, apart from pallor in round goby (Groocock *et al*., 2007), or swim bladder haemorrhage in muskellunge (*Esox masquinongy*) (Elsayed *et al*., 2006). The causes of many mortality events are multifactorial even when VHSV is detected in dead fish. Mortality in wild Pacific herring (*Clupea pallasii*)

was associated with numerous pathogens, including VHSV (Marty *et al*., 1998). In Conesus Lake (New York state), dead walleye (*Sander vitreus*) had lesions consistent with VHSV and the virus was isolated from the fish, but affected fish also had lesions typical of branchial columnaris (Al-Hussinee *et al*., 2011b), a bacterial disease caused by *Flavobacterium columnare* (see Chapter 16).

Histological lesions vary, but are primarily necrotizing and affect almost all tissues. Patterns of the lesions can depend on the genotype and fish species. For example, VHSV Ia in rainbow trout will produce necrotizing lesions in the kidney and liver, but the spleen, heart, brain and other tissues may also be affected (de Kinkelin *et al*., 1979; Wolf 1988; Evensen *et al*., 1994). Turbot (*Scophthalmus maximus*) infected with VHSV genotype Ib (Brudaseth *et al*., 2005) and some species affected with IVb from the Great Lakes had the most severe lesions in the heart, though the liver and haematopoietic tissues were also affected (Lumsden *et al*., 2007; Al-Hussinee *et al*., 2011b). The cardiac and vascular lesions in freshwater drum (*Aplodinotus grunniens*) were so spectacular that the likelihood of a foreign agent being responsible was readily apparent (see Figs 3.1 and 3.2). The infection produces a severe vasculitis in numerous tissues, but it can also be subtle (see Fig. 3.3). Freshwater drum had a florid, often fibrinoid, vasculitis in the liver, kidney (Fig. 3.4), brain and spleen, and both this lesion and a tropism for endothelium have also been documented in rainbow trout infected with Ia and turbot infected with Ib (Brudaseth *et al*., 2002, 2005). However, infected muskellunge had an equivocal lesion. Experimental reproduction of the disease by intraperitoneal injection in fathead minnow (*Pimphales promelas*), rainbow trout or walleye did not produce a notable vasculitis and heart lesions were minimal (Al-Hussinee *et al*., 2010, 2011b; Grice *et al*., 2016). Walleye that were bathed in formaldehyde, health checked

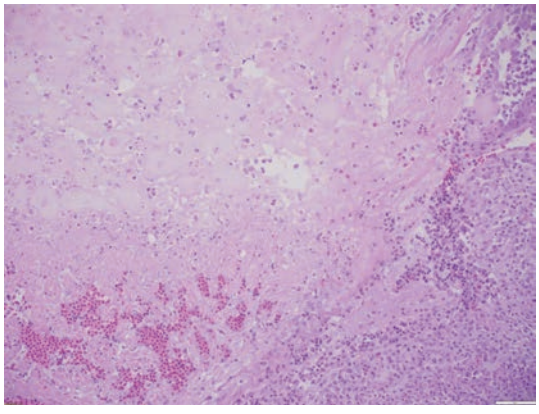

Fig. 3.1. Ventricle of a freshwater drum (*Aplodinotus grunniens*) infected with viral haemorrhagic septicaemia virus (VHSV). There is massive subacute necrosis and inflammation. The remnants of myocardial trabeculae are at bottom left. H&E (haematoxylin and eosin) staining. Bar = 50 µm.

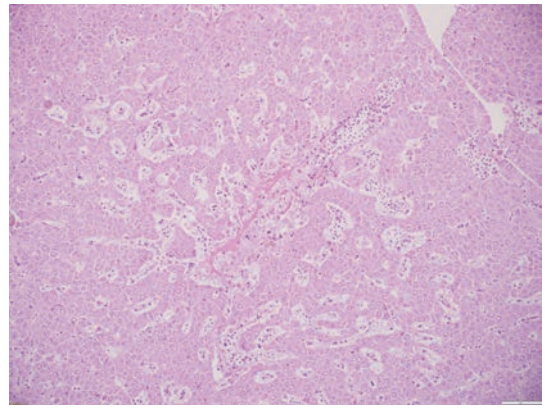

Fig. 3.2. Fibrinoid vasculitis in the liver of a freshwater drum (*Aplodinotus grunniens*) infected with viral haemorrhagic septicaemia virus (VHSV). The necrosis is centred on the venule, not the hepatocytes. H&E (haematoxylin and eosin) staining. Bar = 50 µm.

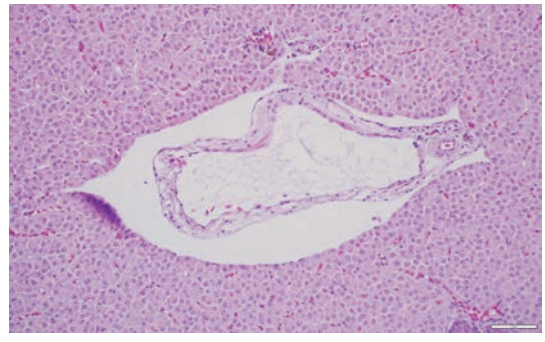

Fig. 3.3. Mild vasculitis in the liver of a freshwater drum (*Aplodinotus grunniens*) infected with viral haemorrhagic septicaemia virus (VHSV). There are a few necrotic cells within the wall of the vessel. H&E (haematoxylin and eosin) staining. Bar = 50 µm.

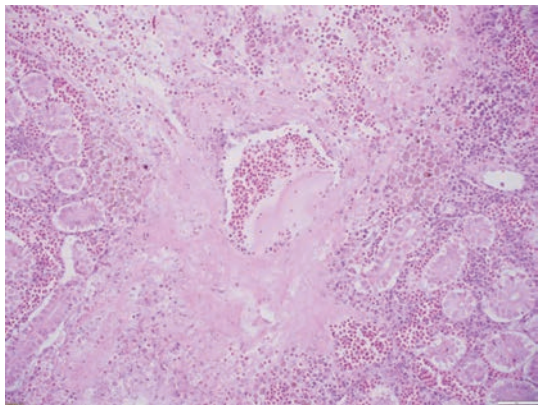

Fig. 3.4. Severe vasculitis in the kidney of a freshwater drum (*Aplodinotus grunniens*) infected with viral haemorrhagic septicaemia virus (VHSV). H&E (haematoxylin and eosin) staining. Bar = 50 µm.

and acclimatized to high quality 18°C well water developed VHS and columnaris shortly after injection with VHSV (Grice *et al*., 2016).

Histopathology can be complemented by immunolocalization of the virus (Evensen *et al*., 1994; Brudaseth *et al*., 2005; Al-Hussinee *et al*., 2010, 2011b; Grice *et al*., 2016). Co-localization of the virus with the majority of lesions using immunohistochemistry (IHC) solidifies the association of the virus with pathogenesis (Al-Hussinee *et al*., 2010, 2011b), but the sensitivity of this method is limited (Evensen *et al*., 1994). Additionally, RNA probes can be used to localize VHSV (Al-Hussinee *et al*., 2011a). The heart of freshwater drum with profound necrotizing myocardial lesions was surprisingly free of virus, at least as detected using IHC (Al-Hussinee *et al*., 2011b); this is in contrast to turbot, where the virus was readily identified in affected hearts (Brudaseth *et al*., 2005).

3.4 Pathophysiology, Pathogenesis and Virulence Factors

Aspects of the pathophysiology and pathogenesis of VHSV have been reviewed by Smail and Snow (2011). Briefly, genotype Ia is virulent in rainbow trout (Brudeseth *et al*., 2002, 2008), while marine Ib isolates are virulent in turbot (Brudeseth *et al*., 2005) but avirulent in rainbow trout (Brudaseth *et al*., 2008). Many Ib isolates were avirulent in rainbow trout challenged by immersion, but their virulence varied when the virus was injected (Skall *et al*., 2004). Genotype IVa was virulent in Pacific herring (*Clupea pallasi*) (Kocan *et al*., 1997), but rainbow trout were resistant to IVa (Meyers and Winton, 1995) and to IVb (Al-Hussinee *et al*., 2010; Kim and Faisal, 2010), although IVa showed better replication and cytopathic effect (CPE) in rainbow trout gill and splenic macrophage cells than IVb (Pham *et al*., 2013).

Horizontal transmission of VHSV via the gill has been detailed (see Brudeseth *et al*., 2008; Pham *et al*., 2013; Al-Hussinee *et al*., 2016). Viral replication (Ia) was detected early after infection in rainbow trout gill epithelium (Brudeseth *et al*., 2002), whereas the endothelium of the kidney was the site of first detection of Ib in turbot (Brudeseth *et al*., 2005) and the gill had virus only in a few fish, late in the infection. Several studies have correlated the capability of VHSV isolates to replicate in gill or skin epithelium with virulence. The avirulence of Ib in rainbow trout correlated with its inability to infect and translocate across the primary gill epithelial cells and to replicate in head kidney macrophages, in contrast to a rainbow trout-virulent Ia isolate (Brudeseth *et al*., 2008). A IVa isolate with low virulence in rainbow trout replicated to a limited degree in excised fin tissue but not in excised gill tissue (Yamamoto *et al*., 1992). Replication in excised fin tissue was correlated with resistance to waterborne infection in rainbow trout (Quillet *et al*., 2001).

The macrophage plays a significant role in the virulence and pathogenesis of VHSV. The virus is detected in macrophages and melanomacrophages following natural and experimental infections by genogroups Ia (Evensen *et al*., 1994; Brudeseth *et al*., 2002), Ib (Brudeseth *et al*., 2005) and IVb (Al-Hussinee *et al*., 2010, 2011b); it has also replicated in primary cultures of macrophages *in vitro* (see Brudeseth *et al*., 2008). Macrophages will phagocytose cells lysed by VHSV and, therefore, immunochemical detection of the virus inside macrophages may not indicate replication. Similarly, primary macrophage cultures of rainbow trout are heterogeneous and only a portion of the cells can support viral replication, even with a virulent Ia isolate (Tafalla *et al*., 1998; Brudeseth *et al*., 2008); hence, significant CPE may (Brudeseth *et al*., 2008) or may not occur (Tafalla *et al*., 1998). In the RTS11 cell line from rainbow trout splenic macrophages, replication of VHSV was aborted and

CPE did not occur for genotypes Ia, IVa and IVb isolates (Tafalla *et al*., 2008; Pham *et al*., 2013).

Much of this evidence was from experimental infections or *in vitro* studies; consequently, environmental factors that could predispose fish to mortality were excluded. Genotypes III and IVa are normally not highly virulent to salmonids; none the less, isolates from wild fish caused mortality in Atlantic salmon (*Salmo salar*) in net pens (Dale *et al*., 2009; Garver *et al*., 2013). The mechanism by which VHSV isolates adapt to new hosts is a critical question in the epizootiology of the virus. Five serial passages of a marine isolate in rainbow trout without intervening *in vitro* culture produced increased virulence, although there was no difference in the G gene sequence to explain the increased virulence (Snow and Cunningham, 2000). In a subsequent study, genotype Ib isolates that were >99.4% similar in the G amino acid sequence, but were of markedly different virulence in rainbow trout, were sequenced. Only four predicted amino acid substitutions were noted, one each in the N, G, NV and L proteins, which suggested that small genome mutations could induce large differences in virulence (Campbell *et al*., 2009).

Selective apoptosis or excessive stimulation of autophagy in tissues, e.g. the heart, could help to explain extensive cell death without large numbers of viruses. The prevention or delay of apoptosis via the generation of anti-apoptotic factors is a common strategy of viruses for enhancing their replication (Skaletskaya *et al*., 2001), but some viruses also stimulate apoptosis in late replication to promote viral spread (Hay and Kannourakis, 2002). Rainbow trout infected with VHSV had apoptotic cell death in the renal interstitium but not in the heart (Eleouet *et al*., 2001). The use of NV gene deficient and NV gene knockout recombinant VHSV demonstrated that the NV gene product has an anti-apoptotic effect during early viral replication, and that wild-type VHSV infection triggered apoptosis by inducing caspases (Ammayappan and Vakharia, 2011). In contrast, the matrix protein of IHNV may induce apoptosis (Chiou *et al*., 2000); such data for VHSV are lacking.

Autophagy is a conserved cellular process that involves autophagosome formation and degradation and the recycling of cellular constituents. The process also influences the immunity and survival of intracellular organisms (Deretic, 2011), including piscine viruses (Schiotz *et al*., 2010; García-Valtanen *et al*., 2014). Purified G proteins from SVCV and VHSV stimulated autophagosome formation, and VHSV-induced autophagy inhibited the replication of both viruses in zebrafish (*Danio rerio*) embryonic fibroblasts (García-Valtanen *et al*., 2014). Autophagy is primarily a pro-survival mechanism but, when excessive, it can cause cell death (Ouyang *et al*., 2012).

Immunosuppression due to environmental factors is commonly proposed to be a predisposing factor in VHS; however, VHSV itself can cause immunosuppression. Cell death by any mechanism, including viral lysis/necrosis in haematopoietic tissues, would contribute to immunosuppression. The NV protein has also been proposed to cause immunosuppression, resulting in the downregulation of many acquired and innate immune genes in the spleen and kidney (Chinchilla *et al*., 2015). The virulence of VHSV may be partially due to the NV protein that downregulates the interferon (IFN) response and tumour necrosis factor alpha (TNFα)-mediated nuclear factor (NF)-kβ activation (Kim and Kim, 2012, 2013).

3.5 Protective and Control Strategies

Several disinfecting methods kill VHSV in cold fresh water (see Bovo *et al*., 2005), including the use of UV irradiation, low and high pH and iodophors (disinfectants containing iodine complexed with a solubilizing agent); the latter is widely used for egg disinfection. The assessment of the risk factors, basic sanitation and the implementation of best practices markedly reduce the severity and scope of the disease; nevertheless, they must be in continual practice (see Gregory, 2008). Complete separation from the environment is the single best method to eliminate the transmission of VHSV. Fish in net pens or in land-based operations where surface water is used will always be at risk. In endemic areas, a risk-based approach is the most sensible and cost-effective method to predict outbreaks (Thrush and Peeler, 2013). Some details of appropriate control strategies are given in OIE (2015).

The selection of European rainbow trout with increased resistance to genotype Ia has been demonstrated by Dorson *et al*. (1995) and the resistance shows high heritability (Yanez *et al*., 2014). The identification of extant strains of fish that are VHSV resistant would be an inexpensive management option in endemic areas when operations cannot be isolated from contaminated water. Extant strains of walleye vary in their susceptibility

to experimental infection with genotype IVb (Grice *et al*., 2016). A quantitative trait locus (QTL) was associated with the survival of rainbow trout and innate immunity to VHSV (Verrier *et al*., 2013).

There are no commercial VHSV vaccines despite significant research (see Smail and Snow, 2011). This is partly due to the economic challenges of vaccinating small fish and also because VHSV in many locations requires eradication after confirmatory diagnosis (OIE, 2015). Numerous experimental DNA vaccines have been developed against rhaboviruses, including VHSV (Boudinot *et al*., 1998; Lorenzen *et al*., 2001; McLauclan *et al*., 2003; Sommerset *et al*., 2003; Chaves-Pozo *et al*., 2010a; Martínez-López *et al*., 2013; Sepúlveda and Lorenzen, 2016). The only commercially available DNA vaccine protects Atlantic salmon from IHNV in British Columbia, Canada; however, few of the fish at significant risk from VHSV compare with Atlantic salmon in value. The DNA experimental vaccines are based on VHSV glycoprotein G sequences, and immunity is directed at the protein responsible for cellular attachment (Bearzotti *et al*., 1999). DNA vaccines can confer long-lasting high relative percentage survival with a single injection. The advantage of DNA vaccines over conventional products is that they stimulate not only an antibody response but also cell-mediated and innate immunity, though DNA vaccines to VHSV do not always provide sterile immunity (Chaves-Pozo *et al*., 2010a; Sepúlveda and Lorenzen, 2016). Further, the neutralizing antibody response can be absent, either before it is seen to develop or after it has waned, yet protection is still provided (Lorenzen *et al*., 1998; McLauclan *et al*., 2003). Short-term non-specific protection against heterologous viruses is also afforded by VHSV DNA vaccines (Lorenzen *et al*., 2002; Sommerset *et al*., 2003) as the stimulation of innate immune effectors is vigorous (Byon *et al*., 2005; Cuesta and Tafalla, 2009).

Immunity to rhabdoviruses has recently been reviewed by Purcell *et al*. (2012) and Pereiro *et al*. (2016), while this chapter emphasizes aspects of innate immunity to VHSV. In rainbow trout infected with VHSV and IHNV, neutralizing antibody, when present, is protective and depends on complement (see Lorenzen and LaPatra, 1999; Lorenzen *et al*., 1999). Protection following intraperitoneal injection of a DNA vaccine induced IgT B cells (Martínez-López *et al*., 2013). Rainbow trout had splenic lymphocytes expressing IgM, IgD and IgT, and clonal expansion of IgM and IgT occurred following systemic VHSV infection (Castro *et al*., 2013). Research on cell-mediated immunity has made significant advances (see Laing and Hansen, 2011; Castro *et al*., 2011); nevertheless, functional evidence for T cell-mediated viral resistance – apart from cell-mediated cytotoxicity (CMC) – is sparse. Infection with VHSV and DNA vaccination induced the clonal expansion of T cells and selected for CDR3 (complementarity determining region 3)-based clonotypic T cell diversity (Boudinot *et al*., 2001, 2004). The upregulation of the T cell co-receptor CD3 (cluster of differentiation 3) and T cell activation in the skin of VHSV bath-infected rainbow trout was recently detected (Leal *et al*., 2016). Leucocytes from VHSV-infected rainbow trout demonstrated CMC to VHSV-infected MHC (major histocompatibility complex) I-matched but not xenogenic cells (Utke *et al*., 2007). Both CD8α (the α chain of CD8) and the natural killer (NK) cell enhancement factor were stimulated, although unexpectedly, the temporal response of the T cells preceded that of the NK cells. DNA vaccination with plasmids containing the VHSV genes for G and N stimulated CMC by peripheral blood leucocytes to VHSV-infected MHC I-matched cells, but only vaccination with the G gene also stimulated NK cells that killed xenogenic infected cells (Utke *et al*., 2008).

The induction of interferon and production of interferon-stimulated genes (ISGs) constitute a critical antiviral mechanism in fish (see Zou and Secombes, 2011), but the IFNs are complex. There are two types of IFN, type I and type II (IFNγ), and also two groups of type I IFNs, and many have multiple genes. Their influences on immunity and on rhabdoviruses vary (see Zou and Secombes, 2011; Purcell *et al*., 2012). Recombinant IFN I inhibits the replication of VHSV in rainbow trout ovary, and VHSV infection strongly stimulates an ovarian IFN response (Chaves-Pozo *et al*., 2010b) and several chemokine genes (Chaves-Pozo *et al*., 2010c). This vigorous response may explain the lack of vertical transmission of VHSV. Recently, intracellular IFNs were discovered in rainbow trout and their overexpression in RTG (rainbow trout gonad)-2 cells stimulated Mx protein, an indicator of alpha/beta interferon induction, and resulted in resistance to VHSV (Chang *et al*., 2013).

Numerous ISGs influence the VHSV response (see Verrier *et al*., 2011). The best characterized ISG in fish is Mx. Rainbow trout have three Mx isoforms that are differentially upregulated in

tissues in response to poly(I:C) (polyinosinic–polycytidylic acid, a synthetic analogue of double-stranded RNA) or VHSV (Tafalla *et al*., 2007). Mx protein was detected for 3 weeks at the site of injection following VHSV DNA vaccination of rainbow trout (Acosta *et al*., 2005) along with the upregulation of transcription of the genes for Toll-like receptor 9, TNFα and interleukin 6 in the head kidney and spleen (Ortega-Villaizan *et al*., 2009). The upregulation of Mx expression after vaccination with VHSV DNA was positively correlated with early protection (Boudinot *et al*.; 1998, McLauclan *et al*., 2003). The VHSV-induced gene *vig-1* was highly expressed in rainbow trout lymphoid tissues following VHSV DNA immunization (Boudinot *et al*., 1999). Vigs including *vig-1*, *vig-2*, *ISG15/vig-3* and chemokines were upregulated in VHSV-exposed leucocytes and were expressed in VHSV-infected rainbow trout (O'Farrell *et al*., 2002). Protein kinase R was implicated in preventing poly(I:C)-dependent increases in the mRNA transcription of other ISGs, but did not control viral transcription when rainbow trout cells were infected with VHSV (Tafalla *et al*., 2008). Overexpression of retinoic acid-inducible and mitochondrial antiviral signalling protein in salmonid cell lines resulted in protection from VHSV (Biacchesi *et al*., 2009). Suppressive subtractive hybridization of rainbow trout leucocytes that were VHSV or mock infected revealed that VHSV infection stimulated the genes for the finTRIM proteins (van der Aa *et al*., 2009), which are similar to the tripartite motif (TRIM) protein family, some of which are responsible for mammalian antiviral innate immunity. These and other gene families, e.g. the genes for the NOD (nucleotide-binding oligomerization domain)-like receptors, are likely to be associated with immunity to VHSV and await further characterization.

The production of nitric oxide (NO) was stimulated in turbot kidney macrophages following VHSV infection, and exogenous NO inhibited VHSV replication (Tafalla *et al*., 1999). VHSV infection of turbot macrophages also reduced the production of NO induced by TNFα or macrophage-activating factor (Tafalla *et al*., 2001). Other soluble factors associated with resistance to VHSV include antimicrobial peptides. There was differential expression of the genes for the antimicrobial proteins hepcidin-2 and nk-lysin in VHSV-susceptible and resistant families of turbot (Díaz-Rosales *et al*., 2012). VHSV infection stimulated recruitment and increased the natural cytotoxic cell-like activity of leucocytes in gilthead sea bream (*Sparus aurata*), along with the production of reactive oxygen intermediates and myeloperoxidase (Esteban *et al*., 2008).

The hepatic expression of serum amyloid A (SAA), an acute-phase reactant in fish, was stimulated by VHSV in rainbow trout (Rebl *et al*., 2009; Castro *et al*., 2014), and interleukin 6 (IL-6) was the likely stimulus for SAA (Castro *et al*., 2014). The expression of galectin 9, (a chemoattractant protein with functions linked to immunity), was upregulated in response to VHSV (O'Farrell *et al*., 2002), but evidence for fish lectins interacting with the virus is limited. Rainbow trout ladderlectin (but not intelectin), another lectin with a role in defence, bound to immobilized purified VHSV (Reid *et al*., 2011), though the downstream effects are unknown because ladderlectin cannot fix complement.

3.6 Conclusions and Suggestions for Future Research

The future is relatively bright for technologies such as DNA vaccination despite the regulatory barriers that exist in many countries. The DNA vaccine (Apex-IHN, Elanco) for protection from IHNV that is commercially available in Canada is an example of a case where the benefits of the vaccine justify the high cost of the technology. Atlantic salmon are a high-value product and IHNV is a substantial disease threat to this species (LaPatra *et al*., 2016). DNA vaccines for VHSV stimulate innate and acquired immunity and a prolonged duration of protection sufficient to protect later life stages, however, challenges for their use remain (see Holvold *et al*., 2014). Their wider application for VHSV needs to be explored, particularly in high-value species like muskellunge released for stocking. Improvements in delivery, the enhanced development of immunity using molecular adjuvants, a marked reduction in cost and easing of the regulatory restrictions are also needed. Unfortunately, some of these factors are to likely remain significant barriers in the future. Continued advances in knowledge of teleost immunity are needed, particularly of the interplay between innate and acquired immune systems and of cell-mediated immunity (CMI).

Management of the spread and impacts of VHSV will rely on traditional approaches, including biosecurity, testing and either eradication or some

method of restricting fish movement. Given the costs involved, some differentiation by regulatory authorities between the genotypes of VHSV would be welcome; this would need to occur at level of the OIE. For example, VHSV IVb is endemic in the Great Lakes and while occasional mortality events still occur, its impact is limited. Risk-based approaches based on the potential for exposure with annual or other testing is a practical approach that has been adopted by Canadian and other authorities. Thus, producers who wish to sell products internationally can establish a VHSV-free status within a VHSV endemic zone.

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4 Epizootic Haematopoietic Necrosis and European Catfish Virus

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4.1 Introduction

Epizootic haematopoietic necrosis (EHN) disease is restricted to Australia and is caused by the ranavirus *Epizootic haematopoietic necrosis virus* (EHNV). This systemic disease causes high mortality among naturally infected wild redfin perch (*Perca fluviatilis*), has an impact on farmed rainbow trout (*Oncorhynchus mykiss*) and can threaten populations of native Australian fishes (Whittington *et al*., 2010; Becker *et al*., 2013). *European catfish virus* (ECV) is a closely related ranavirus that was originally isolated from sheatfish (*Silurus glanis*) in Germany (Ahne *et al*., 1989). This virus causes high mortalities in sheatfish and black bullhead catfish (*Ameiurus melas*), and other economically important fishes may also be susceptible (Jensen *et al*., 2009, 2011). Each of these viruses causes a similar disease with non-specific clinical signs and pathology characterized by widespread systemic necrosis that is most noticeable in haematopoietic tissues (Whittington *et al*., 2010). Both diseases occur in wild fishes and in those in aquaculture. EHNV was the first finfish virus listed by the World Organisation for Animal Health (OIE) due to its virulence, lack of host specificity, geographic isolation and the existence of a reliable diagnostic test (OIE, 2015). Additionally, ranaviruses that infect amphibians are listed by the OIE because they have economic and ecological impacts (OIE, 2016a) and also the potential for interclass transmission (Brenes *et al*., 2014). Experimental evidence indicates that a single fish species can be susceptible to multiple ranaviral species in addition to EHNV and ECV (Jensen *et al*., 2009). This chapter describes EHNV and ECV, which belong to a group of emerging pathogens – all of which are relevant to fish health – for which understanding of their epizootiology is incomplete (Gray *et al*., 2009).

4.1.1 Classification

EHNV and ECV belong to the genus *Ranavirus* (Jancovich *et al*., 2012) in the family *Iridoviridae*. Like all viruses in this family, they are large double-stranded DNA (dsDNA) viruses that undergo a complex nucleocytoplasmic replication cycle (Williams *et al*., 2005). The family *Iridoviridae* includes numerous virus species that cause diseases of fish in aquaculture (Whittington *et al*., 2010).

The five ranavirus lineages described by Jancovich *et al*. (2015) provide a framework for ranaviruses (Fig. 4.1). EHNV and ECV, together with the amphibian pathogen *Ambystoma tigrinum virus* (ATV) form a group in lineage 3. Each lineage contains pathogens of fish, and among these closely related viruses there are amphibian pathogens that also cause disease in fish (Waltzek *et al*., 2014).

The genomes of EHNV and ECV contain approximately 127 kilobases (Jancovich *et al*., 2010; Mavian *et al*., 2012) and the dsDNA is circularly permuted and terminally redundant (Jancovich *et al*., 2012). The guanine/cytosine (G/C) content is 54–55% and there is approximately 25% cytosine methylation (Chinchar *et al*., 2011). The genome of EHNV has 88% nucleotide sequence identity with that of the ESV (European

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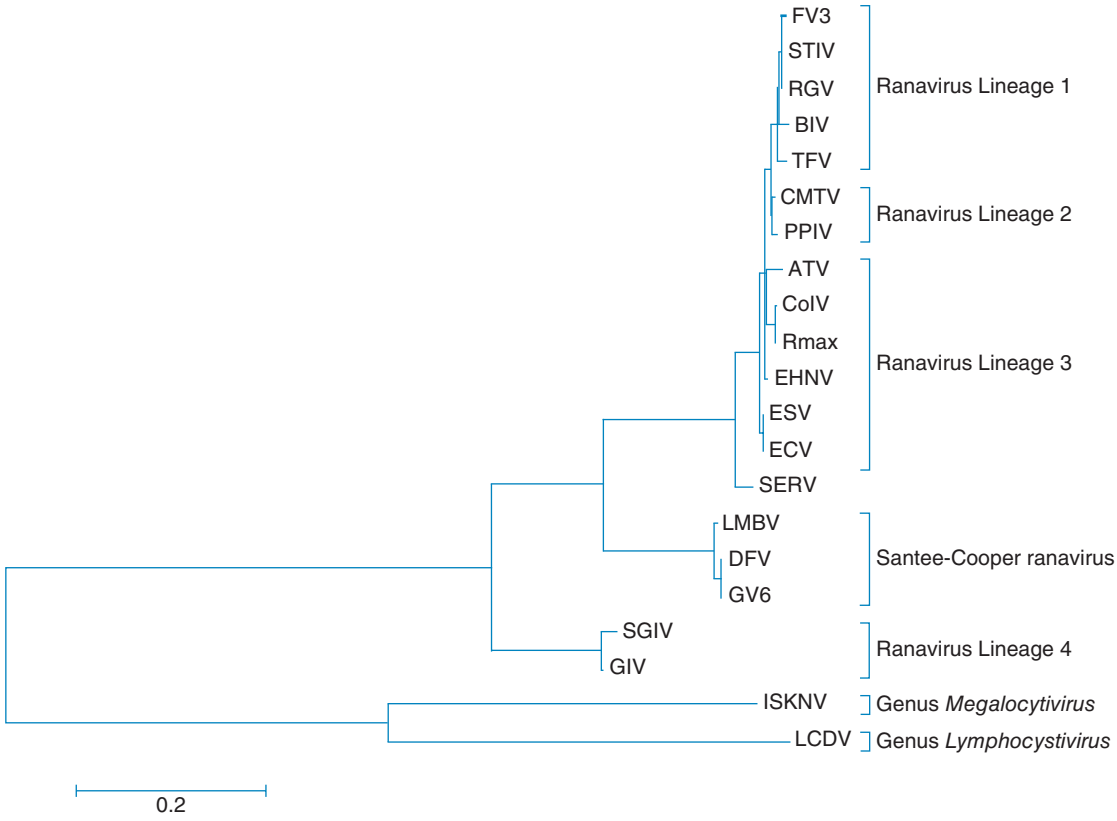

Fig. 4.1. *Epizootic haematopoietic necrosis virus* (EHNV) and *European catfish virus* (ECV) group in ranavirus Lineage 3 (Jancovich *et al*., 2015). Molecular phylogenetics were calculated using 1127 base positions of the major capsid protein gene by the maximum likelihood method in Mega6 (Tamura *et al*., 2013). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Abbreviations (Lineage: virus name, Genbank reference): Lineage 1: FV3 (*Frog virus 3*, AY548484); STIV (soft-shelled turtle iridovirus, EU627010); RGV (Rana grylio iridovirus, JQ654586); BIV (*Bohle iridovirus*, AY187046); TFV (tiger frog virus, AF389451). Lineage 2: CMTV (Common midwife toad virus, JQ231222); PPIV (pike-perch iridovirus, FJ358610). Lineage 3: ATV (*Ambystoma tigrinum virus*, AY150217); CoIV (cod iridovirus, GU391284); Rmax (Ranavirus maxima, GU391285); EHNV (*Epizootic haematopoietic necrosis virus*, AY187045); ESV (European sheatfish virus, FJ358609); ECV (*European catfish virus*, FJ358608). SERV (short-finned eel ranavirus, FJ358612). Isolates of *Santee-Cooper ranavirus*: LMBV (largemouth bass ranavirus, FR682503); DFV (doctor fish virus, FR677324); GV6 (guppy virus 6, FR677325). Lineage 4: SGIV (Singapore grouper iridovirus, AY521625); GIV (grouper iridovirus, AY666015). Type species: genus *Megalocytivirus*, ISKNV (*Infectious spleen and kidney necrosis virus*, NC003494); genus *Lymphocystivirus*, LCDV (*Lymphocystis disease virus 1*, NC001824). Adapted from Jancovich *et al*. (2015).

sheatfish virus) isolate of ECV and the genome structure is very similar. These distinct but closely related species have a similar complement of approximately 100 genes; however, the function of many of the predicted proteins is not known (Grayfer *et al*., 2012). Molecular evolutionary analyses suggest that all ranaviruses might have evolved following relatively recent host shifts from an ancestral fish virus (Jancovich *et al*., 2010). On this basis, new viruses may emerge or be detected and the diagnostic criteria for EHNV and ECV in the future may require consideration of whole genome sequence data.

4.1.2 Structure and replication

The capsids of EHNV and ECV are 150–160 nm in diameter with the icosahedral symmetry that is

common to all iridovirids (Ahne *et al*., 1989; Eaton *et al*., 1991). The major capsid protein (MCP) makes up 40% of a complex virion composed of 36 proteins with structural and enzymatic activities (Chinchar *et al*., 2011). An external lipid and glycoprotein membrane is not essential for infectivity, but appears to enhance it (Ariel *et al*., 1995; Jancovich *et al*., 2012). The unique nuclear and cytoplasmic replication strategy elucidated for the type species, *Frog virus 3* (FV3), has been reviewed (Chinchar *et al*., 2011). There is a coordinated expression of early, delayed early and late genes (Teng *et al*., 2008). Replication of the genome occurs initially in the nucleus followed by the generation of concatermeric copies of the genome and structural proteins in the cytoplasm in morphologically distinct virogenic stroma for the assembly of virions (Eaton *et al*., 1991). EHNV is released by budding from the cell membrane, where an outer envelope is acquired, resulting in virions up to 200 nm in diameter. Alternatively, naked virions that can be released by cell lysis may aggregate in paracrystalline arrays in the cytoplasm (Eaton *et al*., 1991).

4.1.3 Transmission

Further studies on the transmission of EHNV and ECV are required to explain recurrent outbreaks of disease. Vertical transmission has not been reported (Whittington *et al*., 2010). Horizontal transmission of EHNV and ECV occurs via water or the ingestion of tissues from infected fish (Langdon, 1989; Jensen *et al*., 2009; Gobbo *et al*., 2010, Jensen *et al*., 2011; Becker *et al*., 2013; Leimbach *et al*., 2014). Recurrence of EHN in rainbow trout was attributed to reinfection from wild redfin perch (Whittington *et al*., 1996; Marsh *et al*., 2002), and ESV may persist in apparently healthy fish (Ahne *et al*., 1991).

EHNV has been spread from populations of farmed rainbow trout in which disease was active at low prevalence, but practically inapparent (Whittington *et al*., 1999). Fish with subclinical infections are likely to be a source of the virus. EHNV was isolated from apparently healthy redfin perch that survived a natural disease outbreak, suggesting a possible reservoir, though the duration of infection is not known (Langdon and Humphrey, 1987). This suggestion is supported by the re-isolation of infectious virus from apparently healthy fish surviving experimental challenges (Langdon, 1989; Jensen *et al*., 2009, 2011; Gobbo *et al*., 2010; Becker *et al*., 2013; Leimbach *et al*., 2014). These studies also provide evidence that hosts other than those that express disease may be a source of EHNV and ECV. There is no evidence of an amphibian reservoir, although there may be one because of the very broad host range and the capacity for inter-class transmission of ranaviruses (Chinchar and Waltzek, 2014).

The spread of EHNV via fomites or persistence outside a host is also possible because EHNV and similar ranaviruses are extremely stable in the environment under certain conditions. EHNV retained its infective titre for 97 days at 15°C and 300 days at 4°C. The virus was resistant to desiccation, retaining infectivity after drying for 113 days at 15°C (Langdon, 1989), and remained infective in frozen fish for at least 2 years (Whittington *et al*., 1996). Infectivity of a related ranavirus, *Bohle iridovirus* (BIV) declined at 44°C and further declined at 52°C, but treatment at 58°C for 30 min was required for complete loss of infectivity (La Fauce *et al*., 2012).

There is evidence that EHNV has spread by human activity, including the movement of infected rainbow trout fingerlings between farms (Langdon *et al*., 1988; Whittington *et al*., 1994, 1999). Disease outbreaks in redfin perch have occurred, with the progressive spread of EHNV in inland river systems such as the Murrumbidgee River in New South Wales and the Australian Capital Territory, and the Murray River in South Australia (Whittington *et al*., 2010). This may be due to recreational fishing through illegal relocation of redfin perch, the use of frozen redfin perch as bait, or on fomites such as boats and fishing equipment. Birds may also spread EHNV by regurgitating ingesta within a few hours of feeding on EHNV-infected carcasses (Whittington *et al*., 1996).

4.1.4 Geographic distribution

EHNV is found in Australia, while ECV is restricted to continental Europe (Whittington *et al*., 2010). Within Australia, EHNV has a discontinuous distribution over time and throughout freshwater lakes, rivers and impoundments in south-eastern Australia. Its geographic range does not include the entire range of the known susceptible host species (Whittington *et al*., 2010). Only a small number of rainbow trout farms are infected and these are restricted to two water catchment regions, the Shoalhaven River and the Murrumbidgee River

(Langdon *et al*., 1988, Whittington *et al*., 1999). Wild trout stocks, which are present in south-eastern Australia, have not been surveyed for the infection (Whittington *et al*., 1999). Similarly, the farm-level prevalence of EHNV is unclear because not all farms have been tested. The virus has not been detected based on regular surveillance in salmonids in Tasmania and Western Australia.

The first reported outbreak of disease caused by ECV (sheatfish strain) occurred in sheatfish fry from south-east Europe that were reared in a warm-water aquaculture facility in Germany (Ahne *et al*., 1989). Subsequently, the disease has been shown to occur in catfish in France, Italy, Hungary and Poland. Outbreaks have occurred in catfish reared in aquaculture facilities in Italy (Bovo *et al*., 1993) and among brown bullheads (*A. nebulosus*) in Hungary (Juhasz *et al*., 2013) and sheatfish in Poland (Borzym *et al*., 2015). In France, catfish of all sizes in natural lakes were affected during summer months. The pathogen identified in France in 2007 closely resembled that which caused disease in wild catfish 15 years previously (Bigarre *et al*., 2008).

EHNV or ECV could spread to new areas where susceptible hosts are present in permissive environments (Peeler *et al*., 2009). The international trade and the movements of ornamental fish, reptiles and amphibians, and bait have been responsible for the distribution of some ranaviruses and have had serious impacts on populations of naive host species (Jancovich *et al*., 2005; Whittington and Chong, 2007). EHNV is listed by the OIE (2016c) to reduce further impacts on aquaculture and wild fish reduced because of its broad host range and ability to adapt to environmental conditions. Surveillance and disease control zones are important in disease control. The international spread of EHNV has not been documented and a survey by Vesely *et al*. (2011) indicated that ranavirus was not detected in ornamental fish imported into Europe.

4.1.5 Impact of the pathogen on fish production

Recurrent outbreaks of disease occur in wild fish in natural environments and in aquaculture facilities (Ahne *et al*., 1991; Whittington *et al*., 2010). However, EHNV is an infrequent cause of mass mortality in wild redfin perch. All age classes may be affected, but in endemic areas mortality is limited to fingerling and juvenile fish (Langdon *et al*., 1986; Langdon and Humphrey, 1987; Whittington *et al*., 1996); this decline in the fish population has impacts on the recreational fishery (Whittington *et al*., 2010). In contrast to mortality up to 95% in wild redfin perch, the impact of the disease on farmed rainbow trout is less than 5% of the farmed populations (Whittington *et al*., 2010).

Disease caused by ECV also occurs infrequently, but can be recurrent at individual aquaculture facilities. Natural infections of sheatfish have caused 100% mortality in fingerlings and 10–30% mortality in older fish (Ahne *et al*., 1991; Borzym *et al*., 2015). Outbreaks in wild catfish caused high mortality of *A. melas* in French lakes, whereas other fishes were not affected (Bigarre *et al*., 2008). The few documented cases of disease in reared catfish affected fish that included brood stocks (Bovo *et al*., 1993; Juhasz *et al*., 2013). Due to compliance with the European Union (EU) regulations and OIE guidelines on surveillance and reporting (OIE, 2010), EHNV is not found in Europe and the occasional outbreak of ECV disease is well documented.

Disease caused by natural EHNV or ECV infections is known only from a limited number of fish species, but many more species are susceptible in experimental infections (Langdon, 1989; Jensen *et al*., 2009, 2011; Gobbo *et al*., 2010; Becker *et al*., 2013; Leimbach *et al*., 2014). These pathogens therefore have the potential to have a serious impact on wild fish, ecosystems and emerging aquaculture industries. It is important to note that such experimental trials do not provide an estimate of the likelihood or impact of a natural disease outbreak, owing to the complexity of the host and environmental factors that can interact with the pathogen.

4.2 Diagnosis

Disease caused by EHNV or ECV is diagnosed using standard histological techniques with haematoxylin and eosin staining together with confirmation of the presence of the virus. The association of the virus with cellular pathology can be demonstrated using polyclonal antibodies developed against EHNV for immunohistochemical or immunofluorescent staining of fixed tissue sections (Reddacliff and Whittington, 1996; Bigarre *et al*., 2008; Jensen *et al*., 2009). These antibodies, which are available from the OIE Reference Laboratory for EHNV, cross-react with the immunodominant major capsid protein of all ranaviruses so that molecular characterization is required to determine

the viral species (Hyatt *et al*., 2000, OIE, 2016b). Electron microscopy can demonstrate the presence of iridovirids in tissue; however, EHNV and ECV are indistinguishable from each other and from other ranaviruses (Ahne *et al*., 1998). Tests for the viruses can be applied to tissues from clinical cases. These include viral isolation in cell culture, antigen detection using immunological techniques such as enzyme-linked immunosorbent assays (ELISA) and, most commonly, the detection of specific nucleic acid sequence using polymerase chain reaction (PCR) (Whittington *et al*., 2010; OIE, 2016b).

For the purpose of preventing spread of the pathogen, the OIE recognizes infection with EHNV rather than the occurrence of clinical disease or pathological changes. Protocols for sensitive laboratory assays with the highest level of validation are detailed in the OIE *Manual of Diagnostic Tests for Aquatic Animals* for the detection of subclinical infection (OIE, 2016b). Ideally, these tests should be undertaken in laboratories accredited according to international standards for quality control (ISO, 2005). The appropriate samples to test are the viscera; kidneys and the livers and spleens from individual fish are commonly pooled (Whittington and Steiner, 1993). Milt and ovarian fluid are not suitable samples. Tissues can be processed rapidly using the partially automated procedures of bead beating for tissue disruption (this is compatible with both viral isolation and PCR) and magnetic bead based nucleic acid purification for molecular tests (Rimmer *et al*., 2012).

A statistically valid framework for calculating the sample size and selection method is required when undertaking surveillance for freedom from infection (OIE, 2010). Moribund fish are used for the diagnosis of disease, while biased or targeted sampling is recommended for surveillance. The latter is achieved on rainbow trout farms by freezing dead juvenile fish for later testing (Whittington *et al*., 1999).

Viral isolation is the only method of identifying infectious virus. EHNV can be isolated between 15 and 22°C in several fish cell lines, including fathead minnow (FHM), rainbow trout gonad (RTG), bluegill fry (BF-2), and Chinook salmon embryo (CHSE-214) cells (Langdon *et al*., 1986; Crane *et al*., 2005; OIE, 2016b). Similarly, ECV and ESV can be isolated using BF-2 cells or epithelioma papulosum cyprinid (EPC) cells, FHM or channel catfish ovary (CCO) cells (Ahne *et al*., 1989; Pozet *et al*., 1992; Ariel *et al*., 2009). Confirmation of the identity of a virus that can be passaged and causes a cytopathic effect requires immunofluorescent staining (Bigarre *et al*., 2008) or molecular characterization, typically of the MCP gene.

PCR assays most commonly target the MCP gene (Marsh *et al*., 2002; Pallister *et al*., 2007). A conventional PCR assay described in the OIE *Manual of Diagnostic Tests for Aquatic Animals* provides an amplicon that will distinguish EHNV, ECV and other ranaviruses using restriction enzyme digestion (Marsh *et al*., 2002; OIE, 2016b). The real-time quantitative PCR (qPCR) assay described by Jaramillo *et al*. (2012) is convenient and supported by validation data on diagnostic sensitivity and specificity. Polyclonal antibodies that react with EHNV can be applied as an antigen capture ELISA on tissue homogenate samples to provide a lower cost approach to the identification of ranavirus antigen; the antibody reagents and controls can be supplied by the OIE reference laboratory for EHNV (Whittington and Steiner, 1993).

The MCP gene is conserved among ranaviruses, with the sequence identity of EHNV, ECV and BIV being >97.8% (Hossain *et al*., 2008; Jancovich *et al*., 2012). The identification of isolates to species level requires DNA sequencing, restriction enzyme digestion (Marsh *et al*., 2002) or species-specific PCR assays (Pallister *et al*., 2007). Nucleotide sequence analysis improves the diagnosis because isolates cluster in epidemiological events, thereby providing insights for disease control and prevention (Jancovich *et al*., 2010). The nucleotide sequence of the DNA polymerase and neurofilament triplet H1-like protein genes can be used in addition to MCP (Holopainen *et al*., 2009). However, in the future, whole genome sequencing is likely to become more accessible and replace candidate gene analysis for detailed epidemiological studies of ranaviruses (Epstein and Storfer, 2015).

4.2.1 Clinical signs

The clinical signs of disease caused by EHNV and ECV are non-specific and include loss of appetite, reduced activity, ataxia and multifocal haemorrhage, particularly in haematopoietic tissue (Whittington *et al*., 2010). Outbreaks of EHN in wild redfin perch are typically described only as mass mortality (Langdon and Humphrey, 1987). Experimentally infected fish have a dark coloration and show erratic swimming preceding death (Langdon, 1989). Natural disease in rainbow trout

may not be evident because of its low prevalence. In an experimental setting, infected rainbow trout had dark coloration, ataxia and reduced appetite (Reddacliff and Whittington, 1996).

Similarly, outbreaks of ranavirus in wild catfish were evident only as mortality (Bigarre *et al*., 2008). Infected catfish in a culture facility had oedema, petechia of fin bases and viscera, ascites and gill pallor (Pozet *et al*., 1992). Loss of appetite preceded gross signs of disease by several days. In experimental infections, moribund catfish assumed a vertical position with head above water and petechia of the skin and fin bases (Gobbo *et al*., 2010). Disease in sheatfish was sometimes complicated by secondary bacterial infection (*Aeromonas hydrophila*) and there were skin ulcerations on dead and moribund fish (Ahne *et al*., 1989, 1991). No obvious clinical signs of disease were noted in experimental infections, but diffuse subcutaneous haemorrhage in the region of the lower jaw as well as in the fins was found at necropsy (Leimbach *et al*., 2014).

4.3 Pathology

Lesions are attributed to the vascular endothelial and haematopoietic tropism of the pathogen and are similar in all susceptible hosts. EHNV and ECV cause very similar pathology, with widespread systemic necrosis, especially in haematopoietic tissues (Whittington *et al*., 2010). Grossly, there are petechial haemorrhages at the base of fins, excessive amounts of serosanguinous peritoneal fluid and swelling of kidney and spleen (Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). Often, the foci of necrosis are centred around small blood vessels. White to cream foci evident grossly in the liver of redfin perch correspond to necrotic foci. Microscopically, at the margins of necrotic foci in the liver, basophilic intracytoplasmic inclusion bodies may be found in hepatocytes (Fig. 4.2). These inclusion bodies are scarce and difficult to visualize in the kidney and spleen. Immunohistochemical stains reveal widespread localization of EHNV antigen in necrotic areas as well as in the endothelium and in individual circulating leucocytes within blood vessels (Fig. 4.3).

Microscopic lesions in experimentally infected sheatfish and catfish with isolates of ECV were characterized by necrosis of the haematopoietic tissues of the spleen and kidney with degenerate and necrotic cells in the interstitial tissue and tubules of the kidney (Ogawa *et al*., 1990; Pozet *et al*., 1992). A generalized destruction of vascular endothelial cells in both types of fish was also evident, which resulted in diffuse congestion and haemorrhage in the internal organs. As for EHNV, viral antigen can be demonstrated associated with the lesions using

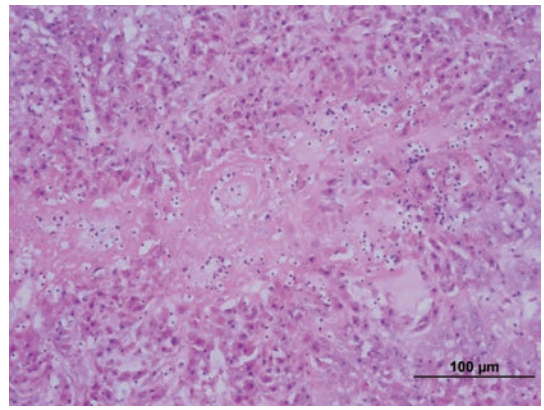

Fig. 4.2. Advanced focal necrosis in the liver of redfin perch. There is widespread dissociation of hepatocytes, many of which are in early stages of degeneration and contain amphophilic intracytoplasmic inclusion bodies. A liver with lesions of this type appears grossly to be darkly discoloured with multiple pale white spots. Stained with haematoxylin and eosin.

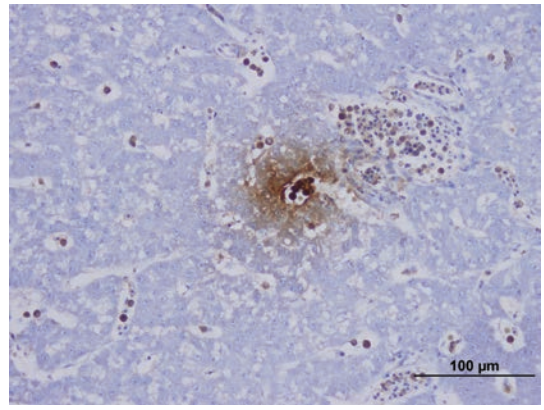

Fig. 4.3. Early focal necrosis in the liver of redfin perch. There is positive staining for EHNV (*Epizootic haematopoietic necrosis virus*) antigen in a focus of hepatocellular degeneration and necrosis adjacent to a large vessel. There is also staining of individual leucocytes within vessels and sinusoids, as well as of endothelial elements. Stained with an immunohistochemistry stain.

indirect fluorescent antibody or immunohistochemical stains (Figs 4.4 and 4.5).

There are often scattered, individual necrotic cells within blood vessels and degenerate vascular endothelial cells. Other lesions include diffuse splenic necrosis, gastrointestinal epithelial necrosis, necrosis of atrial trabeculae, hyperplasia and necrosis of branchial epithelium, focal pancreatic necrosis, oedema and necrosis of the swim bladder and ulcerative dermatitis (Langdon and Humphrey, 1987; Reddacliff and Whittington, 1996). Perivascular mononuclear cell infiltrates may be found in survivors (Becker *et al*., 2013).

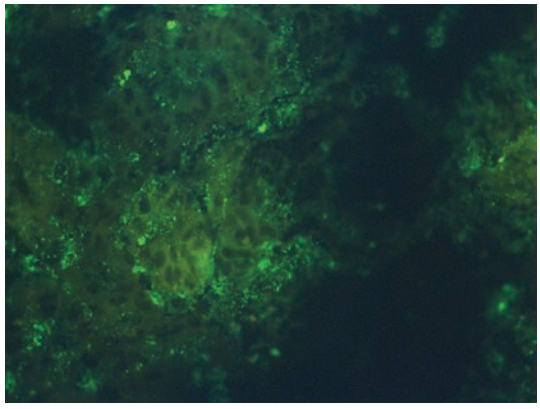

Fig. 4.4. Kidney of black bullhead infected with ECV (European catfish virus). There is bright apple green fluorescence associated with viral antigen in the cells. Stained with indirect fluorescent antibody. Image courtesy of Gobbo F. and Bovo G., Istituto Zooprofilattico Sperimentale delle Venezie, Italy.

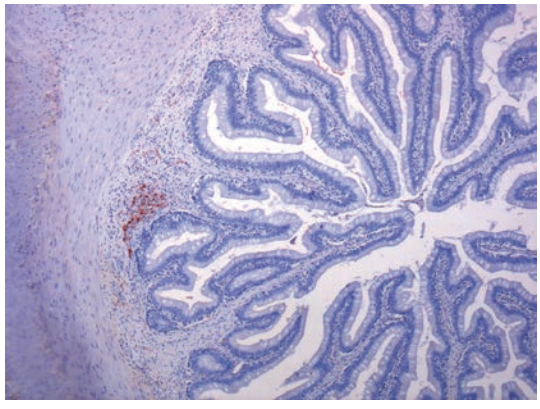

Fig. 4.5. Gut of black bullhead infected with ECV (European catfish virus). There is a large focus of cells containing ECV antigen in the submucosa. Stained with an immunohistochemistry stain. Image courtesy of Gobbo F. and Bovo G., Istituto Zooprofilattico Sperimentale delle Venezie, Italy.

Fishes susceptible to EHNV or ECV only by experimental infection include several native Australian freshwater species as well as pike (*Esox lucius*) and pike-perch (*Sander lucioperca*). The pathology of experimentally infected fishes was similar to that in naturally infected fishes (Jensen *et al*., 2009, 2011; Becker *et al*., 2013).

4.3.1 Pathophysiology

The pathogenesis of EHNV and ECV infection has not been studied in detail. Natural outbreaks of disease occur during warmer periods when large numbers of juveniles are present (Langdon *et al*., 1988; Whittington *et al*., 1994; Bigarre *et al*., 2008, Gobbo *et al*., 2010). Experimental studies have confirmed that transmission and pathogenicity are influenced by water temperature (Whittington and Reddacliff, 1995; Jensen *et al*., 2009, 2011; Gobbo *et al*., 2010; Leimbach *et al*., 2014). Mortality occurred in redfin perch at 12 to 21°C. Disease did not occur below 12°C and an increase in water temperature did not lead to disease in these fish. Rainbow trout were refractory to EHNV bath challenges at 10 and 15°C, with moderate mortalities (14–24%) occurring at 20°C (Langdon, 1989; Whittington and Reddacliff, 1995; Ariel and Jensen, 2009). The incubation period was negatively correlated with water temperature: in redfin perch, it was 10–28 days at 12–18°C compared with 10–11 days at 19–21°C; in rainbow trout, it was 14–32 days at 8–10°C compared with 3–10 days at 19–21°C following intraperitoneal (IP) challenge (Whittington and Reddacliff, 1995). Temperature-dependent replication of EHNV was demonstrated in pike with a peak viral load 7 days post bath challenge at 12°C and after only 3 days at 22°C (Holopainen *et al*., 2011).

Host differences in susceptibility may exist in redfin perch due to different genetics and culture conditions. Australian stocks were highly susceptible to challenge by bath and IP injection, with 100% mortality for both routes at 20°C, and 50% mortality induced by IP injection at 10°C (Langdon, 1989; Whittington and Reddacliff, 1995). Challenge trials of European redfin perch with EHNV via bath exposure did not induce mortality at 10, 15 or 20°C, and IP inoculation induced 47–80% mortality

at 15°C and 28–42% mortality at 20°C (Ariel and Jensen, 2009). In another trial, mortality in juvenile redfin perch was 16% at 11–13°C and 24% at 20–22°C following bath exposure to EHNV (Borzym and Maj-Paluch, 2015). Experimental infections indicated different disease outcomes for EHNV and different isolates of ECV and ESV in the same host species (Jensen *et al*., 2009; Gobbo *et al*., 2010; Leimbach *et al*., 2014). ESV from sheatfish was highly pathogenic to sheatfish, with 100% mortality at 15°C, while ECV only induced 8% mortality in this species (Leimbach *et al*., 2014). Conversely, ECV from catfish was pathogenic to catfish with 82% mortality at 25°C and 30% mortality at 15°C, but this isolate only induced 5 or 1% mortality in sheatfish at these temperatures (Gobbo *et al*., 2010). These findings indicate that although the two ECV isolates were genetically similar, they acted differently in different host species. The dose of virus required to initiate an infection depends on the host species. For instance, Australian bred redfin perch are highly susceptible to EHNV with doses as low as 0.08 $TCID_{50}$ (50% tissue culture infectious dose)/ml by bath exposure, whereas rainbow trout are resistant to bath exposure ($10^{2.2}$ $TCID_{50}$/ml) and IP infection is required for disease expression (Whittington and Reddacliff, 1995). These findings match the divergent clinical presentation of these species in nature.

The prominence of gastrointestinal lesions in fish naturally infected with EHNV and ECV (Ogawa *et al*., 1990; Pozet *et al*., 1992) (Fig. 4.5) is consistent with an oral route of infection (Reddacliff and Whittington, 1996). Viraemia is likely to result in dispersal of the virus, which is reflected in necrosis of circulating leucocytes and vascular endothelium. These processes lead to tissue necrosis at small and large scales, and to the signs of gross pathology (Langdon and Humphrey, 1987; Langdon *et al*., 1988; Whittington *et al*., 1994; Reddacliff and Whittington, 1996).

Currently, there is no evidence of natural infection of any species other than redfin perch or rainbow trout with EHNV, and of catfish or sheatfish with ECV and ESV. However, a number of fish species are susceptible to experimental infection. Ranaviruses have a global distribution and a few viruses that primarily infect amphibians are capable of inter-class transmission (natural or experimental), including to fish (Moody and Owens, 1994; Ariel and Owens, 1997; Duffus *et al*., 2015). For example, seemingly identical ranaviruses were isolated from sympatric, free-living, clinically affected, three-spine stickleback (*Gasterostelus aculeatus*) and red-legged frog (*Rana aurora*) tadpoles (Mao *et al*., 1999). Nevertheless, reports of disease in fish caused by amphibian-associated ranaviruses are scarce (Waltzek *et al*., 2014), with only limited data on the non-piscine host range of EHNV and ECV. The European common frog (*R. temporaria*) is susceptible to the fish ranavirus, pike-perch iridovirus (PPIV), but not to EHNV and ECV (Bayley *et al*., 2013). Thus, experimental evidence and field observations provide support for and against a model in which reservoir species and accidental hosts exchange ranavirus infection.

4.4 Protective and Control Strategies

There is no effective therapy for diseases caused by EHNV and ECV. Therefore, biosecurity measures derived from general best practice recommendations are the focus of disease control (OIE, 2009). These measures include the importation of fish certified free from infection, disinfection of water and control of vectors in endemic regions. Quarantine of incoming fish should be for at least as long as the disease incubation period, which in the most susceptible species can be 32 days (Whittington and Reddacliff, 1995). Active surveillance can be conducted to identify zones that are free from infection to facilitate the trade of live fish (OIE, 2010). Methods for targeted surveillance of aquaculture facilities for pathogens including EHNV are prescribed for the EU (Commission of the European Communities, 2001). Passive surveillance of unusual mortality in wild fish with laboratory tests to identify outbreaks of EHN or ECV disease can be undertaken.

4.4.1 Vaccination

Vaccines are not available for EHNV and there has been limited investigation of the efficacy of alternative prevention measures. Vaccine development is feasible and is practised commercially for iridoviruses in the genus *Megalocytivirus* (Kurita and Nakajima, 2012). Methods to detect a specific EHNV antibody response are available, although further investigation of the immune response is required (Whittington and Reddacliff, 1995; Whittington *et al*., 1999).

4.4.2 Control or elimination of reservoir hosts

Exposure to apparently healthy carrier fish, including survivors of disease outbreaks and potential carrier species, should be prevented. With increasing evidence for a reservoir of many ranaviruses in different taxonomic classes, aquaculture biosecurity requires the rigorous exclusion of non-cultured species from farms.

4.4.3 Environmental modifications to interrupt transmission

The wide thermal range for the disease limits the applicability of temperature manipulation. Mortality might be reduced in cooler water through behavioural self-selection, as in adult redfin perch, which avoid the disease in thermally stratified environments, while juvenile fish succumb to the disease due to feeding in warmer shallow water (Whittington and Reddacliff, 1995).

The importance of the continuous disinfection of intake water is supported by EHNV infection models in which transmission between redfin perch and rainbow trout occurred without direct contact (Langdon, 1989; Ariel and Jensen, 2009). Carryover contamination from an outbreak can be prevented by adequate cleaning and disinfection of aquaculture facilities. General guidelines for decontamination provided by the OIE (2009) can be interpreted using disinfection efficacy data specific for EHNV and related ranaviruses: sodium hypochlorite 200 mg/l; 70% ethanol; 150 mg/l chlorhexidine (0.75% Nolvasan®) for 1 min; 200 mg/l potassium peroxymonosulfate (1% Virkon®); or heating to 60°C for 15 min (Langdon, 1989; Bryan *et al*., 2009, La Fauce *et al*., 2012).

4.4.4 Animal husbandry practices

Optimal husbandry in aquaculture and the maintenance of healthy waterways for wild fish can reduce the impacts of disease in endemic regions. Lower stocking rates and improved water quality reduced mortality in farmed rainbow trout (Whittington *et al*., 1994, 1999). Efforts to diagnose and control bacterial and external parasitic infections are likely to improve disease outcome due to EHNV and ECV (Ahne *et al*., 1991; Whittington *et al*., 2010).

4.5 Conclusions and Suggestions for Future Research

EHNV and ECV cause infrequent but spectacular disease in a few freshwater fishes that are important to aquaculture and wild fisheries. The disease is readily diagnosed using routine histopathological methods together with laboratory tests specific for the pathogens. Surveillance for the viruses and the application of effective biosecurity are the mainstays of disease control because effective treatments and preventive measures are not available. For this reason, the diseases are notifiable in many jurisdictions and EHNV is listed by the OIE to regulate the trade of fish between disease control zones. This is facilitated by an international reference laboratory for EHNV, by recommended and validated laboratory tests and by the international availability of reagents, controls and recommended laboratory protocols (OIE, 2016b). Tests are available for application in high-throughput formats suitable for the certification of freedom from infection at population level.

Experimental studies have demonstrated that EHNV and ECV have the potential to affect many fishes, including those important to food production and conservation. Additionally, the very broad host range of many ranaviruses indicates the possibility for as yet unidentified reservoir and disease hosts for EHNV and ECV. Consequently, these pathogens pose a threat to aquaculture and conservation. Further research is required to develop improved disease control strategies, including effective vaccines. The application of molecular techniques is required to trace the sources of disease outbreaks, routes of transmission and mechanisms of regional and global spread. The possibility of reservoir hosts among fishes, amphibians and reptiles needs to be resolved. Education of the public to the disease threats posed to lower vertebrates via unrestricted trade in ornamental species and baitfish is urgently needed (Jancovich *et al*., 2005; Whittington and Chong, 2007).

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5 Oncogenic Viruses: *Oncorhynchus masou* Virus and Cyprinid Herpesvirus

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5.1 Introduction

Due to their distinctive appearance and obvious pathological nature, tumours of fish have been recognized for centuries. Publications on fish tumours are widely scattered in the scientific literature (Walker, 1969; Anders and Yoshimizu, 1994). The largest registry of tumours in lower animals was established at the Smithsonian Institution in Washington, DC, in 1965. Pathologies range from benign epidermal papillomas to metastatic melanomas and hepatocellular carcinomas in more than 300 species of fish.

Depending upon the season and geographic location, certain types of skin tumours may be prevalent in wild European eel (*Anguilla anguilla*), dab (*Limanda limanda*), European smelt (*Osmerus eperlanus*) (Anders, 1989) and northern pike (*Esox lucius*) from north-east Atlantic coastal areas. However, the occurrence of 'carp-pox' lesion in cultured cyprinids has decreased in importance (Anders and Yoshimizu, 1994). Tumours with a suspected viral aetiology are well documented in mammals, birds, reptiles and fish (Anders and Yoshimizu, 1994). A viral aetiology of papillomas in fish was first suggested by Keysselitz (1908). Owing to the frequent epizootic occurrence of fish tumours, an infectious viral aetiology was suggested (Winqvist *et al*., 1968; Walker, 1969; Mulcahy and O'Leary, 1970; Anders, 1989; McAllister and Herman, 1989; Lee and Whitfield, 1992; Anders and Yoshimizu, 1994), even though viral particles were not always evident. In these cases, evidence was usually based on the exclusion of other potential causative factors. In about 50% of all cases where electron microscopy and virological methods have been applied, viruses or virus-like particles were identified in tumour tissue. Benign tumours such as epidermal hyperplasia, papilloma and fibroma, were mostly caused by herpesviruses infection, and much less related to retroviruses, papovaviruses or adenoviruses. Moreover, retroviruses supposed to cause malignant forms such as sarcomas and lymphosarcomas. Typically, the tumours had just one viral type, in rare cases, different skin tumour types associated with different viruses occurred in the same specimen (Anders and Yoshimizu, 1994).

The significance of these viruses and virus-like particles for tumour induction is mostly speculative and will not be included in the present discussion. Oncogenicity has been clearly demonstrated only with herpesviruses from masu salmon (*Onchorhynchus masou*) (see Section 5.2) and the Japanese Asagi variety of koi carp (itself a domesticated variety of the common carp, *Cyprinus carpio*) (Section 5.3). Although in certain other cases tumour formation was induced experimentally via inoculation of

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cell-free filtrates and/or live tumour cells, attempts to isolate viruses from tumours in cell culture were unsuccessful (Peters and Waterman, 1979). For the two herpesviruses, pathogenicity and oncogenicity have been clearly verified by successful isolation of the causative virus in cell culture and this fulfils River's postulates, but nothing is known about possible oncogenes in these viruses.

5.2 *Oncorhynchus masou* Virus

5.2.1 Introduction

Oncorhynchus masou virus disease (OMVD) is an oncogenic and skin ulcerative condition coupled with hepatitis that occurs among salmonid fishes in Japan. It is caused by *Salmonid herpesvirus 2* (SalHV-2) and was first described from *Oncorhynchus masou* (Kimura *et al*., 1981a,b). The virus is more commonly known as *Oncorhynchus masou* virus (OMV), but other synonyms include the nerka virus Towada Lake, Akita and Amori Prefecture (NeVTA; Sano 1976), yamame tumor virus (YTV; Sano *et al*., 1983), *Oncorhynchus kisutch* virus (OKV; Horiuchi *et al*., 1989), coho salmon tumor virus (COTV; Yoshimizu *et al*., 1995), coho salmon herpesvirus (CHV; Kumagai *et al*., 1994), rainbow trout (*O. mykiss*) kidney virus (RKV; Suzuki 1993) and rainbow trout herpesvirus (RHV; Yoshimizu *et al*., 1995). SalHV-2 is a recognized species in the genus *Salmonivirus*, family *Alloherpesviridae*.

5.2.2 The disease agent

Biophysical and biochemical properties

At or near 15°C, cells infected with OMV show a distinctive cytopathic effect (CPE) within 5 to 7 days. This CPE is characterized by rounded cells followed by syncytium formation, and eventual lysis of RTG (rainbow trout gonad)-2 and other salmonid cell lines (Fig. 5.1A). Cells from non-salmonid species are refractory to infection (Yoshimizu *et al*., 1988b). The maximum infectious

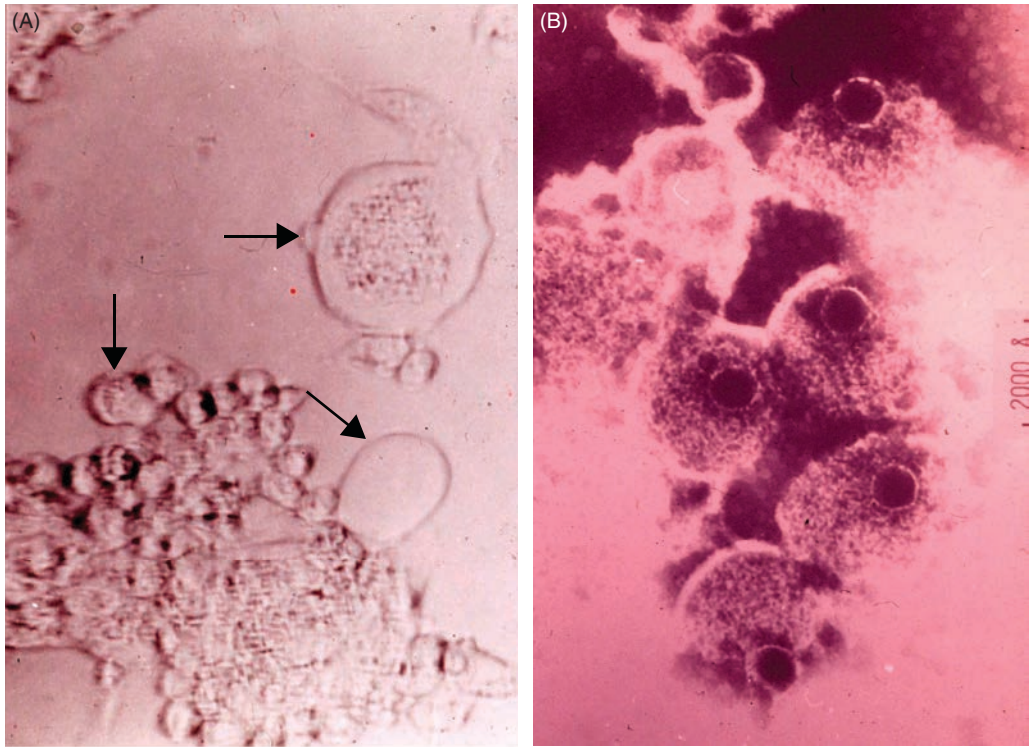

Fig. 5.1. Cytopathic effects of infection by *Oncorhyncus masou* virus (OMV), showing the presence of rounded cells followed by syncytium formation (arrows) produced by OMV in RTG-2 cells incubated at 15°C for 9 days; (B) electron micrograph of negatively stained enveloped virions provided by Dr T. Sano (Yoshimizu and Kasai, 2011).

titre of culture-grown virus is about 10^6 $TCID_{50}$ (50% tissue culture infectious dose)/ml, with some variations depending on the cell line. OMV is heat-, ether- and acid (pH 3)-labile and does not haemagglutinate salmonid blood cells or human O-cells. It is inactivated by ultraviolet (UV) irradiation of 3.0×10^3 μW/s/cm^2 (mJ). Replication is inhibited by 50 μg/ml of the pyrimidine analogue, 5-iodo-2´-deoxyuridine (IUdR) and by anti-herpesvirus agents such as phosphonoacetate (PA), acyclovir (ACV; 9-(2-hydroxyethoxymethyl) guanine), (*E*)-5-(2-bromovinyl)-2´-deoxyuridine (BVdU), and 1-β-D-arabino-furanosylcytosine (Ara-C) (Kimura *et al*., 1981a, 1983b,c; Suzuki *et al*., 1992).

Electron microscopy of infected cells reveals that the intranuclear hexagonal capsids are 115 nm in diameter. An abundance of budding and enveloped virions that are 200 × 240 nm in diameter (Fig. 5.1B) are evident at the surface of and inside cytoplasmic vesicles. The calculated number of capsomeres of negatively stained virions is 162. The optimal growth temperature is 15°C with replication at 18°C and no growth at 20°C or higher. This psychrophilic nature of OMV differs from the temperature sensitivity of channel catfish herpesvirus and other amphibian herpesviruses (Kimura *et al*., 1981a).

Serological relationships

OMV is neutralized by homologous antiserum but not by antisera prepared against other salmonid viruses (e.g. IPNV, infectious pancreatic necrosis virus; IHNV, infectious hematopoietic necrosis virus; CSV, chum salmon virus; and *Herpesvirus salmonis*). All 177 OMV strains isolated from ovarian fluid or tumour tissue of mature masu salmon in hatcheries located in northern Japan (collected from 1978 to 1986) were neutralized by anti-OMV OO-7812 serum, a rabbit antiserum against the reference OMV strain. The ND_{50} (50% neutralization dose) ranged from 1:40 to 1:80 (Yoshimizu *et al*., 1988b). Eleven herpesvirus strains were compared serologically using serum cross-neutralization tests with polyclonal rabbit antisera: NeVTA from kokanee salmon (landlocked sockeye salmon, *O. nerka*); three strains of OMV and YTV from masu salmon; CSTV (coho salmon tumor virus), COTV and two strains of OKV from coho salmon (*O. kisutch*); RKV and RHV from rainbow trout; and *H. salmonis*. The herpesvirus strains in Japan were neutralized by antisera against these viruses and were closely related to *Salmonid herpesvirus 2*, reference strain OMV OO-7812 (Table 5.1). These strains, however, were clearly distinguished from *H. salmonis* (*Salmonid herpesvirus 1*) and OMV is designated as *Salmonid herpesvirus 2* (Yoshimizu *et al*., 1995, after Hedrick *et al*., 1987).

Viral protein and genome

The general properties of OMV are similar to those of *H. salmonis*, *Salmonid herpesvirus 1*, although

Table 5.1. Serological relationship of herpesvirus strains from salmonid fishes using the 1/*r* (relatedness) value[a] based on serum cross-neutralization tests.

		Antiserum[b]								
		OMV								
Species	Virus[b]	I	II	III	YTV	NeVTA	COTV	OKV (M)	RKV	HS
Masu salmon	OMV I	1.00	1.30	0.92	1.42	1.12	1.53	0.85	1.22	>3.16
	OMV II		1.00	0.80	1.00	1.22	1.47	0.85	0.67	>3.47
	OMV III			1.00	1.21	0.94	1.41	1.00	0.83	>3.47
	YTV				1.00	0.84	1.33	0.70	1.19	>5.69
Kokanee salmon	NeVTA					1.00	1.39	0.83	0.89	>4.89
Coho salmon	COTV						1.00	0.96	0.51	>3.16
	OKV (M)							1.00	0.83	>3.47
Rainbow trout	RKV								1.00	>3.47
	HS									1.00

[a]A value of 1 indicates serological identity and greater or lesser values indicate increasing differences (1/r: Archetti and Horsfall, 1950).
[b]Key: COTV, coho salmon tumor virus; HS, *Herpesvirus salmonis*; NeVTA, nerka virus Towada Lake, Akita and Amori Prefecture; OKV (M), *Oncorhynchus kisutch* virus (M); OMV, *Oncorhynchus masou* virus; RKV, rainbow trout kidney virus; YTV, yamame tumor virus.

OMV differs in virion size and in optimal growth temperature. Furthermore, OMV is different from other known fish herpesviruses with respect to the viral-induced polypeptide patterns; 34 polypeptides appear in OMV-infected cells that are virus specific. These polypeptides have molecular weights between 19,000 and 227,000. By contrast, *H. salmonis* induces 25 polypeptides with molecular weights between 19,500 and 250,000 (Kimura and Yoshimizu, 1989). CCV (channel catfish virus) induces 32 polypeptides (Dixon and Farber, 1980), which are distinct from those of OMV. Differences in the electrophoretic migration of two of 34 OMV-specific polypeptides led to the classification of 12 OMV strains into six groups (Kimura and Yoshimizu, 1989).

Restriction endonuclease cleavage patterns of OMV DNAs are different from those of *H. salmonis*. Seven representative OMV strains from ovarian fluids and tumour tissues of wild masu salmon in Hokkaido and Aomori prefectures were analysed with restriction endonuclease. The restriction patterns of OMV strain DNAs were divided into four groups. The restriction profiles of high-passage strains were different from those of low-passage strains when digested with *Bam*HI, *Hin*dIII and *Sma*I. However, no differences were observed between the high- and low-passage viral DNA with *Eco*RI (Hayashi *et al*., 1987). By using ^{32}P-labelled DNA from standard OMV (strain OO-7812) as a probe, most fragments of other OMV DNAs were hybridized (Gou *et al*., 1991). From the results of the DNA homologies, OMV and YTV were considered to be the same virus, while NeVTA was similar but distinct (Eaton *et al*., 1991).

The genome sequence of OMV strain OO-7812 was determined (Yoshimizu *et al*., 2012) and phylogenetic relationships among fish and amphibian herpesviruses were predicted using amino acid sequences from parts of the DNA polymerase and terminase genes. Strain OO-7812 showed 100% identity to strains YTV and NeVTA, and OMV is clearly distinguished from SalHV-1 and SalHV-3 (Fig. 5.2).

DNA polymerase activities were determined in both tumour and normal tissues of masu salmon. High DNA polymerase α-activity was detected in OMV-infected tumour tissues but not in normal tissues, which indicates that the OMV DNA in tumour cells was replicating well. DNA polymerase activity was the same in both tumour and normal tissues. This is the first evidence of the detection of herpesvirus DNA polymerase in tumour tissue in association with herpesvirus (Suzuki *et al*., 1992).

Survivability and immunity

A significant reduction in the infectious titre of OMV occurred within 3 and 7 days in water at 15°C and 10°C, respectively. However, infectivity remained for 7–14 days when the water temperature was below 5°C (Yoshimizu *et al*., 2005) because the activity of bacteria producing antiviral substance(s) in the water decreased at low temperatures (Yoshimizu *et al*., 2014).

5.2.3 Geographical distribution

In the early 1960s, eggs of masu salmon were collected from the rivers around Sea of Japan coast of Hokkaido, and transported to Honshu. With unrestricted fish movements, the virus spread to Gifu, Yamanashi and Niigata Prefectures in Honshu where the first cancerous disease, basal cell carcinoma of masu salmon, was detected (Kimura, 1976). High mortality of kokanee salmon fry occurred in 1972 and 1974, and virus was isolated from moribund fish The virus was classified as a member of the *Herpesviridae* and was named the nerka virus in Towada Lake, Akita and Aomori Prefecture (NeVTA) (Sano, 1976). In 1978, a similar herpesvirus was isolated from the ovarian fluid of masu salmon cultured in the Otobe Salmon Hatchery on the Sea of Japan coast in Hokkaido and named *Oncorhynchus masou* virus (OMV) after the host fish (Kimura *et al*., 1981a,b). OMV showed pathogenicity and oncogenicity towards salmonid fish, masu salmon, chum salmon (*O. keta*), coho salmon and rainbow trout.

From 1978 to 2015, six species of mature salmonid fish, masu salmon, chum salmon, pink salmon (*O. gorbuscha*), kokanee salmon, sockeye salmon (*O. nerka*) and rainbow trout (46,788 females) were collected and surveyed for viral infections in Hokkaido and in Aomori and Iwate prefectures in northern Honshu (Yoshimizu *et al*., 1993; Kasai *et al*., 2004). Herpesvirus was isolated from masu salmon at 13 hatcheries, excepting one hatchery where 60 specimens could not collected. All of the isolates were neutralized with anti-OMV rabbit serum (Yoshimizu *et al*., 1993).

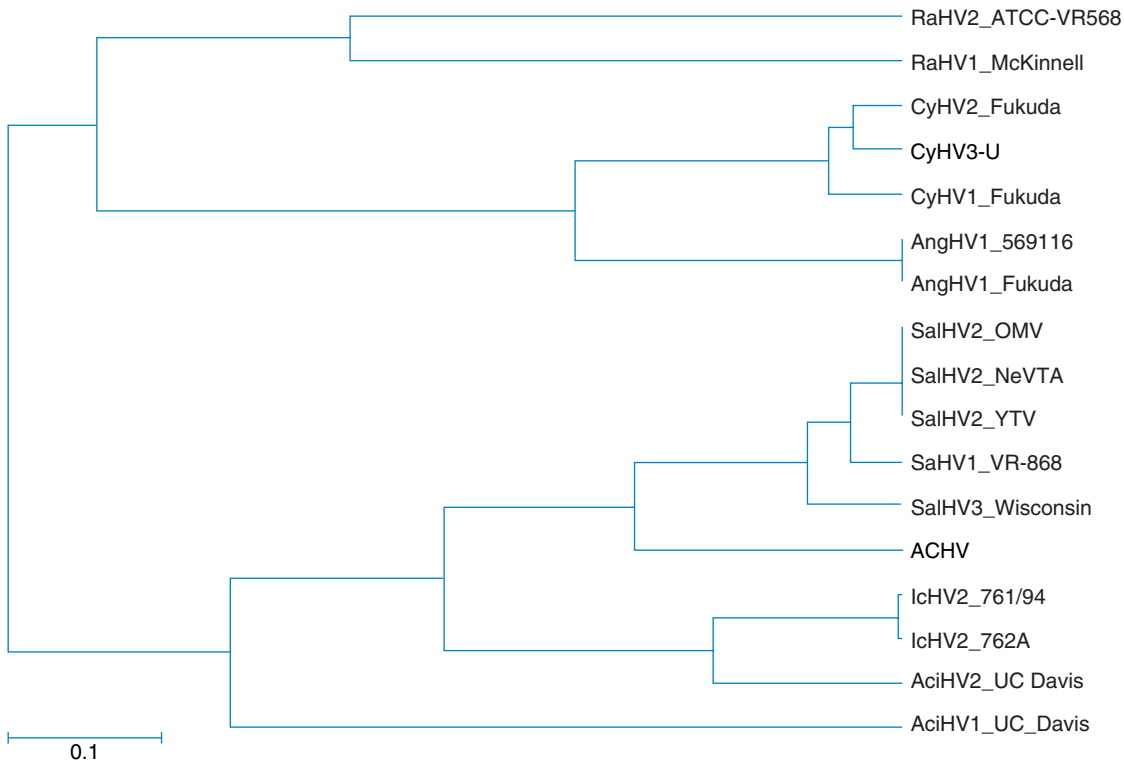

Fig. 5.2. Phylogram depicting the relationship among fish and amphibian herpesviruses based on the concatenated partial deduced amino acid (AA) sequences of the DNA polymerase and terminase genes (247 AA characters including gaps). Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. Key (in order downwards): RaHV, *Ranid herpesvirus* (frog); CyHV, *Cyprinid herpesvirus* (carp and gold fish); AngHV, *Anguillid herpesvirus* (eel); SalHV, *Salmonid herpesvirus*; ACHV, *Atlantic cod herpesvirus*; IcHV, *Ichalurus herpesvirus* (channel catfish); AcHV, *Acipenserid herpesvirus* (white sturgeon).

In 1981, a similar herpesvirus was isolated from the tissues of a basal cell carcinoma on the mouth of yamame (another name for masu salmon) cultured at Koide Branch, Niigata Prefectural Inland Fisheries Experimental Station in Honshu. This virus was named yamame tumour virus (YTV, Sano *et al.*, 1983). Since 1988, herpesvirus has been isolated from pond and pen-cultured coho salmon in Miyagi Prefecture, Tohoku district, Honshu (Kimura and Yoshimizu, 1989). Also, since 1992, a herpesvirus has been isolated from cultured rainbow trout in Hokkaido; this virus was tentatively named rainbow trout kidney herpesvirus (RKV) by Suzuki (1993). From 2000 to 2001, epizootics occurred in rainbow trout weighing 12–1.5 kg at 18 fish farms in Nagano, Shizuoka and Gifu prefectures in central Japan (Honshu). All of the viruses isolated from diseased fish were identified as OMV.

5.2.4 Economic importance of the disease

Many strains of herpesvirus identified as OMV have been isolated from ovarian fluid and tumours of wild and cultured masu salmon in northern Japan (Yoshimizu *et al.*, 1993). Mortality among kokanee salmon ranges from 80 to 100%, while there are no reports of mortality in masu salmon, but the presence of tumours reduces their commercial value. OMVD has been a major problem in the pen culture of coho salmon in the Tohoku district since 1988 and coho salmon culture has been economically damaged by this disease. OMV was found in 1992 pond cultures of rainbow trout in Hokkaido and it has occurred in central Japan since 2000. OMVD is currently regulated and controlled in kokanee salmon, masu salmon and coho salmon (see Section 5.2.7). OMVD outbreaks in rainbow trout remained a major problem in production

farms until 2005, as demonstrated by the high proportion of low-weight dead fish found in an outbreak in 18 fish farms in Nagano Prefecture in 2000–2001 (see Fig. 5.3D; Furihata *et al*., 2003).

5.2.5 Diagnosis

The infectivity of the virus remains unchanged for 2 weeks at 0–5°C, but 99.9% of the infectivity is lost within 17 days at –20°C. Viral isolation should be from fish transported on ice to the laboratory (Yoshimizu *et al*., 2005). For the filtration of OMV, a 0.40 µm nucleopore filter (polycarbonate) is recommended because cellulose acetate membranes trap >99% of the viral particles. For virological surveys, ovarian fluid is collected as described by Yoshimizu *et al*. (1985), diluted with the same volume of antibiotic (Amos, 1985) and incubated at 5°C, overnight. In the case of tumours, a sample of tissue is excised, disinfected with iodophor (50 mg/l, 15 min) washed with Hank's BSS (balanced salt solution) and transported in an antibiotic solution to the laboratory. Tumour tissue is prepared for primary culture or co-culture with RTG-2 cells. After one subculture in primary cells, the culture medium should be inspected for virus (Yoshimizu, 2003b).

Rabbit antiserum or monoclonal antibody against OMV is used in a fluorescent antibody test (Hayashi *et al*., 1993) and a DNA probe is used to detect viral genome (Gou *et al*., 1991). PCR using an F10 primer, GTACCGAAACTCCGAGTC, and R05 primer, AACTTGAACTACTCCGGGG, amplified a 439 base pair segment of DNA from OMV strains from the liver, kidney, brain and nervous tissues of masu salmon, coho salmon and rainbow trout. The size of the amplified DNA of OMV and *H. salmonis* is different, so OMV and *H. salmonis* can be distinguished by their agarose gel profiles (Aso *et al*., 2001).

Presumptive diagnosis

Certain key features such as life cycle, stage, species and stock of fish, as well as water temperature, clinical signs (see Section 5.2.6), and disease history of the facility are evaluated. To isolate OMV, tissue and reproductive fluids are used in standard cell culture techniques. Processed specimens must be inoculated on to RTG-2 or chinook salmon embryo cells (CHSE-214). Cytopathic effects include rounded cells and the formation of giant syncytium. Plaque assay procedures (Kamei *et al*., 1987), which use a methylcellulose overlay, are also used to isolate and enumerate OMVD.

Confirmatory diagnosis

Confirmation of OMV is accomplished using neutralization tests with either a polyclonal rabbit antisera or monoclonal antibody An antigen detection ELISA (Yoshimizu, 2003a), and a fluorescent antibody technique (FAT) for OMV have been developed using either polyclonal or monoclonal antiserum (Hayashi *et al*., 1993). The FAT is specific, reacts with all isolates of OMVD and requires less time for confirmatory diagnosis. PCR can also be used to confirm OMV grown in cell cultures (Aso *et al*., 2001).

Procedures for detecting subclinical infection

The detection of OMV in carrier fish is difficult, but the virus replicates and appears in ovarian fluid at spawning. Antibody detection by a neutralization test or ELISA (Yoshimizu, 2003a) is available for epizootiological studies.

5.2.6 Pathology

Pathogenicity and host susceptibility

The susceptibility of salmonid fry to OMV was studied via immersion in water containing 100 $TCID_{50}$/ml OMV at 10°C for 1 h (Kimura *et al*., 1983a). Kokanee salmon (1 month old) were most sensitive (100% mortality). Masu and chum salmon were also sensitive (87 and 83% mortality, respectively) while coho salmon and rainbow trout were the least sensitive (39 and 29% mortality, respectively). The cumulative mortality of just hatchling chum salmon was 35%, but it was more than 80% in 1–5 month old fry, and in 3-month-old fry it was 98%. At 6 and 7 months old, susceptibility was reduced to 7 and 2%, respectively. There was no mortality among 8-month-old fry that were immersed in virus and then injected intraperitoneally with 200 $TCID_{50}$/fish. By contrast, 1-month-old masu salmon fry were most sensitive and sustained a cumulative mortality of 87%, whereas 3- and 5-month-old masu salmon fry had cumulative mortalities of 65 and 24%, respectively.

Clinical signs and transmission

From June to September of every year since 1970, high mortality (about 80%) of kokanee salmon fry

has occurred in Japan. Clinical signs in fish include a darkened body colour, sluggish behaviour and inappetance. Syncytium-forming virus was isolated from moribund fish in RTG-2 cells incubated at 10°C in 1972 and 1974. The virus was named the nerka virus in Towada Lake, Akita and Aomori Prefecture (NeVTA) (Sano, 1976).

In 1978, a herpesvirus was isolated from the ovarian fluid of an apparently healthy mature masu salmon, cultured in the Otobe salmon hatchery in Hokkaido. This virus was named *Oncorhynchus masou* virus (Kimura *et al*., 1981a, 1981b). The general properties of OMV are similar to those of *H. salmonis* and NeVTA, but it differs in virion size and its optimal growth temperature. It is also distinct from *H. salmonis* with respect to its viral-induced polypeptide patterns, serological properties and in PCR (Kimura and Yoshimizu, 1989; Aso *et al*., 2001). OMV is pathogenic and more significantly, it is oncogenic in masu salmon and several other salmonids (Kimura *et al*., 1981a,b). One-month-old kokanee salmon are most sensitive to the virus. Masu and chum salmon are also highly susceptible to OMV infection, whereas coho salmon and rainbow trout are less susceptible (Tanaka *et al*., 1984). The incidence of tumour-bearing fish approached more than 60%. There were epithelial tumours on 12–100 % of surviving chum, coho and masu salmon, and on rainbow trout beginning at 4 months and persisting for at least a year postinfection (Yoshimizu *et al*., 1987).

Since 1988, herpesvirus had been isolated from the liver, kidney, and developing neoplasia in pond and pen-cultured coho salmon in Miyagi Prefecture (Kimura and Yoshimizu, 1989). Disease signs included white spots on livers, ulcers on the skin and/or neoplasia around the mouth or body surface. The herpesviruses isolated from coho salmon were tentatively named as coho salmon tumor virus (CSTV) by Igari *et al*. (1991), *O. kisutch* virus (OKV) by Horiuchi *et al*. (1989), coho salmon tumor virus (COTV) by Yoshimizu *et al*. (1995) and coho salmon herpesvirus (CHV) by Kumagai *et al*. (1994). These viruses were neutralized by anti-OMV or anti-NeVTA rabbit sera (Yoshimizu *et al*., 1995), and the oncogenicity of CSTV, OKV and COTV was confirmed experimentally. In addition, the restriction endonuclease profiles of CSTV were similar to those of NeVTA and YTV (Igari *et al*., 1991). CHV is highly pathogenic to coho salmon.

Massive mortalities, ranging from 13 to 78%, have occurred among cultured 1-year-old rainbow trout in Hokkaido since 1992. Diseased fish exhibited hardly any external clinical signs, although some fish had ulcerative lesions on their skin. Internally, intestinal haemorrhage and white spots on the liver were observed. No bacteria, fungi or parasites were found and a herpesvirus was isolated from the kidney, liver and skin ulcers. The herpesvirus was tentatively named rainbow trout kidney herpesvirus (RKV) by Suzuki (1993). RKV is highly pathogenic to marketable-size rainbow trout and masu salmon (Sung *et al*., 1996a,b). Epizootics occurred in rainbow trout weighing 1.2–1.5 kg at 18 fish farms in Nagano Prefecture from February 2000 to January 2001. A virus was isolated from diseased fish in RTG-2 cells with CPE syncytia. High infectivity titres (about 10^8 $TCID_{50}$/g) were demonstrated in the internal organs and multiple necrotic foci were observed in the liver (Fig. 5.3A,B,C). The virus was identified as OMV using serological tests and PCR. Based on these results, the epizootic was diagnosed as OMVD. In more than 80% of these cases, outbreaks were linked to introductions of live fish. (Furihata *et al*., 2003, 2004).

The horizontal transmission of OMV was accomplished via cohabitation in specific pathogen-free 5-month-old chum salmon fry. The resulting mortality was similar to the results from the immersion infection of 3 to 7-month-old fry (see above). Clinical signs in infected fish include inappetence, exophthalmia (Fig. 5.4A) and petechiae on the body surface (Fig. 5.4B), especially beneath the lower jaw. Internally, the liver has white lesions (Fig. 5.4C and D), and in advanced cases the whole liver becomes pearly white. In some fish, the spleen may be swollen, and the intestine is devoid of food (Kimura *et al*., 1981a, 1983a).

Histopathology

The kidney of OMV-infected 1- and 3-month old masu salmon, 1-month-old coho salmon and 2-month-old chum salmon is the principal target organ for the virus. Necrosis of epithelial cells and kidney were observed in the early moribund fry, while partial necrosis of the liver, spleen and pancreas was recognized later in moribund 1-month-old masu salmon, with necrosis of the kidney haematopoietic tissue in 3-month-old masu salmon. It was suggested the principal target organ had

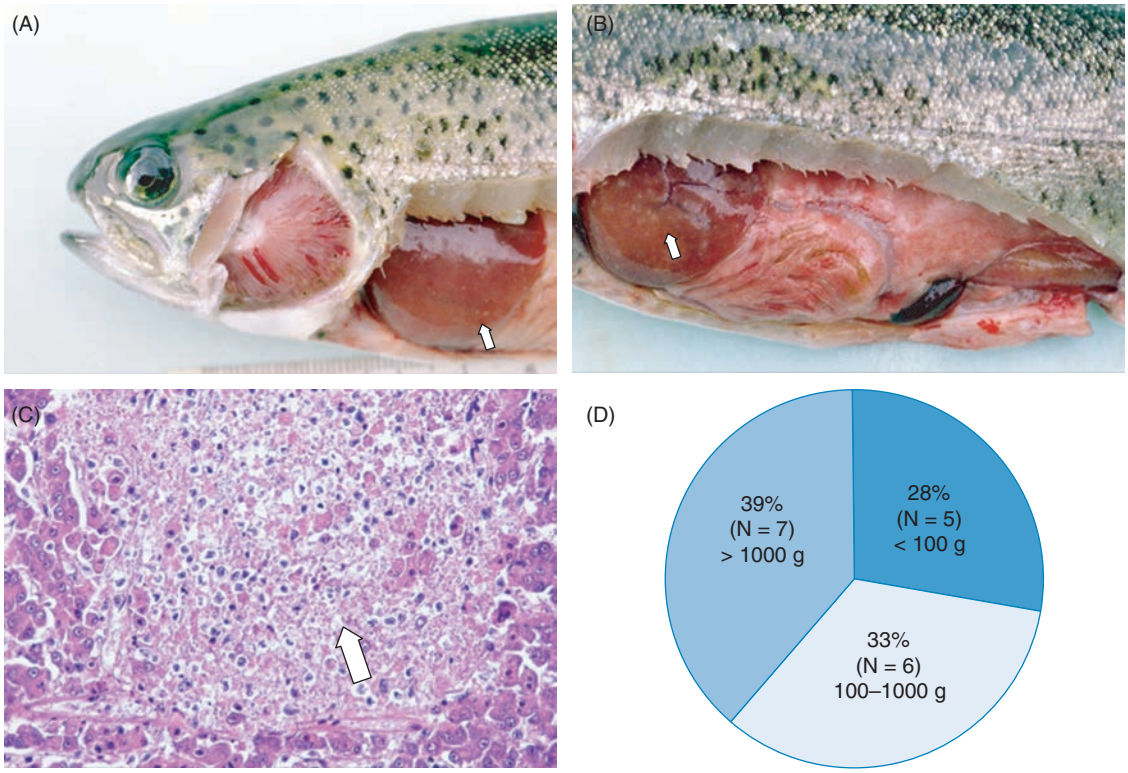

Fig. 5.3. *Oncorhyncus masou* virus (OMV) infection in rainbow trout: (A,B) arrows show necrotic liver symptoms in whole fish, in (B) with white spot lesions; (C) multiple foci of severe necrosis in the liver (arrow); (D) chart showing body weight of dead rainbow trout. Photos and figure were provided by Dr M. Furihata (see Furihata *et al*., *Fish Pathology*, 2003).

moved from the kidney to the liver, and marked histopathological changes were observed in the later stages of the disease. The foci of necrosis in the liver became more severe with longer incubation periods. Hepatocytes showing margination of chromatin were present. Cellular degeneration in the spleen, pancreas, cardiac muscle and brain was also observed (Yoshimizu *et al*., 1988a).

Histopathological changes in rainbow trout, coho salmon and chum salmon were similar to those in masu salmon (Kumagai *et al*., 1994; Furihata *et al*., 2004). In rainbow trout, high infectivity titres were measured in the internal organs and multiple necrotic foci were evident in the liver. The definitive changes were necrosis of OMV-infected cells in the spleen and haematopoietic tissues in the kidney, liver, intestine, heart, gill filaments, epidermis and lateral musculature. In particular, the intestine showed severe necrosis and haemorrhage in the epithelium and underlying tissues of rainbow trout with OMVD (Furihata *et al*., 2004).

Tumour induction

Tumour formation could be induced experimentally via waterborne infection of OMV. At about 4 months postinfection and persisting for at least 1 year, 12–100% of surviving masu, chum and coho salmon, as well as rainbow trout, developed oral epithelial tumours (Fig. 5.5A,B; Kimura *et al*., 1981b). Histopathologically, tumours were composed of proliferative, well-differentiated epithelial cells supported by fine connective tissue stroma. OMV was recovered from the culture medium of one passage of the transplanted tumour cells in primary cultures (Yoshimizu *et al*., 1987). The perioral site was the most frequent area for tumour development. As control fish held under the same conditions showed no tumours, OMV is presumed

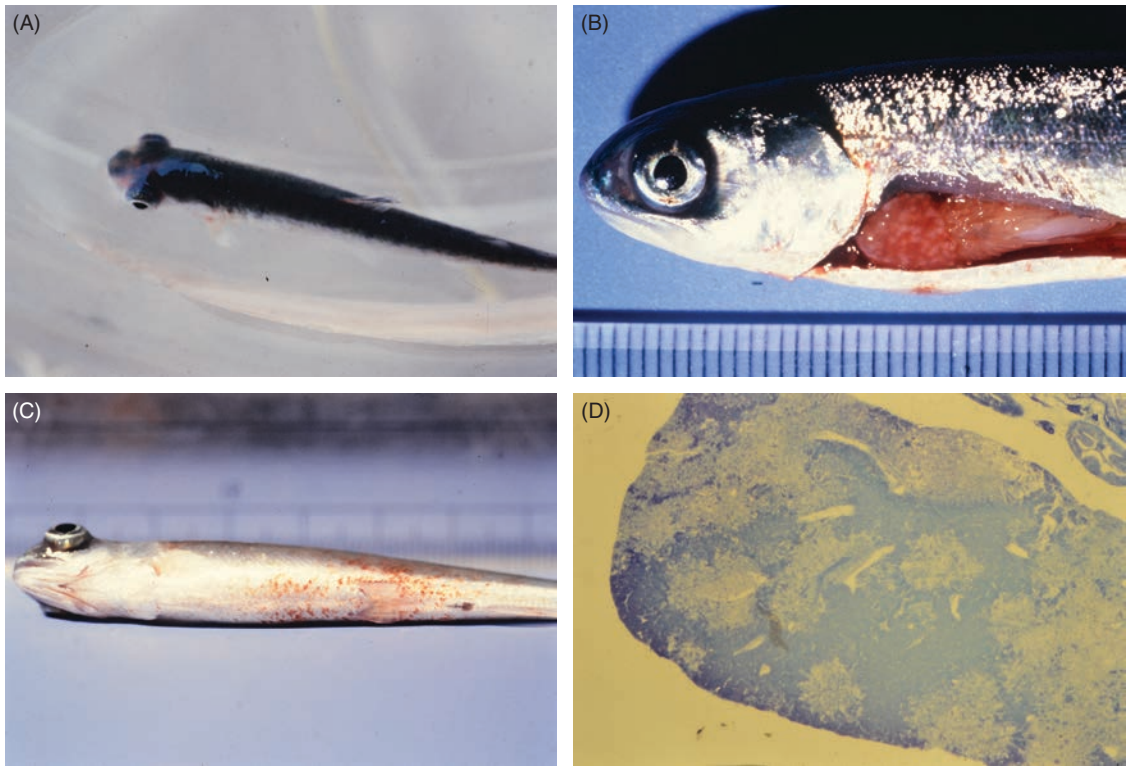

Fig. 5.4. Three-month-old chum salmon fry exposed to *Oncorhyncus masou* virus (OMV). (A) Exophthalmia; (B) white spot lesions on the liver; (C) petechiae in the body surface; and (D) multiple foci of severe necrosis in a liver section (haematoxylin and eosin (H&E) stain). The photographs and figure were provided by Dr T. Kimura (see Kimura *et al*., Fish Pathology, 1981a).

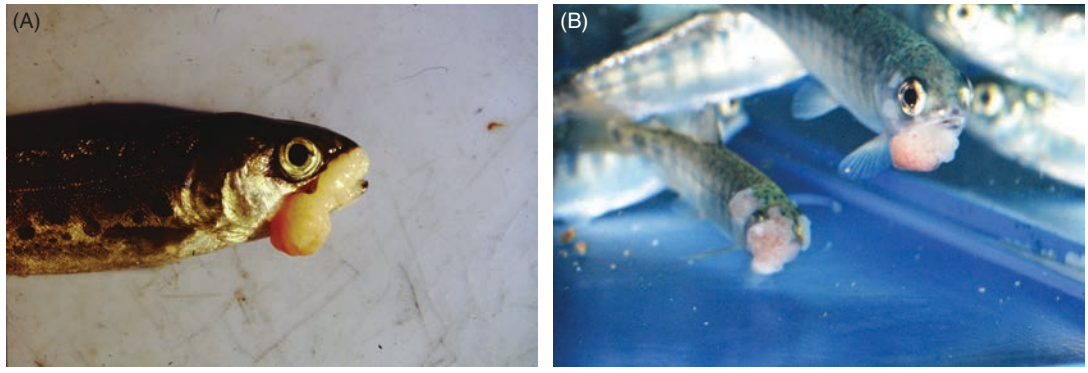

Fig. 5.5. Experimentally induced tumours caused by *Oncorhyncus masou* virus (OMV) in (A) masu salmon and (B) coho salmon. Photos were provided by M. Yoshimizu.

to have caused tumour development. This neoplasia may persist for one year postinfection.

The tumour cells appear to be of epithelial origin. There are several layers of epithelial cells in a papillomatous array and supported by fine connective tissue stroma. Abundant mitotic figures suggested a highly proliferative nature. Tumours appearing on the caudal fin, operculum, body surface,

corneas of the eye and kidney had similar characteristics to those of the mouth (Kimura *et al*., 1981b,c). Tumours in the kidney, as well as those from other sites, such as on the eye, under the operculum and the caudal fin, had similar histopathological lesions.

Electron micrographs revealed that tumour cells had the typical neoplastic feature of variable nuclear size and a loose intracellular connection. However, OMV particles were not found in the nuclei or in the cytoplasm of the tumour cells (Kimura *et al*., 1981b,c; Yoshimizu *et al*., 1987). The virus was isolated from eroded tumour tissue in one fish 9 months after infection, and from a primary culture of tumour cells from another fish 10 months after infection. The primary cultures exhibited continuous growth for 4 days, followed by CPE-like changes. At this time, OMV was isolated from the culture medium. Neutralizing antibody against OMV was detected in individual fish and in pooled sera from tumour-bearing fish.

5.2.7 Protective control strategies

Epizootiology

In the 1980s, OMV was distributed widely among masu salmon in northern Japan. In 1988, OMVD was diagnosed in net-pen cultured coho salmon in the marine environment of Tohoku district, central Japan. The virus in coho salmon was successfully controlled by selecting moribund fish just after transportation from fresh water to seawater, and testing their sera using FAT. If the fish were positive, farmers would disinfect the hatchery pond and equipment before eyed-eggs were transplanted in the following year (Kumagai *et al*., 1994). OMVD was found in rainbow trout in 1991 in Hokkaido, and from 2000, it had become a major problem in pond culture on the mainland. Natural and experimental infections indicated that fish between 1 and 5 months old are the most susceptible. From 2000 to 2005, epizootics were reported in juvenile, yearling and mature rainbow trout (Furihata *et al*., 2003), with morality exceeding 80%. Most OMVD occurs in fresh water at 15°C or lower.

Virucidal effects of disinfectants and antiviral chemotherapy

The virucidal effects of six disinfectants were examined against OMV (Hatori *et al*., 2003). At 15°C for 20 min, the minimum concentrations showing 100% plaque reduction of OMV by iodophore, and by solutions of sodium hypochlorite, benzalkonium chloride, saponated cresol, formaldehyde and potassium permanganate were, 50, 100, 100, 3500 and 16 ppm, respectively.

ACV had high efficacy against the OMV, *Herpesvirus salmonis* and CCV. CPEs induced by 100 $TCID_{50}$/ml OMV in RTG-2 cells was inhibited by 2.5 µg/ml ACV. ACV was more effective than other compounds such as 9-β-D-arabinofuranosyladenine (Ara-A), IUdR and PA. The growth of RTG-2 cells was considerably inhibited by ACV at 25 µg/ml, but no morphological changes were observed in the cells. The replication of OMV in RTG-2 cells inoculated with 100 $TCID_{50}$/ml was completely suppressed by 2.5 µg/ml of ACV. The addition of ACV within 4 days postinfection reduced OMV replication. In order to be effective, ACV must be present continuously (Kimura *et al*., 1983a).

The therapeutic efficacy of ACV was evaluated using OMV and chum salmon fry. Experimentally infected fish were treated with ACV either orally or by immersion. Daily immersion of fish into ACV solution (25 µg/ml, 30 min/day, 15 times) reduced the mortality of the infected fish. Oral administration of the drug (25 µg/fish a day, 60 times) did not affect the survival of chum salmon. In contrast, the group of chum salmon administered IUdR orally showed a higher survival than the ACV-administered group. This study suggests that an effective level of ACV was not maintained in fish that were medicated orally. The daily immersion of infected fish into ACV solution (25 µg/ml 30 min/day, 60 times) considerably suppressed the development of tumours induced by OMV (Kimura *et al*., 1983b).

Vaccination

Mature rainbow trout vaccinated with formalin-inactivated OMV had anti-OMV IgM that showed neutralization activity in the brood and could reduce the ratio of OMV isolation from ovarian fluid (Yoshimizu and Kasai, 2011). Vaccination using formalin-inactivated OMV is also very effective in protecting from OMV infection at the fry stage (Furihata, 2008). Unfortunately, a commercial vaccine for OMVD is not available in Japan.

Control strategy

General sanitation is practised in hatcheries to control and prevent fish diseases (Yoshimizu, 2003b).

Special care should be taken to avoid the movement of equipment from one pond to another and all equipment should be routinely disinfected after use. Methods to sanitize a hatching unit should be carefully developed with respect to chemical toxicity for fish. Workers might be responsible for transferring pathogens; consequently, the proper disinfection of hands and boots is required to prevent the dissemination of the virus. Although it may be difficult to sanitize hatching and rearing units during use, raceways and ponds should routinely be disinfected with chlorine (Yoshimizu, 2009). Pathogen-free water supplies are often essential for success in aquaculture. The water that is commonly used in hatcheries, which comes from rivers or lakes, may contain fish pathogens. Such open water supplies should not be used without filtration and treatment to eliminate and kill fish pathogens. Fish viruses are divided into two groups based on sensitivity to UV irradiation. The sensitive viruses include OMV, IHNV, LCDV (Lymphocystis disease virus) and HIRRV (*Hirame rhabdovirus*), which are inactivated by UV at 10^4 μW s/cm (Yoshimizu et al., 1986).

Effective monitoring and management is very important during the collection of eggs at spawning. As some viruses are transmitted vertically from the adult to progeny via contaminated eggs or sperm, disinfection of the surface of fertilized eggs and eyed eggs can break the infection cycle for herpesvirus and rhabdovirus (Yoshimizu *et al*., 1989; Yoshimizu, 2009). Health inspections of mature fish are conducted to ensure that fish are free from certifiable pathogens. Routine inspections and specialized diagnostic techniques are required to ensure specific pathogen-free brood stock. For salmonid fishes, ovarian fluid is collected by the method of Yoshimizu *et al*. (1985) and routinely inspected following cell culture. Fertilized eggs are disinfected with iodophore at 25 mg/l for 20 min or 50 mg/l for 15 min. The region inside the egg membrane of eyed eggs is considered pathogen free (Yoshimizu *et al*., 1989, 2002). Since 1983, iodophore treatment to disinfect eggs in all hatcheries in Hokkaido has helped to eliminate outbreaks of OMV (Yoshimizu *et al*., 1993; Kasai *et al*., 2004; Yoshimizu, 2009).

Fry with abnormal swimming or disease signs should be removed immediately and brought to the laboratory for analysis. Moreover, health monitoring should be done regularly using cell culture isolation, FAT, the immunoperoxidase technique (stain) (IPT), antigen-detecting ELISA and PCR tests (Yoshimizu *et al*., 2005).

Breeding

Tetraploid female rainbow trout and sex-reversed diploid male brown trout (*Salmo trutta)* were crossed in Nagano Prefectural Fisheries Experimental Station to obtain triploid salmon (Kohara and Denda, 2008), which was named 'Shinsyu Salmon'. These triploid salmon display fast growth, good survival rates during the spawning season and are resistant to infection by both OMV and IHNV.

5.3 Cyprinid herpesvirus 1 (CyHV-1)

5.3.1 Introduction

Whitish or pinkish papillomatous lesions are sometimes seen on the skin and fin of carp, and these have been recognized since the Middle Ages in Europe as 'carp pox'. This condition has a worldwide distribution. The prevalence of these tumours may differ among strains or varieties of carp. A report by Calle *et al*. (1999) found a higher prevalence of tumours in carp with no scales, although this report constitutes the only evidence of this. The tumour tissue is organized with well differentiated cells and epidermal peg interdigitates with the papillae of the dermal connective tissue and capillary vessels (Sano *et al*., 1985a).

5.3.2 The disease agent

The disease agent of carp pox was originally named *Herpesvirus cyprini* (CHV). It is a cyprinid herpesvirus that was first isolated in fathead minnow (FHM) cells from the papillomatous tissue of the Asagi variety of koi carp (*C. carpio*) reared in Japan (Sano *et al*., 1985b), and it is currently classified as the species *Cyprinid herpesvirus 1* (CyHV-1) in the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*. The enveloped virion ranges from 153 to 234 nm in diameter with a capsid 93–126 nm in diameter (Sano *et al*., 1985a). The virus grows in FHM cells at 10–25°C, but not at 30°C, and maximum infectious titres of 10^4–10^5 $TCID_{50}$/ml were recorded at 15 and 20°C (Sano *et al*., 1985a, 1993a). Other cell lines derived from carp and cyprinid fishes such as koi fin (KF-1), common carp (*C. carpio*) brain (CCB) and epithelioma papulosum cyprini (EPC) cell lines are susceptible to the

virus (Sano *et al*., 1985a; Adkison *et al*., 2005). CPE is characterized by the formation of cytoplasmic vacuolation and also of Cowdry type A intranuclear inclusion bodies. The sequence of the whole viral genome, which is a linear double-stranded DNA of 291 kbp has been deposited in GenBank as JQ815363 (Davison *et al*., 2013). Restriction endonuclease cleavage profiles of isolates from Japan showed minor differences, suggesting that there is variation of the genome DNA sequence of the virus (Sano *et al*., 1991a).

5.3.3 Diagnosis

Whitish or pinkish papillomatous lesions on the fin, skin or mandible of common carp or koi might be suspected as viral papilloma caused by CyHV-1. Isolation of the virus from the papillomatous tissue in cell culture using cell lines such as FHM or KF-1 is difficult because the appearance of the infectious virus depends on the developmental stage of the papilloma. The indirect immunofluorescent antibody test (IFAT) using specific rabbit antiserum can detect viral antigens in tissues (Sano *et al*., 1991b), and *in situ* hybridization using a DNA probe has also been developed (Sano *et al*., 1993b). The whole genome sequence of the virus is known, and some of the genes, including DNA polymerase, have been annotated (Davison *et al*., 2013), so that specific primers for PCR detection can be designed after alignment of the sequences of related cyprinid herpesvirus, including CyHV-3 and CyHV-2, which cause mortality of the goldfish *Carassius auratus* and gibelio carp *C. auratus gibelio*.

5.3.4 Pathology

Pathogenicity and oncogenicity

Sano *et al*. (1985a, 1990) first reported the pathogenicity and oncogenicity of CyHV-1 to carp, although the virus did not cause mortality in the grass carp, *Ctenopharyngodon idella*, crucian carp, *Carassius auratus*, or willow shiner, *Gnathopogon elongatus*. Experimental infection with CyHV-1 showed high and weak lethality to 2-week-old and 4-week-old carp fry, respectively, but no mortality occurred in 8-week-old fry (Sano *et al*., 1991b). Furthermore, infected carp fry (2 week old) showed cumulative mortality of 60, 16 and 0% at 15, 20 and 25°C, respectively, (Sano *et al*., 1993a). During acute disease, the virus was detected in the gills, liver, kidney and intestine, demonstrating the occurrence of systemic infection, but the detection rate and concentration of the virus in the infected fish at 25°C were lower than those in the fish at 15 and 20°C. Subsequently, papillomas (Fig. 5.6) on the skin, fin or mandible were induced in 35, 72.5 and 27.5% of the survivors at 15, 20 and 25°C, respectively (Sano *et al*., 1993a). Histopathological lesions in experimentally induced papillomas (Fig. 5.7A) were similar to those in natural infections (Sano *et al*., 1991b).

Viral antigens and infectious titres could not be detected in fish infected with CyHV-1 using polyclonal antibodies to the virus or FHM cells 8 weeks after the inoculation when the water temperature was around 25°C or higher. However, viral DNA was detected in the brain, spinal cord, liver and subcutaneous tissues of the survivors using *in situ* hybridization with a fragment of the virus genome (Sano *et al*., 1992, 1993b). This shows that CyHV-1 can latently infect carp at high water temperatures. When the water temperature was lowered, papillomas appeared and the viral genome was detected both in the papilloma and in normal epidermis and subcutaneous tissue, spinal nerves and the liver.

Davison *et al*. (2013) reported that the JUNB family, which encodes a transcriptional factor involved in oncogenesis, is confirmed in the viral genome. Further study is needed to determine the gene(s) responsible for the induction of papillomas.

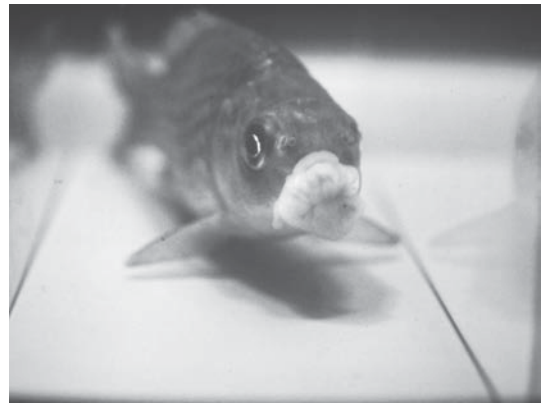

Fig. 5.6. Cyprinid herpesvirus 1 (CyHV-1)-induced papilloma on the mandible of carp that had survived from an infection with the cultured virus. Photograph provided by M. Sano.

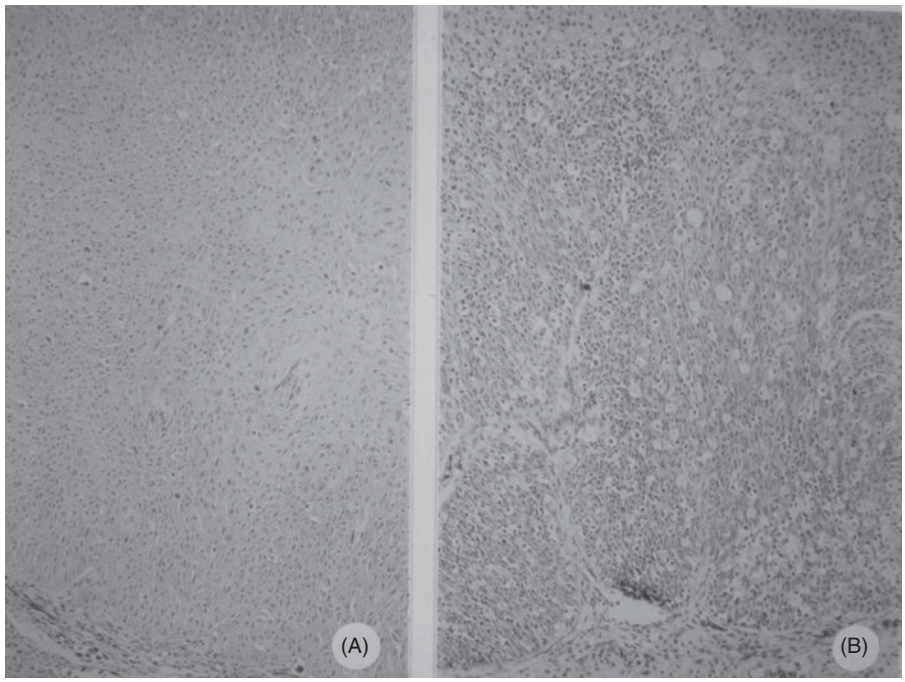

Fig. 5.7. Histopathology of (A) induced papilloma on the mandible of carp surviving infection by cyprinid herpesvirus 1 (CyHV-1) at 2 weeks old, and (B) virus-induced papilloma on the mandible of the fish 7 days after being treated with a water temperature shifting from 10 to 20°C for the induction of regression of the disease. Photographs were provided by N. Sano.

Regression and recurrence of tumours

Spontaneous regression of papillomas occurs in the spring as the water temperature rises. The regression also occurs in experimentally CyHV-1-induced papillomas when the water temperature was increased from 14 to 20, 25 and 30°C. At higher water temperatures (20–30°C), the regression is rapid, resulting in desquamation on the surface of the skin of all fish by 9 days.

After regression, papillomas frequently recur spontaneously (Sano *et al*., 1991b); they recurred in 83% of survivors 7.5 months after desquamation because the virus is present latently. Subsequently, the virus may reactivate and induce papilloma development in the following year (Sano *et al*., 1993a).

Leucocyte inflammation and oedema occur in the first stage of papilloma regression (see Fig. 5.7B). The subsequent stages include massive inflammation and oedema, cell necrosis that results in sponge-like tissues and, finally, desquamation from the epidermis. The injection of anti-carp peripheral blood lymphocyte (PBL) rabbit serum, which depressed the *in vitro* cytotoxic activity of normal carp PBLs, retarded the regression in experimentally CyHV-1 induced papilloma at 20°C (Morita and Sano, 1990). Moreover, the injection of anti-PBL serum induced recurrence of papillomas within 10 days of injection at 20°C on three out of eight fish whose papillomas had naturally desquamated 3 months earlier (N. Sano, unpublished data). This suggests that the cytotoxic activity of carp PBLs is important to the development and regression of papillomas (Morita and Sano, 1990).

5.3.5 Control strategies

In papillomas on sub-adult and adult fish, the mode of transmission of the virus seems to be horizontal from fish bearing the papillomas. Separation of papilloma-bearing fish can be a realistic way to control the occurrence of papillomas at the farm level. Also, in koi farms, treatment with elevated temperature is carried out routinely to induce desquamation of the papillomas (see above) before fish are shipped. However, little effort has been expended on developing and implementing hatchery-based

control strategies as there is no mass mortality of fry caused by CyHV-1 in farms. The effects of disinfectants and physiological treatments (such as heat and UV) on the nature of the infection and survival of the virus are not known.

5.4 Conclusions and Suggestions for Future Research

Oncorhynchus masou virus disease (OMVD) is an oncogenic and skin ulcerative condition coupled with hepatitis among salmonid fishes in Japan. The causative agent is the *Salmonid herpesvirus 2* (SalHV-2), which was first described as an oncogenic virus isolated from *O. masou*. Other synonyms (acronyms) include OMV, NeVTA, YTV, OKV, COTV, CHV, RKV and RHV. SalHV-2 belongs to the genus *Salmonivirus* and family, *Alloherpesviridae*. The main susceptible fish species are masu salmon, coho salmon and rainbow trout. Economic losses caused by this virus have been recognized among coho salmon and rainbow trout. OMVD is successfully controlled by disinfection of all equipment and eggs with iodophore just after fertilization and again at the early-eyed stage, and culture in a clean facility using well water or disinfected river water. Pathogenicity and oncogenicity are different among the OMV strains isolated from masu salmon, coho salmon and rainbow trout. Further studies are needed to determine the similarities and differences of these strains; their pathogenicity and oncogenicity, and to elucidate the mechanisms of infection and tumour formation, and their gene sequences.

The oncogenicity of cyprinid herpesvirus 1 (CyHV-1) has been confirmed and viral dynamics, including systemic infection, latent infection and papilloma development and regression in fish have been demonstrated in experimental studies. The fish immune system can also play an important role in the development and regression of tumours induced by the virus. Unlike experimental infections using cultured virus, there is no mass mortality of fry caused by CyHV-1 in farms in Japan. Latent or persistent infection occurs in adult carp during the warmer season even when fish have no apparent tumours, and these carp can be a major source of infection. Further epizootiological studies are needed of CyHV-1, especially of how its life cycle relates to the development of papillomas. As the viral genome sequence predicts the presence of oncogenic genes, further studies on the identification and function(s) of the proteins encoded in the genes should be carried out. The development and regression of the papillomas seem to rely on the state of the immune system, so it would be productive to more precisely determine the roles of that system in the host–pathogen relationship and ways to exploit it to the benefit of the host. It would also be helpful to better understand the disease process and to promote breeding programmes to develop less susceptible fish because on an empirical basis there may be differences in susceptibility to the virus among koi strains on farms.

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6 Infectious Salmon Anaemia

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6.1 Introduction

Infectious salmon anaemia (ISA) is a significant infectious viral disease of farmed Atlantic salmon, *Salmo salar* L., that was first reported in Norway during 1984 (Thorud and Djupvik, 1988). Outbreaks of ISA have an impact on the economy of the Atlantic salmon aquaculture industry, and this has led to the implementation of large-scale biosecurity measures. Outbreaks have now been reported in most Atlantic salmon farming areas, including the east coast of Canada and the USA, Scotland, Norway, the Faroe Islands and Chile (Rimstad *et al*., 2011). In Chile and the Faroe Islands, the disease caused major economic setbacks and left the entire industry with an uncertain future (Mardones *et al*., 2009; Christiansen *et al*., 2011) in a manner similar to that in Norway in and after 1989 (Håstein *et al*., 1999; Rimstad *et al*., 2011). In the early 1990s, ISA was listed as a notifiable disease by the World Organisation for Animal Health (OIE) (Håstein *et al*., 1999; OIE, 2015a).

Following its initial detection, ISA spread rapidly throughout the Norwegian aquaculture industry, resulting in a peak with more than 90 outbreaks in 1990 (Håstein *et al*., 1999). Although the causative virus was not identified until 1995 (Dannevig *et al*., 1995), biosecurity measures were introduced to combat and control the disease. These measures included early detection of the disease and the slaughter of diseased populations, regulations on transport, the disinfection of offal and waste from slaughterhouses, year class separation at farming sites and improved health control and certification. The effect was remarkable as only two new outbreaks were reported in 1994, demonstrating that ISA can be controlled without the use of drugs and vaccines, and even without knowledge of the aetiological agent, the epidemiology or the pathogenesis of the disease. ISA still occurs at a low prevalence in Norway, while the other previously affected countries seem to have controlled the disease with no, or only a few sporadically occurring, disease outbreaks. Epidemiological information on ISA may be found in the OIE's World Animal Health Information System (WAHIS) Database – WAHID (http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home).

ISA does not usually cause high mortalities at the start of an outbreak, and if proper control measures are implemented, an outbreak may be controlled with only minor mortalities. Even so, economic losses may be significant due to the various measures and restrictions implemented. However, if inadequate measures are implemented, the outbreak may develop into a serious disease problem both in the salmon farm concerned and in adjacent farms (Lyngstad *et al*., 2008). Indeed, there are multiple examples from Norway where single ISA outbreaks have developed into small endemics. The only solution in such cases has been to fallow entire areas.

The causative agent of ISA is infectious salmon anaemia virus (ISAV), the only member of the genus *Isavirus*, family *Orthomyxoviridae* (Palese and Shaw, 2007). The two glycoproteins embedded in the ISAV envelope, the haemagglutinin esterase (HE) glycoprotein and the fusion (F) glycoprotein, are important for virus uptake and cell tropism (Falk *et al*., 2004; Aspehaug *et al*., 2005; Aamelfot *et al*., 2012) . In addition, the virion is formed by two other major structural proteins, the nucleoprotein (NP) (Aspehaug *et al*., 2004; Falk *et al*., 2004) and the matrix (M) protein (Biering *et al*., 2002; Falk *et al*., 2004). The HE proteins bind the cellular

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receptors of ISAV, which are 4-*O*-acetylated sialic acids (Hellebo *et al*., 2004), expressed on endothelial cells and red blood cells (RBCs) in the host (Aamelfot *et al*., 2012).

The segmented ISAV genome is highly conserved. The two gene segments with the highest variability are those coding for the HE and F proteins. Phylogenetic analyses of ISAV isolates, based on the HE gene, revealed two major clades, one European and the other North American. In addition, ISAV has been characterized and typed based on the amino acid patterns of a highly polymorphic region (HPR) consisting of 11–35 amino acid residues in HE (Rimstad *et al*., 2011), just upstream of the transmembrane domain. The virulent HPR variants may be explained as differential deletions (Mjaaland *et al*., 2002) of a putative non-virulent full-length ancestral sequence (HPR0). The HPR0 variant was first identified in wild salmon in Scotland (Cunningham *et al*., 2002). Whereas all ISAV isolates from ISA disease outbreaks have deletions in the HPR region, which are often denoted HPR-deleted, the HPR0 subtype has not been associated with clinical or pathological signs of ISA (Cunningham *et al*., 2002; Mjaaland *et al*., 2002; Cook-Versloot *et al*., 2004; McBeath *et al*., 2009; Christiansen *et al*., 2011). Epidemiological studies show that ISAV HPR0 variants occur frequently in sea-reared Atlantic salmon. The HPR0 strain seems to be more seasonal and transient in nature and displays a cell and tissue tropism with prevalence on gill epithelial cells (Christiansen *et al*., 2011; Lyngstad *et al*., 2012; Aamelfot *et al*., 2016) and possibly also on the skin (Aamelfot *et al*., 2016). A peculiarity of the ISAV HPR0 type is that, unlike HPR-deleted ISAV, it cannot be replicated in currently available cell cultures (Christiansen *et al*., 2011). The risk of emergence of pathogenic HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low, but not negligible (Christiansen *et al*., 2011; EFSA Panel on Animal Health and Welfare (AHAW), 2012; Lyngstad *et al*., 2012).

Other gene segments may also be important for the development of ISA. A putative virulence marker has been identified in the F protein. Here, a single amino acid substitution, or a sequence insertion, near the putative cleavage activation site of the protein, is a prerequisite for virulence (Kibenge *et al*., 2007; Markussen *et al*., 2008; Fourrier *et al*., 2015). Indeed, Fourrier *et al*. (*2015*) recently demonstrated that the combination of deletions in the HPR and certain amino acid substitutions in the F protein may influence the proteolytic activation and activity of the F protein.

Outbreaks of ISA have only been detected in farmed Atlantic salmon, and the majority of cases have occurred during the seawater stage of the salmon life cycle. However, experimental infection trials have demonstrated that Atlantic salmon of different developmental stages are equally susceptible to the infection in both fresh water and salt water. Viral replication without clinical disease has been demonstrated experimentally in other fish species, including brown trout (*S. trutta* L.), rainbow trout (*Oncorhynchus mykiis* (Walbaum)), Arctic charr (*Salvelinus alpinus* L.), chum salmon (*O. keta* (Walbaum)), coho salmon (*O. kisutch* (Walbaum)), herring (*Clupea harengus* L.) and Atlantic cod (*Gadus morhua* L.) (Rimstad *et al*., 2011). ISAV has also been detected in healthy wild Atlantic salmon and sea trout (*S. trutta* L.) (Raynard *et al*., 2001; Plarre *et al*., 2005). These wild fish or fish of other species may act as carriers of, or reservoirs for, the virus.

The infectivity of ISAV is anticipated to be retained for a long time outside the host. In an experiment using both fresh water and seawater at different temperatures, Tapia *et al*. (2013) found the virus survives for 5 days in seawater at 20°C up to 70 days in fresh water at 10°C, but there is no knowledge of viral survival under natural conditions, where it may adhere to, and be protected by, organic matters. Waterborne transmission has been demonstrated in cohabitation experiments, indicating this is an important route for the spread of ISA within and between nearby farms (Thorud and Djupvik, 1988; Lyngstad *et al*., 2008). The virus may be shed into the water via various routes, including the skin, mucus, faeces, urine and blood, and within waste from dead fish (Totland *et al*., 1996). The main route of entry is thought to be the gills (Rimstad *et al*., 2011), although recent studies have demonstrated that early replication occurs in both the gills and the skin (Aamelfot *et al*., 2015).

Horizontal spread of the virus is well documented during outbreaks both within and between farms, but in most cases, investigators could not document how the infection was initially introduced. Infections/outbreaks after the transfer of infected smolt have been demonstrated in a few cases, while transfer by various farming equipment, including well-boats, is important (Vågsholm *et al*., 1994; Jarp and Karlsen, 1997; Murray *et al*., 2002).

The transition of virulent HPR-deleted virus from the frequently occurring HPR0 type has been

suggested (Cunningham *et al*., 2002; Mjaaland *et al*., 2002; Lyngstad *et al*., 2012), though the significance and frequency of this has not been documented. A reservoir in the marine environment is also a possibility, as several fishes can have subclinical infections.

Finally, a number of reports have suggested the possibility of vertical transmission (Melville and Griffiths, 1999; Nylund *et al*., 2007; Vike *et al*., 2009; Marshall *et al*., 2014), but there is no confirmation of this (Rimstad *et al*., 2011). Experience-based information from Norwegian salmon farming operations has suggested that vertical transmission is not a significant concern.

6.2 Diagnosis

Field outbreaks of ISA in Atlantic salmon vary considerably in the development of the disease, its clinical signs and histological changes. This reflects the complex interaction between the virus, host and environment. A peculiarity of ISA is that clinical disease often spreads in a non-systematic way, slowly from net pen to net pen within a farm, possibly reflecting the extended time from infection to the development of severe anaemia and clinical disease. The incubation period in natural outbreaks varies from a few weeks to several months (Vågsholm *et al*., 1994; Jarp and Karlsen, 1997).

Diseased fish are usually lethargic, and often display abnormal swimming behaviour. Daily mortality is typically 0.05 to 0.1%. If nothing is done to limit disease development, the disease may spread, and the accumulated mortality in a farm can reach more than 80% over several months. In a disease outbreak with low mortality, the clinical signs and macroscopic pathological changes may be limited to anaemia and circulatory disturbances, including haemorrhages. The chronic disease phase with low mortality can easily be overlooked. Occasionally, episodes of acute, high mortality over a couple of weeks may ensue, especially if no measures are taken. The pathology is then more severe, with ascites and haemorrhages dominating. The disease appears throughout the year though outbreaks are more frequently detected in spring or early summer and in late autumn.

The most prominent clinical signs of ISA include pale gills (except in the case of blood stasis in the gills), exophthalmia, distended abdomen, blood in the anterior eye chamber and, sometimes, skin haemorrhages, especially of the abdomen (Fig. 6.1), as well as scale pocket oedema. Severe anaemia

Fig. 6.1. Atlantic salmon with infectious salmon anaemia (ISA) showing typical skin bleedings.

with haematocrit values below 10% is common (Thorud and Djupvik, 1988; Evensen *et al*., 1991; Thorud, 1991; Rimstad *et al*., 2011).

The diagnosis of ISA is based on the combined evaluation of clinical signs, macroscopic lesions and histological changes, supplemented with immunohistochemical (IHC) examinations for endothelial infection (see Fig. 6.4). IHC examination is of particular importance as this method may establish a direct link between the virus and the disease. Positive IHC findings are confirmed using quantitative (real-time) reverse-transcription PCR (qRT-PCR) and viral isolation. Usually, the HE and F genes are also sequenced for use in epidemiological evaluations and to determine the viral type. A description of the methods used is given in the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2015b).

Differential diagnoses include other anaemic and haemorrhagic conditions, including erythrocytic inclusion body syndrome, winter ulcer and septicaemias caused by infections with *Moritella viscosa*. Disease cases in Atlantic salmon with haematocrit values below 10% are not a unique finding for ISA; nevertheless, cases with low haematocrits without any obvious cause should always be tested for ISAV (OIE, 2015b).

6.3 Pathology

Fish infected with HPR-deleted ISAV may vary from showing no gross pathological changes to having severe lesions, depending on the size of the infective dose, viral strain, water temperature, and

age and immune status of the fish. The most prominent sign is anaemia, often with a haematocrit below 10%, and circulatory disturbances. External signs include pale gills, localized haemorrhages of the eyes and skin (Fig. 6.1), exophthalmia, and scale oedema. However, the disease may appear in different manifestations, and development may appear as an acute form or as a slowly developing chronic disease (Rimstad *et al*., 2011).

Ascites, swollen spleen, oedema and petechial bleeding on the serosa are common. More variable, but very obvious when present, are severe haemorrhagic lesions in the liver, kidney, gut or gills. The 'classical' liver manifestation of ISA is characterized by dark liver due to haemorrhagic necrosis (Evensen *et al*., 1991).

Clinical signs and pathology may be more subtle in the slowly developing chronic form of the disease. The liver may appear pale or yellowish and the anaemia may not be as severe as in the acute disease. Less ascites fluid is found than in the acute form, but haemorrhages in the skin and swim bladder and oedema in the scale pockets and swim bladder can be more pronounced than in acutely diseased fish (Evensen *et al*., 1991; Rimstad *et al*., 2011).

Histological changes in the liver include zonal hepatocellular degeneration and necrosis (Fig. 6.2) (Evensen *et al*., 1991; Speilberg *et al*., 1995; Simko *et al*., 2000). The lesions result in extensive congestion of the liver with dilated sinusoids, and in later stages, the appearance of blood-filled spaces. Speilberg *et al*. (1995) investigated the liver pathology of experimentally infected salmon, and detected sinusoidal endothelial degeneration and loss preceded by the degeneration of hepatocytes and multifocal haemorrhagic necrosis; the virus was not observed in affected hepatocytes. These observations were confirmed using IHC examinations, during which no virus was detected in the hepatocytes in the pathological lesions (Aamelfot *et al*., 2012).

The haematopoietic tissue in the kidney is primarily affected by the haemorrhages but, in severe cases, the kidney tubules become necrotic (Fig. 6.3) (Byrne *et al*., 1998). The kidney haemorrhages are most easily detected via histology as the kidney is diffusely dark in colour. Intestinal haemorrhages may resemble haemorrhagic enteritis. In fresh specimens, there is no blood in the gut lumen, but histological sections are characterized by extensive bleeding within the lamina propria with no inflammation. In some cases, the only pathological findings are anaemia and general circulatory disturbances. Although

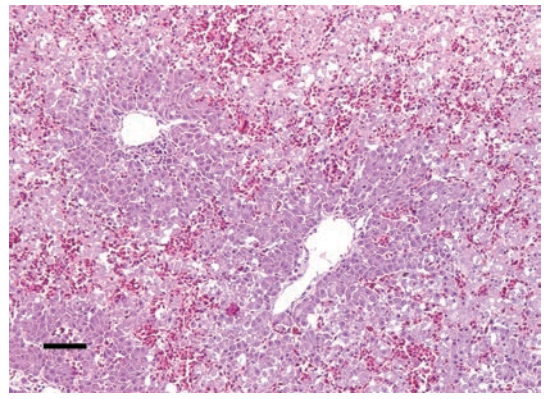

Fig. 6.2. Histological section of Atlantic salmon with infectious salmon anaemia (ISA) showing liver zonal haemorrhagic necrosis (haematoxylin–eosin stain). Scale bar = 50 µm. Courtesy of Dr Agnar Kvellestad.

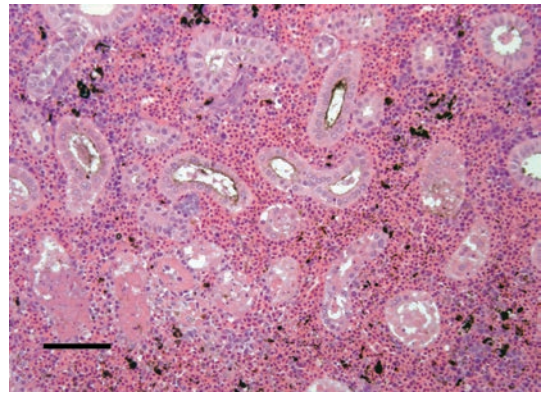

Fig. 6.3. Histological section of Atlantic salmon with infectious salmon anaemia (ISA) showing kidney haemorrhages and tubular necrosis (haematoxylin–eosin stain). Scale bar = 50 µm.

virus-infected endothelial cells are in all organs, a striking feature is the limited inflammatory cellular response (Aamelfot *et al*., 2012).

Haemorrhages may be divided into different manifestations, i.e. liver, kidney, gut and gill manifestations, while the spleen is more constantly swollen and dark (Rimstad *et al*., 2011; Aamelfot, 2012). The kidney manifestation is characterized by moderately swollen kidneys with interstitial haemorrhaging and some tubular necrosis (Byrne *et al*., 1998; Simko *et al*., 2000). The gut manifestation is characterized by a dark red gut due to haemorrhaging within the intestinal wall, but not in the

lumen (in fresh specimens). The gill manifestation is an exception to the pale anaemic gills as blood has accumulated, especially in the central venous sinus of the gill filaments. The haemorrhagic organ lesions are visible on autopsy in organs such as the liver and gut, but are less obvious in the gills and kidney. In any particular ISA outbreak, one of the haemorrhagic organ manifestations may dominate, whereas in other outbreaks all manifestations can be found, even within the same fish. Outbreaks dominated by either the liver or kidney manifestation are most common. However, the haemorrhagic organ lesions can be absent or very rare in the initial stages of an outbreak, leaving only the anaemia and the more subtle circulatory disturbances as initial clues to the aetiology of the disease.

6.4 Pathogenesis

The disease is a generalized and lethal condition of farmed Atlantic salmon, with terminal ISA characterized by anaemia, bleeding and circulatory disturbances. The exact mechanisms behind this pathology are still somewhat obscure, though possible factors are starting to emerge (Aamelfot *et al*., 2014). Significant differences in disease appearance and severity are common, even with the assumed same isolate. Thus, when evaluating the pathogenesis of ISA, interactions between the host, the infectious agent and the environment (i.e. the aetiological triad) must be considered.

Thorud (1991) who examined anaemic fish from ISA field outbreaks, found increased RBC fragility, increased numbers of immature RBCs and increased proportions of RBC ghost (or smudge) cells, all indicating a haemolytic anaemia. However, neither jaundice nor other signs of excess haemoglobin breakdown were observed, possibly because the anaemia occurs in the terminal stages of the disease. Leucopenia, including lymphocytopenia and thrombocytopenia, were observed, and these were attributed to general stress due to the disease. Other plasma parameters revealed an increase in aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase, which indicates organ damage. Finally, the increase in plasma osmolality indicated impaired osmoregulation.

Viral virulence is multifactorial as it depends on factors such as receptor binding, cellular uptake, replication rate and shedding of new virions, modulation of the host immune response and the ability to spread to new hosts (McBeath *et al*., 2007; Purcell *et al*., 2009; Medina and Garcia-Sastre, 2011; Peñaranda *et al*., 2011; Wargo and Kurath, 2012; Cauldwell *et al*., 2014; McBeath *et al*., 2015). Thus, evaluating pathogenesis may involve several features in addition to host and environmental factors. By comparison to the related influenza viruses, ISAV has equivalent functional features, including viral receptor binding, fusion activity, receptor destroying activity, replication efficiency promoted by the viral polymerases, and the ability to modulate the host immune response, all of which are important factors for disease development (Palese and Shaw, 2007; Rimstad *et al*., 2011).

Major target cells are the endothelial cells lining the blood vessels of all organs, including the sinusoids, endocardium (Fig. 6.4), scavenger endothelial cells in the anterior kidney and, possibly, the endothelium of the secondary vessel system (Rummer *et al*., 2014) and RBCs. Evensen *et al*. (1991) had earlier suggested endothelial cells as possible target cells. Using electron microscopy, it was demonstrated that endothelial damage followed by hepatocellular degeneration preceded anaemia in ISAV-infected salmon. In addition, Evensen *et al*. (1991) found increased splenic phagocytosis. This was corroborated by Aamelfot *et al*. (2012), who detected generalized haemophagocytosis, in addition to the *in situ* haemadsorption of RBCs to ISAV-infected endothelial cells. Aamelfot *et al*. (2012) also demonstrated intact endothelium with no cytopathic effects or significant perivascular infiltration of leucocytes, i.e. inflammation and

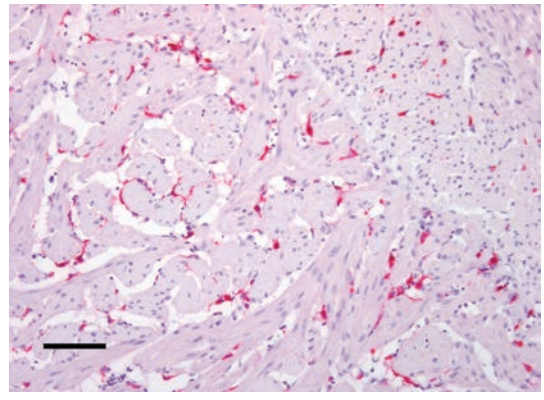

Fig. 6.4. Immunostained histological section of heart of Atlantic salmon with infectious salmon anaemia (ISA). The red coloured endothelial cells are infected with infectious salmon anaemia virus (ISAV). Scale bar = 50 µm.

apoptosis were not detected, indicating that ISAV infection does not incite inflammation or cause cytopathic effects in infected cells. This is not consistent with findings reported from systemic influenza in poultry, where apoptosis and inflammation are common (Kobayashi *et al*., 1996; Schultz-Cherry *et al*., 1998). Similar findings were reported for other endotheliotropic viruses such as filoviruses (i.e. Ebola and Marburg), in which infection of the endothelial cells does not appear to disrupt the cell architecture (Geisbert *et al*., 2003). Nevertheless, the permeability of the infected endothelial cells may still be compromised.

Aamelfot *et al*. (2012) also investigated the pathological lesions in the liver and kidney of severely anaemic moribund Atlantic salmon from confirmed ISA outbreaks. The necrotic lesions of the parenchyma appeared not to be infected by ISAV. A possible explanation is that the lesions are not directly caused by the infection. They may rather be indirect or secondary and caused by the host response, which destroys the RBCs and creates hypoxia in severely anaemic fish. Such hypoxic conditions may start a vicious circle leading to hypovolaemic shock and death, similar to that suggested for Ebola virus endothelial infection (Schnittler *et al*., 1993).

While classical ISA is characterized by an endothelial infection of the circulatory system, the cell tropism during infection with non-pathogenic ISAV HPR0 is different. Although this virus is in both the gills and skin during infection, only epithelial cells become infected (Aamelfot *et al*., 2016). The transition from ISAV HPR0 to HPR-deleted virus results in a modified fusion protein activity (Fourrier *et al*., 2015), which highlights the fusion protein as a factor determining cell tropism and virulence during ISAV infections, in addition to the role of the receptor-binding HE protein. In addition, viral fusion activity also influences virus cell uptake and replication efficiency.

ISAV uptake has been studied via immersion experiments using viruses of high and low virulence (Aamelfot *et al*., 2015; McBeath *et al*., 2015). In both cases, a transient initial infection of epithelial cells was demonstrated; with the low virulence virus, early epithelial infection was more pronounced and lasted longer. It also disseminated more quickly to internal organs and induced a more rapid systemic host immune response. This response may have offered some protection to the fish, and at least partly explains the lower virulence and mortality caused by this virus isolate.

Viruses of the family *Orthomyxoviridae* can bind to or haemagglutinate RBCs. ISAV haemagglutinates RBCs from Atlantic salmon, rainbow trout, horse, donkey and rabbit, but not RBCs from brown trout. The expected elution of the *in vitro* haemagglutination reaction due to the viral esterase (i.e. the virus receptor-destroying enzyme on the HE protein), is observed on RBCs from all species examined, except Atlantic salmon (Falk *et al*., 1997).

ISAV binds extensively to circulating RBCs, and RBCs attach to infected endothelial cells, which may be regarded as *in vivo* haemadsorption (Aamelfot *et al*., 2012). These observations may have significant importance for disease pathogenesis and may explain several observations. First, the attachment of virus to RBCs may compromise cell membrane integrity, which increases RBC fragility as observed by Thorud (1991). This is supported by experimental data demonstrating that the occurrence of large amounts of virus on RBCs coincides with increased RBC fragility (K. Falk, 2016, unpublished results). Secondly, virus-coated RBCs may be regarded as foreign by the host scavenger system, which explains the increased haemophagocytosis observed. Indeed, canine RBCs with attached influenza virus survive only a fraction of their expected lifespan (Stewart *et al*., 1955). Thirdly, the thrombi observed in early reports of ISA pathology (Evensen *et al*., 1991) and the attachment of RBCs to ISAV-infected cells (Aamelfot *et al*., 2012) can also be explained by *in situ* haemagglutination and haemadsorption. Fourthly, the viral receptors on the RBC surface may act as decoy receptors, and interact with virus dissemination, as noted by Baum *et al*. (2002). Whether this will promote spread of infection, as reported for HIV (Baum *et al*., 2002), or delay the infection, as reported for parvovirus (Traving and Schauer, 1998), needs further examination. In addition, ISAV is not released from Atlantic salmon RBCs under in *vitro* conditions (Falk *et al*., 1997). Although the significance of this latter observation is unknown, we have identified a couple of ISAV isolates that were eluted under *in vitro* conditions from Atlantic salmon RBCs. When one of these isolates was tested in an experimental infection, the isolate had low virulence, resulting in lower mortality and less pathology than a highly virulent virus (McBeath *et al*., 2015).

Briefly, these observations indicate that the interaction between ISAV and RBCs is important in pathogenesis, and particularly explains the anaemia, but potentially also other clinical signs. Further examination of this interaction may contribute to

understanding of the pathogenesis of other haemagglutinating viruses.

Both innate and adaptive cellular and humoral immune responses against ISAV have been experimentally demonstrated in Atlantic salmon. This includes specific antibody responses (Falk and Dannevig, 1995; Lauscher *et al*., 2011) and interferon-related responses (McBeath *et al*., 2007; LeBlanc *et al*., 2010; McBeath *et al*., 2015). Furthermore, the ISAV non-structural proteins have interferon antagonistic activities (McBeath *et al*., 2006; Garcia-Rosado et al., 2008), though the significance of this in relation to pathogenesis and virulence is not known.

6.5 Protective and Control Strategies

ISA outbreaks often develop slowly and the disease spreads locally separated in time and space, which suggests horizontal transmission (Lyngstad *et al*., 2008, 2011). However, the origin of the infection in the farmed salmon population is unknown. Unidentified reservoirs, the maintenance in vectors such as salmon lice (*Lepeophtheirus salmonis*) (Krøyer, 1837), subclinical infections and/or vertical transmission are all possible. In addition, the emergence of virulent HPR-deleted ISAV through transitions from the prevalently occurring non-virulent HPR0 ISAV is considered likely.

The incidence and impact of ISA may be greatly reduced by the implementation of general biosecurity measures that reduce horizontal transfer and infection pressure. These measures include early detection, the isolation and slaughter of diseased fish, general restrictions on transport, the disinfection of offal and waste from slaughterhouses, year-class separation at farm sites, and improved health control and certification (Håstein *et al*., 1999). In Norway, the result of these actions was an improvement in the sanitary situation in the fish farming industry. Together with significant improvements in husbandry practices, better laboratory identification and subsequent restrictions imposed on farms with ISA, a remarkable and rapid reduction in the number of ISA outbreaks was obtained (Håstein *et al*., 1999).

Hence, ISA can be controlled if it is detected early, taken seriously and the correct measures are implemented. There are numerous examples showing that if initial outbreaks are not treated correctly, they will develop into devastating epidemics that are very costly. Both the Faroese and the Chilean Atlantic salmon farming industry have experienced significant production and economic setbacks due to ISA outbreaks (Rimstad *et al*., 2011).

Norway, Scotland, the east coast of Canada, the Faroe Islands and Chile have experienced major ISA epidemics. The strategies adopted to control or combat the disease are slightly different in these countries. In Norway, ISA is accepted as endemic in most areas, with 2–20 annual outbreaks. When detected, strict control measures, including slaughtering and zoning, are implemented to limit dissemination. Recently, vaccination has become more common. Following the ISA epidemic in Scotland in 1998, an eradication procedure was applied, and Scotland is still ISA free. Canada, the Faroe Islands and Chile have implemented a combination of biosecurity procedures and vaccination following their outbreaks, and are currently experiencing either no outbreaks or very few occasional outbreaks.

As ISA is slow to develop, the biosecurity is important for control, and an efficient vaccine will supplement other control strategies. The currently available vaccines are inactivated whole virus grown in cell culture added to mineral oil adjuvants. These vaccines are commonly used on the east coast of Canada, in the Faroe Islands and Chile and, to some extent, in Norway. Due to European Union (EU) regulations, vaccination in European aquaculture is not allowed in areas that have been declared free of ISA and Scotland is an example.

Reports on ISA vaccines are scanty in the scientific literature. Jones *et al*. (1999) reported a relative percentage survival (RPS) of 84–95% in experimental trials, using an adjuvanted, inactivated ISA virus from culture preparations. More recently, Lauscher *et al*. (2011) also tested a virus cell culture preparation against experimental infections, and found a RPS of 86%. Protection depended on the amount of vaccine antigen injected and correlated with the anti-ISAV antibodies. There are also several conference abstracts and Internet publications that support these findings.

Vaccination against ISAV was first attempted in Canada in 1999, where this was combined with control programmes and general biosecurity measures. However, ISA outbreaks were recorded in vaccinated populations, so the efficacy of the vaccine was questioned. There are no reports documenting the field performance of ISAV vaccines. Another example of the use of ISA vaccine includes the successful control programme following multiple ISA outbreaks in the Faroe Islands from 2000 to 2005. Almost all the Faroese farms were fallowed, and a strict control regime based on general hygienic principles was

implemented. Most fish were vaccinated with an oil-adjuvanted inactivated cell culture grown ISA vaccine (Christiansen *et al*., 2011). No ISA outbreaks have been recorded since 2005, though an extensive surveillance programme has frequently found the non-virulent ISAV HPR0 type. It was also speculated that vaccination might have prevented the transition of the ISAV HPR0 virus to the virulent HPR-deleted virus (Christiansen *et al*., 2011).

Although commercial vaccine development has mainly concentrated on inactivated ISAV cell culture preparations, vaccine research using molecular principles has been reported. Mikalsen *et al*. (2005) injected plasmids coding for the HE protein intramuscularly (IM) three times at 3 week intervals; there was modest protection, with an RPS of 40–60%. After vaccination, the fish were challenged via intraperitoneal (IP) injection, which may explain the poor protection achieved. Wolf *et al*. (2013) vaccinated fish by IM injection of an ISAV haemagglutinin-esterase-expressing salmonid alphavirus replicon, followed by cohabitation challenge. The RPS ranged from 65 to 69%, compared with 80% in controls immunized with an inactivated ISAV cell culture antigen.

More interesting are two reports from Chilean research groups that describe oral vaccination. Both groups based their vaccines on chitosan-based encapsulation. Rivas-Aravena *et al*. (2015) used cell culture grown virus and also included a novel adjuvant – the DNA coding for the replicase of alphavirus – in the chitosan particles. They demonstrated a RPS of 77%. Caruffo *et al*. (2016) used recombinant expressed ISAV HE and F proteins for the chitosan encapsulation, and reported an RPS of 64%. Thus, in both reports, the vaccines were targeting the mucosal surface of the gut. However, in both studies, the fish were challenged via injection, which might have reduced the efficacy of the vaccines. The advantages of oral vaccination are: (i) it avoids serious side effects related to oil-adjuvanted vaccines; and (ii) it allows for booster vaccination.

There are no reports on the use of antiviral agents, chemotherapeutics or other pharmaceutical treatments to treat ISA.

6.6 Conclusions and Suggestions for Future Research

Infectious salmon anaemia is a slowly developing, generalized and lethal disease that may cause detrimental effects in farmed Atlantic salmon. It is an OIE reportable disease, and control programmes have been implemented in most Atlantic salmon farming regions. The diagnosis is usually made based on pathological examination, including IHC and RT-PCR.

The disease is mainly characterized by anaemia, with infection of endothelial cells throughout the circulatory system. The mechanism of pathogenesis is still obscure; however, the observed binding of virus to RBCs is suggested to be an important factor.

Outbreaks are relatively easy to control if they are detected early and appropriate and strict biosecurity measures are implemented immediately. Although experimental vaccines work well under laboratory conditions, their efficacy has not been documented in the field. The production of vaccines based on cultured virus has limitations because production of virus in cell cultures is generally low and this limits the amount of antigen in the vaccine. Vaccines based on ISAV virulence factors should be developed and their efficiencies in field situations should be tested and documented.

How ISA is introduced into, and maintained in, the salmon population need further study. Possible mechanisms include the existence of marine reservoir(s), vertical and/or horizontal transmission, the occurrence of low-virulence viruses causing undetected subclinical infections and the development of virulent ISA virus from the frequently occurring and non-virulent HPR0 type.

The current major challenges associated with ISA control are questions related to risk and factors affecting the transition of non-virulent HPR0 ISAV to virulent HPR-deleted ISAV. These questions have become important not only to the understanding of ISA epidemiology, but also in relation to the international Atlantic salmon trade, as both virus types are notifiable (OIE, 2015a). Thus, the detection of ISAV HPR0 can be, and has already been, used to limit salmon export. To address and solve the key question of the transition of non-virulent HPR0 ISAV to virulent HPR-deleted ISAV we need to elucidate how and why the transition occurs. As this transition is expected to be a stepwise process involving several mutations, there is also a need to know more about ISAV virulence disease mechanism(s).

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7 Spring Viraemia of Carp

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7.1 Introduction

Spring viraemia of carp (SVC) is often a fatal haemorrhagic disease of common carp, *Cyprinus carpio*, and other fishes. It is caused by the spring viraemia of carp virus (SVCV), a rhabdovirus (Fig. 7.1) of the type species, *Carp sprivivirus*, in the genus *Sprivivirus* (Stone *et al*., 2013; Adams *et al*., 2014). SVC is reportable to the World Organisation for Animal Health (OIE), but the virus is serologically related to other non-reportable viruses.

7.2 Hosts

SVC has been described in numerous hosts, although some early reports of the disease may actually have been carp erythrodermatitis, a bacterial disease. In addition to the common and koi carp (*C. carpio*), hosts include the following cyprinids (where no citation is referenced, check Dixon, 2008): bighead carp, *Aristichthys nobilis*; crucian carp, *Carassius carassius*; grass carp, *Ctenopharyngodon idella*; silver carp, *Hypophthalmichthys molitrix*; goldfish, *Carassius auratus*; orfe, *Leuciscus idus*; tench, *Tinca tinca*; roach, *Rutilus rutilus*; bream, *Abramis brama* (Basic *et al*., 2009); and emerald shiner, *Notropis atherinoides* (Cipriano *et al*., 2011). Non-cyprinid hosts include: sheatfish (other names, European catfish, wels), *Silurus glanis*; pike, *Esox lucius*; Siberian sturgeon, *Acipenser baerii* (Vicenova *et al*., 2011); largemouth bass, *Micropterus salmoides*; and bluegill sunfish, *Lepomis macrochirus* (Cipriano *et al*., 2011; Phelps *et al*., 2012).

SVCV has been isolated from rainbow trout, *Oncorhynchus mykiss* (Stone *et al*., 2003; Jeremic *et al*., 2006; Haghighi Khiabanian Asl *et al*., 2008a; I. Shchelkunov, personal communication) and SVCV nucleotides have been found in trout (Shchelkunov *et al*., 2005). The virus was not pathogenic to rainbow trout following experimental bath infection and the virus isolated by Jeremic *et al*. (2006) was also not pathogenic to rainbow trout following intraperitoneal (IP) injection (P.F. Dixon, J. Munro and D.M. Stone, unpublished data), though both strains of the virus were pathogenic to common carp. In contrast to the aforementioned results, a recent study (Emmenegger *et al*., 2016) has shown that rainbow trout were moderately susceptible to a North American isolate of SVCV following IP injection.

Viruses isolated from the black bullhead catfish, *Ictalurus melas* (preferred name *Ameiurus melas*) (Selli *et al*., 2002) and Nile tilapia, *Sarotherodon niloticus* (preferred name *Oreochromis niloticus*), were identified as SVCV based on tissue immunohistochemistry; Soliman *et al*., 2008). As antibodies against SVCV will cross-react with other viruses, the aforementioned species should not be considered hosts without more robust confirmation. A reverse transcription polymerase chain reaction (RT-PCR) apparently identified nucleotide sequences of SVCV in tissues from three diseased Indian carp species (rohu, *Labeo rohita*; merigal, *Cirrhinus mrigala*; catla, *Catla catla*), but the virus was not isolated (Haghighi Khiabanian Asl *et al*., 2008b). However, the nucleotide sequence data deposited at Genbank shares <80% nucleotides with known SVCV nucleotide sequence (D.M. Stone, unpublished data).

A rhabdovirus of penaeid shrimps was isolated from *Litopenaeus stylirostris* and *L. vannamei* in

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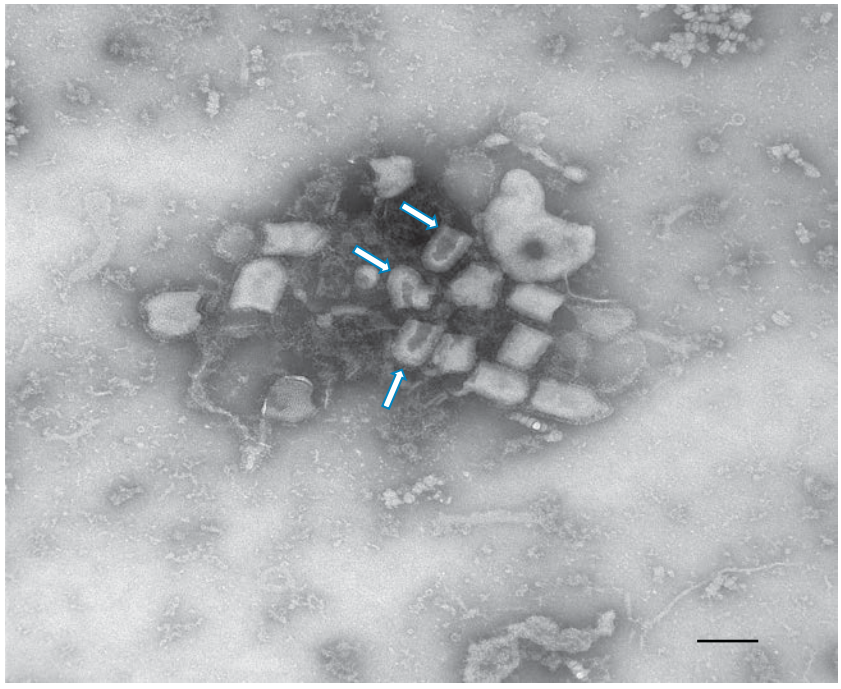

Fig. 7.1. Negatively stained spring viraemia of carp virus (SVCV) seen by transmission electron microscopy showing numerous bullet-shaped particles with a nucleocapsid surrounded by an envelope with prominent spikes on the surface. Bar = 100 nm. Image courtesy Dr J V Warg.

Hawaii, and replicated in both species (see Dixon, 2008). The nucleotide sequence of its G (glycoprotein) gene is identical to that of SVCV, and the two viruses should be considered genuine SVCV. Hence, SVCV has been in Hawaii even though the disease has not been reported. It is also not known whether the virus is still present there.

Other fish species are susceptible to SVCV under experimental conditions, but because there is insufficient supporting data, these will not be discussed. The zebrafish, *Danio rerio*, is important in that it is increasingly used to model SVCV–host interactions (Sanders *et al*., 2003).

7.3 Geographic Range

SVC was for a long time only recorded in the carp-producing countries of Europe (see Dixon, 2008; Rexhepi *et al*., 2011), but since 2000, the geographic range of SVCV isolations has expanded, though the number of isolations has not mirrored the occurrence of the disease. For example, the virus has been isolated from cultured common and koi carp in China and found in wild carp in Canada, but the disease has not been observed in either country (Garver *et al*., 2007; Zhang *et al*., 2009). However, SVCV has been associated with mortality in common and koi carp in the USA (see Warg *et al*., 2007; Phelps *et al*., 2012). SVCV antigens were detected in goldfish tissues using an enzyme-linked immunosorbent assay (ELISA) in Brazil, but viral isolation was not attempted (Alexandrino *et al*., 1998), but further confirmation is needed before Brazil is included within the geographic range of SVC.

7.4 Mode of Transmission

SVC has a wide temperature range but field mortalities in carp usually occur between 5 and 18°C (Fijan, 1988; Ahne *et al*., 2002). Progress of the disease is often rapid above 10°C, and it occurs in spring, particularly following a cold winter. Experimentally, a rise in temperature is not needed to cause disease, but higher mortalities or a more rapid disease course occurred in fish that had overwintered compared with those infected in autumn; the poor condition of the overwintered fish was a possible risk factor (Baudouy *et al*., 1980a,b,c).

In aquaculture, 9–12 and 21–24 month old carp are routinely affected, although all ages are susceptible (Fijan, 1988). In experimental studies, grass carp and common carp became more resistant with age (Shchelkunov and Shchelkunova, 1989), but adult wild common carp was affected (Marcotegui *et al*., 1992; Dikkeboom *et al*., 2004; Phelps *et al*., 2012). Also, under experimental conditions, wild common carp were more susceptible to SCV than farmed carp (Hill, 1977).

The transmission of SVCV is horizontal (Fijan, 1988). The virus has been isolated from ovarian fluid (Békési and Csontos, 1985), but vertical transmission has not been demonstrated. SVCV appears to enter via the gills (see Dixon, 2008) and then spreads to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks, high titres of virus are detected in the liver and kidneys of infected fish, whereas lower titres are found in the gills, spleen and brain (Fijan *et al*., 1971).

The virus is released into the environment from carcasses and by excretion from infected fish (see Dixon, 2008). There is an inverse relationship between temperature and the duration of viral survival outside the host, e.g. 35 days in river water at 10°C and 42 days in pond sludge at 4°C (Ahne, 1982a,b).

SVCV shed by survivors is probably the main way the virus is transmitted, but not much is known about persistence of the virus in infected fish, the duration of viral shedding or amounts of virus shed. Virus may be shed following a stressful event, particularly from fish in poor condition in the spring following a harsh winter (Fijan, 1988).

The mechanical transmission of SVCV by herons (Peters and Neukirch, 1986), carp louse, *Argulus foliaceus*, and leech, *Piscicola geometra* (Ahne, 1978, 1985a; Pfeil-Putzien, 1978) has been demonstrated experimentally, but is not known whether such transmission occurs in nature. Pfeil-Putzien and Baath (1978) isolated SVCV from carp lice taken from naturally diseased fish, which indicates uptake of the virus from an infected host. Pike preying upon SVCV-infected pike fry became infected (Ahne, 1985b). SVCV may be translocated through the movement of bait fish (Goodwin *et al*., 2004; Misk *et al*., 2016), and invertebrates may be reservoirs of the virus, but there is no evidence to support either mode of transmission.

7.5 Impacts on Fish Production

SVC has caused major losses in carp production, particularly in European countries and in states of the former USSR (Ahne *et al*., 2002), although precise figures are hard to obtain because epizootics are sporadic and losses fluctuate annually (Fijan, 1999). A historic figure from 1980 estimated that SVC caused an annual loss of 4000 t of 1-year-old carp (10–15% of that age group) in Europe (see Sano *et al*., 2011). In some countries, carp are bred for sport fisheries, which is a lucrative leisure industry. Estimated figures from the UK collated in 1997 showed that the cost of the disease was £20,000–230,000 for fish farms, upwards of £30,000 for fisheries and £20,000–30,000 for retail outlets (see Taylor *et al*., 2013). Further losses of production occur in control programmes that require the slaughter of infected fish (Taylor *et al*., 2013), and any control programme will have a detrimental financial impact (see Dixon, 2008).

7.6 Clinical Signs of Disease

The clinical signs of SVC are non-specific, and not all fish will exhibit all of the signs. Initially there may be increasing mortality, often with a rapid onset. Infected fish may swim slowly and erratically, lose balance and swim on their sides. Two of the most obvious and consistent features are abdominal distension and haemorrhages. The latter may occur on the skin, fin bases, eyes and gills, which may be pale (Fig. 7.2). The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. The abdomen is usually filled with a clear fluid, sometimes bloodstained. The spleen is often enlarged, most internal organs are usually oedematous, and organs adhere to each other and to the peritoneum. There are petechial haemorrhages in the musculature and the swim bladder (see Dixon, 2008), though these are uncommon in SVC caused by Asian strains of SVCV (Goodwin, 2003; Dikkeboom *et al*., 2004).

Gaafar *et al*. (2011) conducted a detailed histopathological study in common carp and compared their findings with those of other authors, and Misk *et al*. (2016) recently reported the histopathology of SVC in three experimental hosts. Histopathological studies commonly reveal oedema, inflammation, haemorrhage and necrosis in the liver, pancreas, intestine, spleen, kidney, heart, muscles and air bladder, but again, not all fish will exhibit each feature.

Early in disease development, there is hyperplasia in the spleen, especially of the reticuloendothelium,

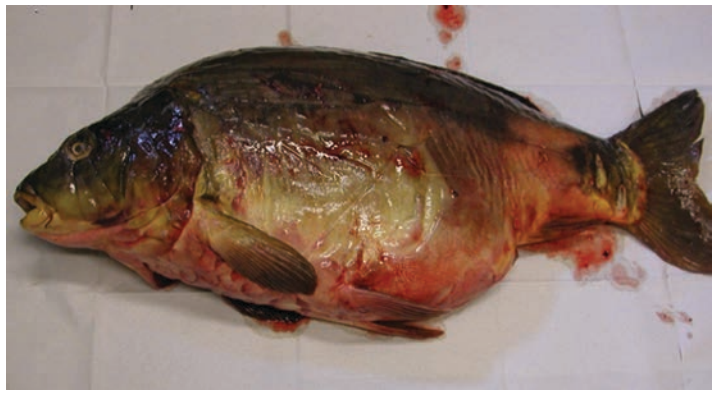

Fig. 7.2. Gross appearance of a common carp (*Cyprinus carpio*) showing the abdominal distension, extensive haemorrhage and exophthalmia consistent with an infection of spring viraemia of carp virus (SVCV).

and the pulp can fill with erythrocytes, while in the later stages of the disease there is dystrophy and cellular degeneration. There can be increased pigmentation, because of larger numbers of iron-containing macrophages (Fig. 7.3A), and degenerative and proliferative changes in the kidney. In severe disease, there is marked oedema, with dissociation of the cells and liquid in the renal glomeruli and clogging in some tubuli. There is focal exfoliation of the epithelium of the urinary tract and nephrosis and peritubular oedema. Haemopoietic tissue is oedematous and its cells show dystrophy and degeneration with haemorrhaging (Fig. 7.3B). In less severe cases, there is an increase in blood filling the kidney and hyperplasia of the haemopoietic tissue, with increased numbers of melanomacrophages.

Periglandular oedema occurs in the islets of the pancreas with infiltration by leucocytes. Hyperplasia of the hepatocytes, and abnormal hepatocytes may be present. There can also be proliferative changes. In liver oedema of the parenchyma, focal or diffuse erythrodiapedesis (extravasation of erythrocytes) and fatty degeneration of hepatocytes are often seen. The hepatic cells are enlarged and exhibit granular or vacuolated dystrophy. The intestine may have degenerative and proliferative changes (Fig. 7.3C). There may be perivascular inflammation of intramural vessels, mucoid degeneration, necrosis and desquamation of the epithelium. Late in infection, the villi may be atrophied. Focal cellular infiltration may occur in the heart in the epicardium and myocardium, followed by focal degeneration and necrosis (Fig. 7.3D). Muscle bundles are friable with the elimination of striations.

The blood vessels of the brain may enlarge with pericellular oedema of the neurons. Eosinic inclusions have been observed in a small number of glial cells. Inflammation of the peritoneum can occur. There can be dilated lymph vessels filled with detritus, macrophages and lymphocytes. Internal mucus may thicken and contain foci of tissue degeneration where the epithelium is exfoliated and desquamated. The gills may show degenerative and proliferative changes, necrosis and infiltrations. There may be diffuse lamellar fusion and hyperplasia of the epithelial lining at the base of cellular lamellae and diffuse branchial necrosis. The numbers of melanomacrophages may increase.

7.7 Diagnosis

Definitive diagnosis of SVC is made by isolation of the virus in cell culture followed by its identification using serological and/or molecular methods. The OIE, in its *Manual of Diagnostic Tests for Aquatic Animals*, includes comprehensive methodology to diagnose and identify SVC (OIE, 2015a). It recommends either the epithelioma papulosum cyprini (EPC) (Fijan *et al.*, 1983) or fathead minnow (FHM) (ATCC CCL-42) cell lines for isolation. When there are high levels of virus in clinical fish, their tissues can be directly tested using serological or molecular methods to provide a rapid presumptive diagnosis while awaiting the results of viral isolation. The isolation of SVCV from survivors, or those with a sublethal infection, is not readily achieved, which can make tracking the distribution of virus-exposed fish difficult. The viral

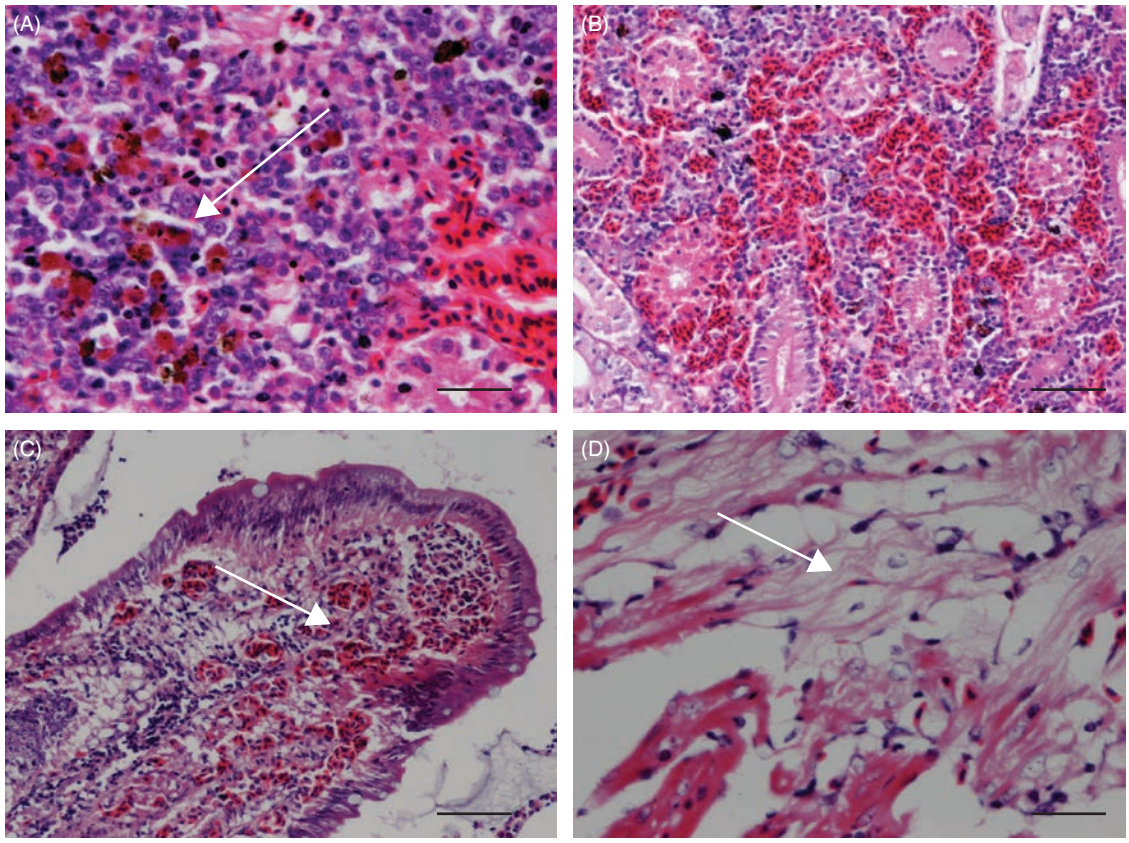

Fig. 7.3. Haematoxylin and eosin stained tissue sections from carp infected with spring viraemia of carp virus (SVCV) showing: (A) a region of cardiomyocytic necrosis (arrow) with loss of cytoplasmic staining and nuclear degeneration; (B) diffuse haemorrhaging within the renal interstitium; (C) haemorrhaging within the lamina propria of an intestinal villus (arrow); and (D) increased numbers of melanomacrophages in the renal interstitial tissue (arrow), Bar = 50 μm in panels A–C and 25 μm in panel D. Images courtesy Dr S.W. Feist.

screening of apparently healthy carp in China and Canada has identified SVCV in both countries (Liu *et al*., 2004; Garver *et al*., 2007). Viral screening in cell culture after each of two consecutive 7 day periods could be required for concluding that a population is negative. Although there is no evidence that viral propagation takes more than 7 days, the cultures can also be checked using molecular methods to enhance the robustness of a negative result.

Virus isolated in cell culture must be identified, but some antisera cross-react with other viruses, such as pike fry rhabdovirus (PFRV). Antisera may also react with some isolates and not others (see Dixon, 2008). Hence definitive identification of SVCV in culture is best achieved by using RT-PCR-based methods. The assay adopted by the OIE targets a partial nucleotide sequence of the G gene of a wide range of SVCV isolates and other closely related viral isolates that could be misidentified as SVCV serologically (Stone *et al*., 2003). According to Stone *et al*. (2003), there are four genogroups: genogroup I comprises SVCV isolates, genogroup II is a single isolate from grass carp, genogroup III comprises the reference PFRV isolate, and genogroup IV, also called the tench rhabdovirus group, comprises unassigned isolates and isolates previously identified as PFRV. However, the International Committee for Taxonomy of Viruses (ICTV) recognizes only two species, SVCV and PFRV, with the grass carp rhabdovirus and tench rhabdovirus considered to belong within the PFRV species (Adams *et al*., 2014).

According to Stone *et al*. (2003), isolates of genogroup I have significant nucleotide sequence divergence, and can be divided into four subgroups by geographic origin. Subgroup Ia isolates are from Asia, the USA and Canada (Garver *et al*., 2007; Phelps *et al*., 2012). Subgroup Ib is from Moldova and Ukraine. Subgroup Ic is from Ukraine and Russia. Subgroup Id is from Europe, plus one isolate from Moldova and one from Ukraine. A potential fifth subgroup, Ie, comprises isolates from Austria (D.M. Stone unpublished data). Phylogenetic comparisons of isolates based on the G gene may become a valuable tool for tracking the source of SVCV isolates (Stone *et al*., 2003).

Additional conventional RT-PCR, quantitative real-time PCR and loop-mediated isothermal amplification assays are available for detecting and confirming SVCV infections (Koutná *et al*., 2003; Liu *et al*., 2008a,b; Yue *et al*., 2008; Zhang *et al*., 2009; Shimahara *et al*., 2016). With the exception of the conventional PCR assay developed by Shimahara *et al*. (2016), the rest of these assays were not validated against representatives from each of the recognized SVCV genogroups, and in the case of the real-time assays (Liu *et al*., 2008a; Yue *et al*., 2008), they failed to detect isolates from at least one subgroup (D.M. Stone, unpublished data). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera, and this can be used to screen a virus culture (Ruane *et al*., 2014).

7.8 Pathophysiology

Infected carp produce a temperature-dependent humoral response against SVCV. Carp infected at 13–14°C responded more slowly and had lower neutralizing antibody titres than those at 25°C (Fijan *et al*., 1977). At 10–12°C, mortalities due to SVCV in carp reached 90% with no detectable neutralizing antibody, while there were no mortalities at 20–22°C and neutralizing antibody was detected 30 days postinfection (Ahne, 1980). Interferon was produced within 24 h of infection (Baudouy, 1978). In separate experiments, IP injection of carp with SVCV resulted in: (i) the upregulation of several antiviral molecules (Feng *et al*., 2011); (ii) significant increases in the expression of the natural killer cell enhancing factor beta gene in the blood cells, gills, intestine and spleen (Huang *et al*., 2009); and (iii) significant upregulation of prothymosin alpha transcripts in the kidney, peripheral blood, spleen and intestine, and upregulation of thymosin beta transcripts in the intestine, peripheral blood, liver and spleen (Xiao *et al*., 2015) – both of these peptides stimulate the immune response.

The pathophysiology of SVC is not well studied. What is known was derived from studies of natural infections and from one experimental infection. Řehulka (1996) studied the development of SVC in carp that had been taken from a State Fishery overwintering pond in the Czech Republic and found to be susceptible to SVC at the end of their hibernation. They were transferred to an experimental pond early in April, fed on pellets, and the course of disease development studied until early June. In diseased carp (as confirmed by virus isolation and neutralization), there was a decrease in lymphocytes, an increase in neutrophils, particularly myelocytes and metamyelocytes, and an increase in monocytes, These changes were associated with the worsening health of the fish and were accompanied by a change in morphology of the cells, particularly vacuolization of the nucleus and cytoplasm. There were also indications of anaemia. Overall, the disease caused almost 60% mortality. During the course of the disease, Osadchaya and Rudenko (1981) reported a marked decrease in glycogen synthesis in the liver as indicated by a practically complete absence of glycogen granules in hepatocytes and a very small quantity of iron-containing pigment in the splenic pulp. According to Fijan (1999), the multiplication of SVCV in capillary epithelium, and in haematopoietic and excretory kidney tissues affected osmoregulation, which could be fatal.

Jeney *et al*. (1990) showed that following experimental infection of fingerling sheatfish, haematocrit values and haemoglobin concentrations significantly decreased, suggesting that haemopoesis was affected. Serum glutamic oxaloacetic transaminase and glutamic pyruvic transaminase levels increased significantly following infection, indicative of tissue necrosis. In black carp, *Mylopharyngodon piceus*, there was an increase in mitochondria antiviral signalling protein (part of the innate immune response) mRNA in the intestine, muscle and liver at 33 h postinjection, but a decrease occurred in the spleen (Zhou *et al*., 2015).

7.9 Prevention and Control

Both the prevention and control of SVC are currently enacted through legislation and good management.

Prevention has also been attempted by vaccination and the use of resistant strains of carp. For example, Kirpichnikov *et al*. (1993) reported that the hybrid line of Ukrainian-Ropsha carp had increased resistance to SVC compared with other stocks or strains of carp.

Commercial vaccines underwent field trials in the former Czechoslovakia in the 1970s and 1980s (see Fijan, 1984, 1988; Dixon, 1997; Sano *et al*., 2011). However, the vaccine is no longer available, and problems with interpretation of the trials have been discussed by Dixon (1997). Oral vaccination using low virulence SVCV, or virus attenuated by passage through cell cultures, has been attempted. Laboratory experiments showed promise, but a large-scale field trial in pond-cultured fish was unsuccessful (Fijan, 1988). Oral vaccination utilizing different attenuated viruses was also investigated (Kölbl, 1980, 1990). Survival in field trials was encouraging (15% mortality in vaccinated fish versus 49% mortality in control fish). The vaccine was modified by an unspecified method and vaccination in the field was successful as judged by no SVC being observed along with an increase in yield; for example, there was an increase from a low yield of 15 t at one site which was attributed to SVC, up to 50 t in the first year following vaccination, and to 70 t the year after; the vaccine did not go on to be commercialized.

Different approaches to vaccine production have subsequently been undertaken, but none have produced a commercial vaccine. Recombinant SVCV glycoprotein injected into carp produced neutralizing antibodies but did not protect fish against SVCV (see Dixon, 1997). A genetically engineered *Lactobacillus plantarum* with a surface-expressing SVCV glycoprotein and cyprinid herpesvirus-3 (CyHV-3) ORF81 protein was incorporated into pelleted feed as an oral vaccine (Cui *et al*., 2015). Under experimental conditions, there was 29% mortality in vaccinated carp compared with 78 and 89% mortality in control fish challenged with SVCV. The vaccine also protected koi carp against CyHV-3 (see Chapter 10). There have been laboratory trials with DNA vaccines based on the G gene of SVCV. Different combinations of SVCV DNA plasmids containing partial or complete G gene fragments were compared (Kanellos *et al*., 2006). Two vaccinated groups of common carp had relative percentage survival (RPS) values of 33 and 48% in challenges that produced >60% mortality in control fish. Emmenegger and Kurath (2008) used a single DNA vaccine to immunize koi carp against SVCV and the RPS ranged from 50 to 70%.

Interferon-inducer double-stranded RNA injected into carp prior to challenge with SVCV reduced mortality from 100% to 22–40% (Masycheva *et al*., 1995; Alikin *et al*., 1996). The use of liposomes containing poly(I:C) (polyinosinic–polycytidylic acid, a synthetic interferon inducer) and bacterial lipopolysaccharide as an immunostimulant prior to SVCV infection has also been investigated (Ruyra *et al*., 2014). The administration of the liposomes to zebrafish by immersion or IP injection reduced mortality (RPS of 33.3 and 42.3%, respectively) following challenge. Methisoprinol (an antiviral agent) inhibited the replication of SVCV *in vitro*, but its efficacy was not tested in fish (Siwicki *et al*., 2003).

Other approaches to preventing or ameliorating SVC include the identification of multipath genes in zebrafish surviving experimental SVCV infection in order to develop drugs that might prevent the disease (Encinas *et al*., 2013), investigating autophagy or RNA inhibition as a method to suppress SVCV replication (Garcia-Valtanen *et al*., 2014a; Gotesman *et al*., 2015; Liu *et al*., 2015), investigating the use of plasmid constructs expressing glycoproteins of viral haemorrhagic septicaemia virus as a molecular adjuvant for an SVCV G gene DNA vaccine (Martinez-Lopez *et al*., 2014), and investigating the immunomodulatory properties of zebrafish β-defensin and its role as a DNA vaccine molecular adjuvant (Garcia-Valtanen *et al*., 2014b).

Without commercial vaccines or therapeutic substances, biosecurity strategies remain the most effective ways of preventing SVC. These range from national or international legislation or standards to good management practices at the farm level. The latter include on-site quarantine of new fish stocks, the disinfection of equipment, use of footbaths, and reducing stress and other diseases, etc.

Several studies have identified disinfectants and other chemical or physical means for inactivating the virus (see Kiryu *et al*., 2007; Dixon, 2008; Dixon *et al*., 2012), although the results from these studies must be interpreted with care. For example, 99.9% of SVCV was inactivated in hydrochloric acid after 2 h at pH 3.0 (Ahne, 1976), but in formic acid at pH 4.0 (to mimic the procedure used to dispose of dead fish), the virus survived for at least 28 days (Dixon *et al*., 2012).

Legislation to control the introduction and spread of disease within a country is the most important way of preventing and controlling SVC. Such legislation is often strengthened by international standards

or agreements. The OIE's *Aquatic Animal Health Code* (OIE, 2015b) outlines approaches to biosecurity that can be implemented from the national level down to a production site. Håstein *et al.* (2008) have outlined an overview of the implementation of biosecurity strategies, which uses SVC as a disease-specific case study. Oidtmann *et al.* (2011) reviewed the international standards relating to the movement of fish and fish products, particularly with emphasis on national measures to combat fish disease. The UK has implemented a fish health strategy to establish freedom from SVC, which has been outlined by Taylor *et al.* (2013). Legislative controls may have led to freedom from SVC in Croatia, and reduced the incidence of SVC in Hungary (Molnár and Csaba, 2005; Oraic and Zrncic, 2005). However, the epizootiology of the virus in individual countries will determine the feasibility of establishing an SVC-free country. For example, SVC occurs predominantly in wild fish in the USA (Phelps *et al.*, 2012), where establishing freedom from disease is difficult to achieve. Jeremic *et al.* (2004) were also pessimistic about an SVC-free Serbia because of repeated transfer of the disease from cultivated to wild carp and vice versa. None the less, even though SVC is widespread within a country, it may still be possible to establish disease-free zones, provided that fish movements are strictly controlled. Legislation to prevent the introduction of SVC via contaminated fish or fish products is an important approach. All movements, whether they involve live fish or processed contaminated products, would be strictly controlled. Non-approved movements of such products may have introduced or reintroduced SVC into the USA and the UK (Håstein *et al.*, 2008; Taylor *et al.*, 2013). Therefore, continual surveillance of imported fish and fish products is essential.

7.10 Conclusions and Future Research

SVC has caused major losses in carp production, but obtaining accurate figures is complicated by the sporadic nature of the disease and annual fluctuations in losses. Clinical signs are non-specific and diagnosis is via isolation of the virus and subsequent identification using serological and/or molecular biology methods. The OIE provides comprehensive methodology for the diagnosis and identification of SVC using the EPC or FHM cell lines cultured at 20°C for isolation of the virus. The confirmation of SVCV is best achieved using RT-PCR-based methods followed by sequence analysis. Analysis of the partial G gene sequence allows the assignment of SVCV into one of four distinct genogroups.

Effective prevention and control of the disease are currently best achieved by a combination of legislation and good management practices. Vaccination and the use of resistant strains of carp are possible, but no commercial vaccine is available. Non-approved movement of fish products may have been responsible for the introduction or reintroduction of SVC into the USA and the UK, and emphasizes the need for the continual surveillance of imported fish and fish products.

The disease has not been reported in China, although the virus has been detected on several occasions. Apparently healthy SVCV-infected common carp were also found in Canada, which is in stark contrast to high mortality attributed to the Asian SVCV in common and koi carp in the USA. This suggests a significant degree of variation in the pathogenicity of Asian SVCV strains, which needs further investigation.

The transmission of SVCV and the permissive temperatures for this are generally well understood, but the survival and persistence of SVCV is quite variable and needs further study. Host age and genetics, the water temperature and general welfare may determine the progression of the disease.

The use of *in situ* hybridization and real-time qPCR techniques on archived material (e.g. formalin fixed organs) will aid future investigations into the persistence of SVC in carrier fish and the potential re-emergence of the disease. These tools are also invaluable for detecting potential alternative hosts.

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8 Channel Catfish Viral Disease

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8.1 Introduction

Channel catfish viral disease (CCVD) is an acute viraemia that occurs primarily among young (0–4 month old) channel catfish (*Ictalurus punctatus*) in aquaculture. CCVD outbreaks occur almost exclusively in the summer when water temperatures exceed 25°C and may exceed 90% mortality in less than 2 weeks. Older fish may experience a more chronic outbreak, often with secondary *Flavobacterium columnare* or *Aeromonas* infections that can mask the underlying CCVD (Plumb, 1978). Pond-to-pond spread is often reported within fingerling production facilities. The disease was first described by Fijan *et al*. (1970) and the most notable clinical signs were exophthalmia, abdominal distension, disoriented swimming and rapidly increasing mortality.

The causative agent, *Ictalurid herpesvirus 1* (IcHV1, commonly known as channel catfish virus, CCV), was characterized by Wolf and Darlington (1971). This virus is the type species of the *Ictalurivirus* genus and it is one of the best characterized members of the family *Alloherpesviridae*, which includes most herpesviruses of fishes and amphibians. IcHV1 is similar in virion structure and replication to other members of the order *Herpesvirales* (Booy *et al*., 1996). However, at the molecular level (genome sequence), IcHV1 is substantially different from members of other alloherpesvirus genera and shows almost no homology to members of the *Herpesviridae* (Davison, 1992; Waltzek *et al*., 2009; Doszpoly *et al*., 2011a).

A closely related virus in the genus *Ictalurivius* is *Ictalurid herpesvirus 2* (IcHV2), which causes a disease similar to CCVD that has devastated the aquaculture of the black bullhead catfish (*Ameiurus melas*) in Italy (Alborali *et al*., 1996; Doszpoly *et al*., 2008, 2011b; Roncarati *et al*., 2014). Channel catfish fingerlings and juveniles are extremely sensitive to IcHV2, and in experimental infections they develop a CCVD-like disease at 24–25°C, which is cooler than the temperatures commonly seen with IcHV1 infections (Hedrick *et al*., 2003).

IcHV1 is host specific and only produces infections in channel catfish, blue catfish (*Ictalurus furcatus*) and channel × blue hybrids (Plumb, 1989). Early fears that CCVD would limit the catfish industry have not been realized. Producers have adjusted management by reductions of stocking densities and by minimizing stress during the critical first summer of production. Consequently, the industry-wide impact of CCVD mortality has become manageable. Nevertheless, when sporadic outbreaks do occur, the individual operational losses can be devastating. Such outbreaks often occur in multiple ponds and this can result in catastrophic loss of production. Furthermore, CCVD survivors have reduced growth (McGlamery and Gratzek, 1974).

8.1.1 Prevalence and transmission

IcHV1 is present in most locations where channel catfish are produced commercially. The virus establishes latency, is vertically transmitted and can remain undetected (with no sign of disease) for many generations. Using polymerase chain reaction (PCR), Thompson *et al*. (2005) evaluated the prevalence of IcHV1 in 3–5 day old fry in commercial hatcheries in Mississippi. They found that

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breeding populations at all five hatcheries tested had the virus. These hatcheries produced 20% of the fingerlings for the industry. The prevalence of latent IcHV1 in the fry ranged from 11.7 to 26.7%, but replicating virus was not detected by cell culture and CCVD did not occur within the production facilities during the year of the study. When fish populations were tracked during their first production season, latent IcHV1 infection increased from 13 to >35%. Still, no disease occurred and viral replication remained undetected by cell culture. These results suggested that vertical transmission commonly occurs between parents and their progeny, and that subclinical horizontal transmission is common within fingerling populations.

8.1.2 Factors that promote CCVD outbreaks

Factors that influence CCVD outbreaks include high temperatures, stress, population density and age (Plumb, 1978, 1989). The effect of temperature was experimentally demonstrated in infection studies; increasing the water temperature from 19 to 28°C had a profound effect on the progression of CCVD, whereas reducing the temperature from 28 to 19°C substantially curbed losses (Plumb, 1973a).

Circumstantial evidence indicates susceptibility is inversely related to age and almost all outbreaks in commercial production systems occur in young of the year populations. The apparent effect of age may be related to the development of immunity due to sublethal natural exposure or to changes in behaviour as the fish age. Within the most susceptible period, experimental evidence suggests that very young fry are more resistant to CCVD than are fish >1 month of age, which might be related to the presence of maternal antibodies (Hanson *et al*., 2004). Also, differences in the susceptibility of channel catfish strains have been demonstrated (Plumb *et al*., 1975).

The influence of stress on CCVD outbreaks is less clear. Circumstantial evidence suggests that poor water quality and handling stress correlate with CCVD outbreaks in natural systems, but low water stress and the oral administration of cortisol did not influence experimentally induced CCVD losses (Davis *et al*., 2002, 2003). The effect of stress hormones may be more related to the amount of virus recrudescence than it is to the susceptibility of fish to immersion infections. In cold temperature-exposed adult channel catfish, the administration of the synthetic corticosteroid dexamethazone resulted in a higher level of viral recovery from isolated leucocytes (Bowser *et al*., 1985). Arnizaut and Hanson (2011) found that the administration of dexamethazone to carrier juvenile channel catfish caused an increase on viral DNA loads and increased viral gene expression. An increase in circulating IcHV1-specific antibodies suggested that some viral recrudescence had occurred, although infectious virus was not detected. Also, many outbreaks have occurred without a clear predisposing stress event.

The importance of IcHV1 infection to large juvenile and adult channel catfish is less well understood. Hedrick *et al*. (1987) demonstrated that naive adult channel catfish are susceptible to CCVD in experimental immersion infections. Additionally, seasonal increases of antiviral antibody suggested that at least some antigen expression occurs in adults during the summer (Bowser and Munson, 1986). IcHV1 has been isolated from diseased adult channel catfish in the winter when the water temperature was 8°C; the virus was also isolated from non-diseased fish in the population (Bowser *et al*., 1985). The detection of replicating virus in fish during the winter is very rare and the associated disease is economically insignificant.

8.2 Diagnosis

Presumptive diagnosis of CCVD is usually based on rapidly progressing mortality of young channel catfish with the fish displaying the typical clinical signs (abdominal distension, exophthalmia and disoriented swimming), gross pathology, histopathology and the production of the typical cytopathic effect (CPE) of IcHV1 in cell culture.

IcHV1 is easily cultured in catfish cell lines but does not produce CPE on most non-catfish cell lines. Briefly, the culture is started by homogenizing whole fry, viscera from small fingerlings or posterior kidney from larger fingerlings in serum-free cell culture medium. Then microbial components of the homogenates are removed using a 0.22 µm filter and this filtrate is diluted in cell culture medium at a 1:100 final dilution of tissue to medium on channel catfish ovary (CCO) and/or brown bullhead (BB) cell lines at 28–30° C. Both cell lines are available from the American Type Culture Collection (Manassas, Virginia). Cultures from acutely CCVD-affected fish produce high levels of infectious virus and develop a rapidly progressive CPE within 24–48 h. The characteristic

CPE in positive cultures is syncytial formation due to fusion of cells. These syncytia subsequently dislodge themselves from the culture flask leaving radiating cytoplasmic projections to attachment points (see Fig. 8.1). Cultures without CPE should be observed daily for 7 days and then blind passaged for an additional 7 days before they are considered negative for IcHV1.

The channel catfish reovirus (Amend *et al*., 1984) and IcHV2 are capable of infecting North American catfish species and will also cause CPE on CCO and BB cells at 28–30° C. The catfish reovirus causes limited syncytia but the CPE spreads more slowly. This virus is not associated with high losses in channel catfish. However, IcHV2 is of more concern because it can cause CCVD-like disease in experimental infections of North American catfish. It also produces a CPE that is similar to that of IcHV1 (Hedrick *et al*., 2003). In the original description of IcHV2 disease outbreaks, the CPE was seen in the bluegill cell line, BF2, and the EPC (epithelioma papulosum cyprini) cell line of fathead minnow origin (Alborali *et al*., 1996). This demonstrates that ICHV2 has a broader cell host range that may be of diagnostic value.

The confirmation of virus isolated using cell culture requires serology or molecular analysis. The use of neutralizing monoclonal antibody (Arkush *et al*., 1992) distinguishes IcHV1 from IcHV2 (Hedrick *et al*., 2003). Polyclonal antibodies are also useful, but IcHV1 induces neutralizing antibodies poorly in rabbits. Additional neutralizing monoclonal and monospecific polyclonal antibodies have been developed (Wu *et al*., 2011; Liu *et al*., 2012), but their ability to distinguish IcHV1 from IcHV2 has not been determined. These diagnostic antibodies are not commercially available.

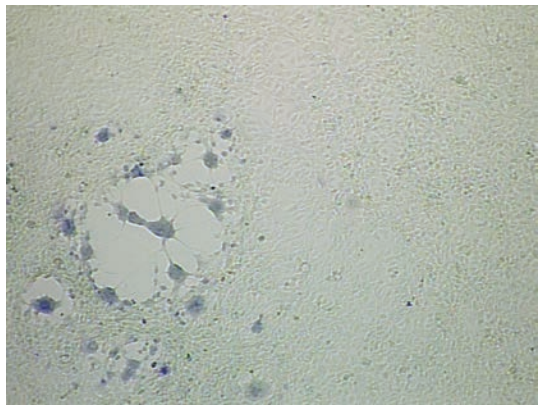

Fig. 8.1. A plaque caused by *Ictalurid herpesvirus 1* (IcHV1) on a monolayer of the Channel catfish ovary cell line. Note the contracting syncytium leaving cytoplasmic projections (100× magnification).

The most common molecular method for confirming a presumptive diagnosis is the PCR assay. Several sensitive traditional PCR assays have been described (Boyle and Blackwell, 1991; Baek and Boyle, 1996; Gray *et al*., 1999; Thompson *et al*., 2005), but their ability to distinguish IcHV1 from IcHV2 has not been reported. A hydrolysis probe (TaqMan)-based quantitative PCR has been developed specifically for IcHV2 (Goodwin and Marecaux, 2010). We use two different TaqMan-based quantitative PCRs (qPCRs) that distinguish between two genomovars of IcHV1 and do not detect IcHV2 (see Table 8.1) (Hanson, 2016). As with any PCR assay, these methods are very sensitive to genomic contamination from previous PCR products, so assays should be set up in separate locations and use separate equipment. Furthermore, negative control cultures must be processed to ensure that contamination did not occur during sample preparation.

The detection of latent infections is needed to identify carrier populations of fish. Direct cell culture from carrier fish has no diagnostic value because no infectious virus is produced when the virus is latent. Although the use of dexamethazone injection to induce recrudescence and the subsequent co-culture of leucocytes with CCO cells has been successful in demonstrating the presence of IcHV1 in adult catfish (Bowser *et al*., 1985), this was only successful in fish from cold water during the winter. Other workers have been unsuccessful in reactivating culturable latent IcHV1 in known carrier fish using the same protocol; consequently, this is not a reliable diagnostic method (Arnizaut and Hanson, 2011). The detection of antibodies to IcHV1 in brood fish has been used to successfully identify carrier populations (Plumb, 1973b; Amend and McDowell, 1984; Bowser and Munson, 1986; Crawford *et al*., 1999). The enzyme-linked immunosorbent assay (ELISA) is more sensitive than serum neutralization (Crawford *et al*., 1999). Serological assays are an indirect detection method; their reliability depends not only on the presence of the pathogen but also on the immune status of the fish. IcHV-1-specific antibody concentrations in carrier fish have been shown to vary substantially between fish within a population (Arnizaut and Hanson, 2011). What is more, the mean concentrations vary

Table 8.1. Primers, probes and amplification parameters for qPCR assays of *Ictalurid herpesvirus 1* (IcHV) and *Ictalurid herpesvirus 2* (IcHV2).[a]

Assay, primers and probes	Sequence#	Cycle parameters
IcHV1 TaqMan		
Upper primer	CTCCGAGCGATGACACCAC	60 s at 64°C
Lower primer	TGTGTTCAGAGGAGCGTCG	15 s at 72°C
IcHV1a probe[b]	FAM-CCCATCCCTTCCCTCCTCCCTG-BHQ-1[c]	15 s at 95°C
IcHV1b probe[b]	FAM-CCCATCCTTCCCCTCCTCCCTG-BHQ-1	
IcHV2 TaqMan		
Upper primer	ATACATCGGTCTCACTCAAGAGG	45 s at 59°C
Lower primer	TAATGGGTATTGGTACAAATCTTCATC	45 s at 72°C
Probe	FAM-CGC+CTG+AGA+ACC+GAGCA –BHQ-1[d]	30 s at 95°C

[a]The IcHV2 assay is from Goodwin and Marecaux (2010).
[b]Probe IcHV1a detects genome type A, Probe IcHV1b detects genome type B. Both assays use the same primers and reaction conditions.
[c]FAM designates 6-FAM (6-carboxyfluorescein) fluorescent dye and BHQ-1 indicates black hole quencher.
[d]+ indicates the use of locked bases to increase the melting point.

substantially over the year, with the highest levels of antibody seen primarily in the autumn (Bowser and Munson, 1986). In addition, catfish that carry IcHV1 latently as a result of vertical transmission, but have not experienced virus antigen expression, have no detectable antibodies. Because of the variation in antibody levels, direct detection of the latent genome in carrier fish is preferred.

The conventional PCR and qPCR methods described above are very sensitive and can detect latent IcHV1 in the tissues of carriers. However, even though the use of PCR to detect carrier populations is very sensitive, the concentration of IcHV1 genome in latently infected tissue can be close to the minimal limit of detection. The tissues sampled for PCR evaluation depend on the life stage and the health status of the fish. Sac fry should be processed whole, but without their yolk (Thompson *et al*., 2005). To evaluate clinically diseased fish for direct CCVD diagnosis, posterior kidney tissue is preferred. For latency evaluation of larger fish, caudal fin biopsies are reliable (Arnizaut, 2002). Samples can be prepared using commercially available DNA extraction kits such as the Gentra Puregene Tissue Kit (Qiagen). When using qPCR on 1 µg DNA from tissues, the number of threshold cycles for a CCVD clinically affected fish or an infected cell culture is below 27, whereas for latently infected fish it is 33–38. In studies among brood stock, we found that PCR-negative parents could still produce IcHV1-positive offspring (Y. Habte and L. Hanson, unpublished). Although PCR assays are useful in detecting carrier populations, they are not sensitive enough to definitively cull positive individuals from a population.

8.3 Pathology

8.3.1 Clinical signs and gross lesions

The original descriptions and reviews of CCVD (Fijan *et al*., 1970; Plumb, 1978, 1986; Hanson *et al*., 2011; Plumb and Hanson, 2011) have provided detailed descriptions of the clinical signs as well as the gross pathology of the disease. Clinical signs include abnormal swimming behaviour (i.e. rotating along the longitudinal axis) and convulsions followed by quiescence when the affected fish breathe rapidly at the bottom of the water column. Fish may then posture themselves vertically in the water column with their heads at the surface shortly before they expire. This vertical posture in the water was once thought to be diagnostic for the disease. Grossly, the affected fish may show external haemorrhages at the base of the fins and ventral abdomen, exophthalmia, abdominal distention, and pale or haemorrhagic gills. Recent clinical submissions of naturally affected CCVD fish to our laboratory usually have the bilateral exophthalmia and distended abdomens, but often lack the external haemorrhagic lesions (Fig. 8.2A).

Unfortunately, with clinical submissions, there is often no accurate means of determining the chronicity of the disease, and the lack of haemorrhagic external lesions may reflect a different point/phase of the disease continuum. Historically described

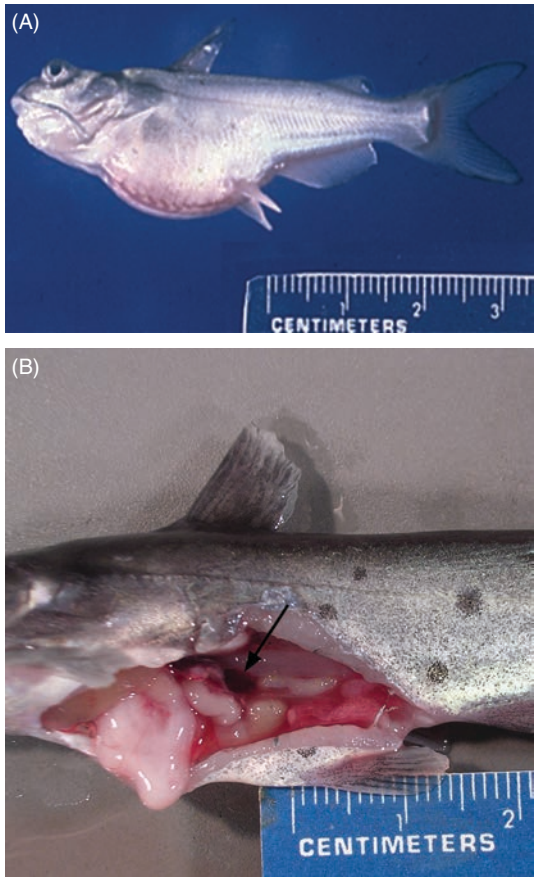

Fig. 8.2. Images of channel catfish fingerlings with channel catfish viral disease (CCVD). (A) External gross pathology; note the markedly distended abdomen. (B) Internal gross lesions; the arrow indicates a congested spleen that is adjacent to a slightly swollen posterior kidney, the gastrointestinal tract is slightly hyperaemic and the organs are glistening from the ascites that flowed out of the incised abdomen.

gross internal lesions include haemorrhagic areas in the musculature, liver and kidneys. Pale livers, pale enlarged posterior kidneys, an enlarged congested spleen and gastrointestinal tracts devoid of feed but filled instead with a yellow mucoid fluid are lesions that have been ascribed to this disease. Plumb (1986) described a general hyperaemia in the visceral or coelomic cavity as well as a straw/yellow-coloured ascites which caused the abdominal distension. The ascites, exophthalmia and congested spleen are almost always seen in clinical submissions. Haemorrhage in the musculature, liver and posterior kidney, and renomegaly, are less apparent and may be underappreciated owing to the diminutive size of the affected fish (Fig. 8.2B).

8.3.2 Histopathology

Early descriptions of the histopathological lesions were based mainly on experimentally infected fish that were injected with the virus (Wolf *et al*., 1972; Plumb *et al*., 1974). Kidneys (anterior and posterior) were often the most severely affected tissue, followed by the gastrointestinal tract, liver and skeletal muscle. Plumb *et al*. (1974) detected increased numbers of lymphoid cells in the posterior kidney in addition to haemorrhage and necrosis of epithelial cells of the proximal tubules. There was also extensive necrosis of the hematopoietic tissue of the kidney. Necrosis of the epithelial cells surrounding the pancreatic acinar cells, with limited or minimal pancreatic acinar cell necrosis, was evident. The marked haemorrhage/congestion in the spleen obscured the white pulp. Haemorrhaging was also visible in the submucosa and villi of the gastrointestinal tract.

Major *et al*. (1975) studied both experimentally and naturally infected fish. Lesions were more severe in the younger naturally infected fish. They also found lesions in the brain and pancreas in addition to the lesions that were reported by Wolf *et al*. (1972) and Plumb *et al*. (1974). In addition, Major *et al*. (1975) described vacuolation of neurons with oedema of the surrounding nerve fibres and necrosis of the pancreatic acinar cells. Oedematous changes were not limited to the brain but were also present in the heart, spinal cord, gills, kidneys and gastrointestinal tract. Hepatocellular necrosis was present in younger naturally infected fish, while oedema and multifocal necrosis were more common in older fish. They also detected eosinophilic intracytoplasmic inclusion bodies in hepatocytes of experimentally infected fish, which were less frequent in naturally infected fish.

The naturally infected CCVD fish that have been submitted to our laboratory have histopathological lesions consistent with those described by Major *et al*. (1975). In addition, these fish also have multifocal to locally extensive necrosis of the hematopoietic elements within the anterior kidney (Fig. 8.3). It is difficult to appreciate the increased lymphoid elements in these samples as compared with those in experimentally infected fish (Plumb *et al*., 1974). Similarly, there is moderate-to-severe multifocal

necrosis of the interstitial portions of the posterior kidney (Fig. 8.4), which only occasionally extends to the renal tubular epithelium. Rare multinucleated syncytia are sometimes present in these necrotic foci (Fig. 8.4A). Eosinophilic intranuclear inclusions are sometimes present in cells presumed to be lymphocytes (the nucleus is markedly distended with only a thin rim of basophilic, and these cells appear to lack cytoplasm) within the renal vessels of the posterior kidney, and rarely in other organs such as the spleen (Fig. 8.4B). Intranuclear inclusion bodies are rarely seen.

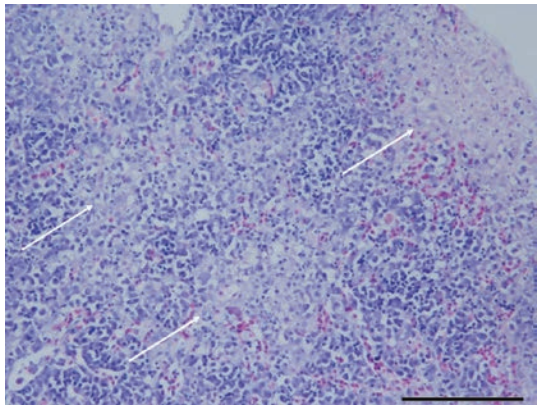

Fig. 8.3. Histopathology of the anterior kidney of a channel catfish viral disease (CCVD)-affected channel catfish fingerling. The arrows point to the more eosinophilic necrotic foci with pyknotic nuclei and karyorrhectic debris (haematoxylin and eosin (H and E) stain; Bar ~ 100 µm)

The hepatocellular necrosis is multifocal and mild, and the pancreatic involvement is minimal when present (Fig. 8.5). The severe congestion/haemorrhage of the spleen masks the normal splenic architecture as well as any necrosis that may be present (Fig. 8.6). There is necrosis present in the glandular portions of the stomach (Fig. 8.7A) which can extend into the proximal intestine. In the intestinal mucosa, the necrosis is multifocal; it is regionally extensive in the submucosa. Small aggregates of mononuclear inflammatory cells are sometimes present at the base of the villi (Fig. 8.7B). There may also be local extensive congestion in portions of the gastrointestinal tract. No significant microscopic lesions are seen in the other tissues including the brain, muscle and heart. Lesions in clinical cases correlate well with what has already been reported in the literature (Plumb *et al.*, 1974; Wolf *et al.*, 1972; Major *et al.*, 1975) Differences may reflect the chronicity of the disease, varying environmental conditions, and perhaps the strain of virus.

8.4 Pathophysiology

The kidney appears to be one of the major organs affected by CCVD, and renal damage results in

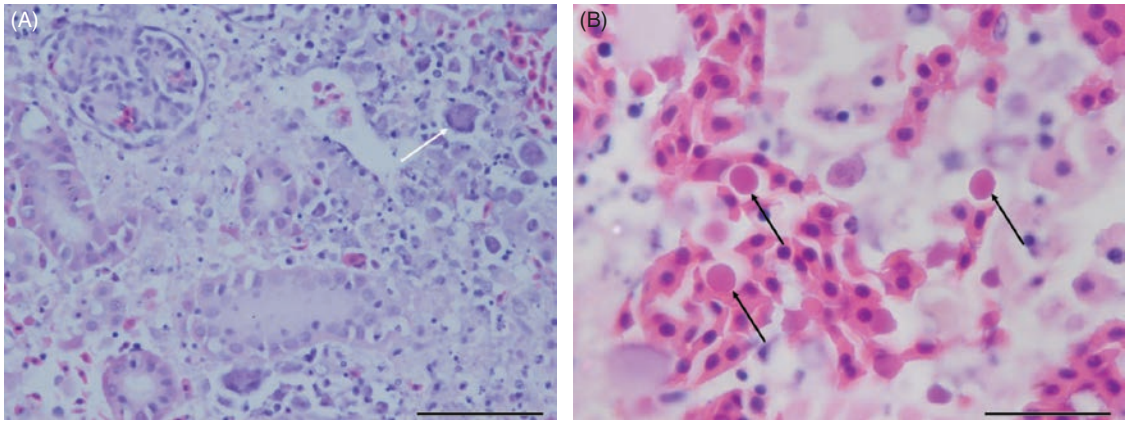

Fig. 8.4. Histopathology of the posterior kidney of a channel catfish viral disease (CCVD)-affected fingerling revealing: (A) interstitial necrosis with a multinucleated syncytium as indicated by the arrow (haematoxylin and eosin (H and E) stain; Bar ~ 50 µm); and (B) cells, presumably lymphocytes, with eosinophilic inclusion bodies and only a thin rim of chromatin as indicated by the arrows (H and E stain; Bar ~ 20 µm).

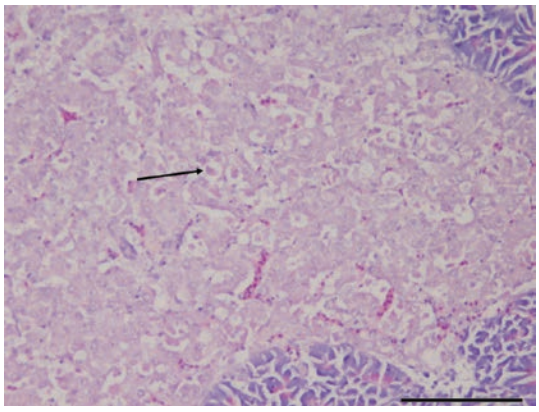

Fig. 8.5. Focus of necrosis within the liver of a channel catfish viral disease (CCVD)-affected channel catfish fingerling. The arrow points to one of several slightly more eosinophilic shrunken hepatocytes (haematoxylin and eosin (H and E) stain; Bar ~ 100 µm).

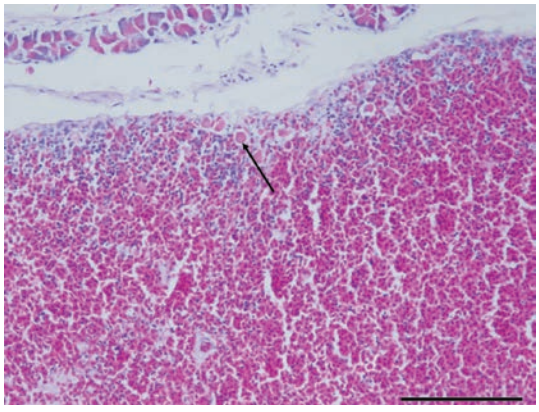

Fig. 8.6. Histopathology of the severely congested spleen of a channel catfish viral disease (CCVD)-affected channel catfish fingerling. The arrow points to a cell with an intranuclear inclusion, as seen in Fig. 8.4A. The white pulp is obscured by the red blood cells. (haematoxylin and eosin (H and E) stain; Bar ~ 100 µm).

fluid imbalances, which would explain the gross lesions (e.g. ascites, which produces abdominal distension, and exophthalmia). However, the colour of the ascites is more indicative that this may be a modified transudate with leakage from the vasculature. Lesions in the gastrointestinal tract and liver may also contribute to the ascites and oedema due to decreased absorption and protein synthesis and thus reduced oncotic pressure of the plasma. The pale gills are most likely the result of both haemorrhaging and congestion in multiple organs, but especially in the spleen. Additionally, there is necrosis within the anterior kidney and in the interstitium of the posterior kidney, which would affect haematopoiesis and cause anaemia. While the cellular tropism of IcHV1 has not been established, most alloherpesviruses are epitheliotropic (reviewed in Hanson *et al*., 2011). The necrosis of the mucosal epithelium of the gastrointestinal tract and the epithelium surrounding the pancreas indicates that IcHV1 has some epitheliotropism. Virus quantification and tracking experiments demonstrate peak and earliest virus production in renal tissue, followed by the liver, intestine and skin (Plumb, 1971; Nusbaum and Grizzle, 1987; Kancharla and Hanson, 1996). Cell culture assays demonstrate that IcHV1 replicates well in fibroblast-derived cell lines as well as B lymphocytes and causes CPE in other leucocytes (Bowser and Plumb, 1980; Chinchar *et al*., 1993). Peak virus production in the kidney occurs at 3–4 days postexposure to the virus at 28°C in both sublethal and lethal doses, and this is also the period of peak mortality (Kancharla and Hanson, 1996).

The specific pathogenic mechanisms of IcHV1 have not been well defined. The genome encodes over 76 genes (Davison, 1992), and most are non-essential for replication. The non-essential genes that have roles in virulence include the thymidine kinase (TK) gene and gene 50. Virally encoded TK probably facilitates virus replication in non-replicating cells (Zhang and Hanson, 1995). The TK mutant of CCV replicates to similar levels as the wild type virus and in experimentally infected fish also had early kinetics that were similar to those of the wild type virus, but the mutant virus is cleared more quickly (Zhang and Hanson, 1995; Kancharla and Hanson, 1996). The product of gene 50 is a secreted mucin of unknown function, and viruses that are mutated in this gene are attenuated in virulence (Vanderheijden *et al*., 1996, 1999, 2001).

Temperature and dose effects due to crowding are the most important factors that influence CCVD outbreaks. The influence of temperature appears to be a common factor that regulates the expression of alloherpesviruses and promotes or limits the associated viral diseases. Temperature has also been shown to regulate disease outbreaks caused by *Cyprinid herpesvirus 1*, *Cyprinid herpesvirus 3*, *Salmonid herpesvirus 3* (epizootic epitheliotropic disease of lake trout), Percid herpesvirus 1 (diffuse epidermal

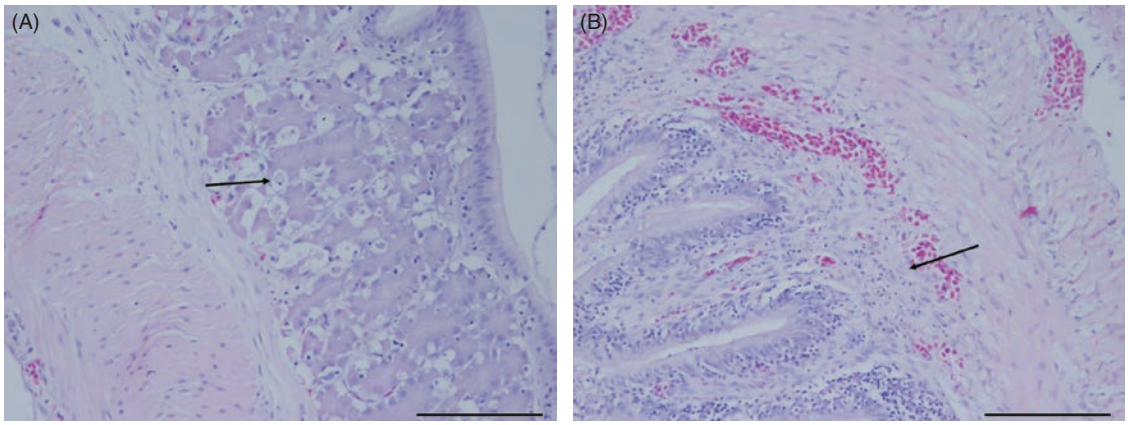

Fig. 8.7. Pathology associated with channel catfish viral disease (CCVD) in the gastrointestinal tract of an affected channel catfish fingerling. (A) Section of the stomach; the arrow points to one of the many necrotic cells within the glandular portion of the stomach (haematoxylin and eosin (H and E) stain; Bar ~ 100 µm). (B) Section of a portion of the proximal intestine; the arrow points to the focus of inflammation necrosis in the submucosa. The necrotic regions in the mucosa are within the more vacuolated areas and there are small aggregates of mononuclear inflammatory cells at the base of the villi. Several of the blood vessels are also congested in this figure (H and E stain; Bar ~ 100 µm).

hyperplasia of walleye) and Esocid herpesvirus 1 (blue spot disease of northern pike) (reviewed in Hanson *et al*., 2011; Plumb and Hanson, 2011). The effect of temperature is best characterized in diseases caused by *Cyprinid herpesvirus 3* infections: koi herpesviral disease is limited to temperatures of 18–28°C and temperatures outside this range appear to cause the virus to establish a latent infection; this has been demonstrated in cell cultures as well as in the host (Gilad *et al*., 2003; Dishon *et al*., 2007; Ilouze *et al*., 2012).

8.5 Protective and Control Strategies

The relatively low incidence of CCVD outbreaks in catfish fingerling production facilities is due to active and intentional management. Production ponds are typically stocked at less than 250,000 fry/ha. Fingerling producers avoid crowding or harvesting fingerlings when water temperatures are above 25°C. Ponds are carefully monitored and aerated to keep oxygen levels above 3 mg/l. Chloride levels are generally maintained above 100 mg/l to avoid nitrite toxicity and only quality fresh feed is provided. When producers vaccinate fry against other diseases, temperature and oxygen levels are carefully monitored. Fry are generally stocked only in the cool morning hours and any batches of fry that show unusual elevated mortality are discarded and their tanks are disinfected. Many producers avoid collecting eggs and hatching fry late in the season to avoid the elevated water temperatures that would occur when the fingerlings are ready to be stocked into ponds.

Vaccination, the selection of resistant strains and establishing IcHV1-free breeding stock are prevention and control methods that show promise, but are not currently implemented. Attenuated virus vaccine candidates include a natural mutant developed by culturing IcHV1 in a *Clarias bactrachus* cell line (Noga and Hartmann, 1981), a recombinant TK-deleted IcHV1 (Zhang and Hanson, 1995) and a gene 50-deleted recombinant IcHV1 (Vanderheijden *et al*., 2001). Both of the recombinant attenuated viruses showed the potential to express foreign genes and induce immunity to the gene products, which indicates that they could be used as vaccine vectors (Zhang and Hanson, 1996; Vanderheijden *et al*., 2001). The development of bacterial artificial chromosomes with IcHV1 inserts allows researchers to use efficient bacteria-based recombination and selection methods to produce recombinant IcHV1 (Kunec *et al*., 2008, 2009). A limiting factor in the implementation of CCVD vaccines is the need to vaccinate fish at an age when they are immunologically competent and can develop protective immunity (Petrie-Hanson and Ainsworth, 2001), but still young enough to be

most susceptible to CCVD. Moreover, the current management methods preclude applying immersion vaccines after 8 days of age because this is when fry are stocked into the fingerling ponds. Other immune-potentiating treatments that increase channel catfish resistance are the application of interferon inducers such as poly(I:C) (polyinosinic–polycytidylic acid) (Plant *et al*., 2005), or pre-exposure to catfish reovirus as has been demonstrated in cell culture (Chinchar *et al*., 1998). Practical application methods of these immune stimulants and their utility in populations of channel catfish have not been studied.

As some strains of channel catfish are more resistant to CCVD, selective breeding could be used to control outbreaks (Plumb *et al*., 1975). Early studies suggested that channel × blue hybrids may be more resistant to CCVD (Plumb and Chappell, 1978), but later studies did not support this finding; it was suggested that the strain of channel catfish used influences the susceptibility of the hybrid (Silverstein *et al*., 2008). The selection of IcHV1-free brood stock to produce virus-free fingerlings is possible using aggressive screening and culling methods. However, the fish must be raised in more biosecure environments than are used in current aquaculture facilities. The virus is most likely endemic because brood fish have not been routinely screened for the virus.

If a CCVD outbreak occurs, there are a few practical measures that reduce losses. If practicable, reducing water temperatures will reduce mortality. Otherwise, management after an outbreak is limited to preventing transmission by avoidance. For example, fish, water or equipment from an infected area should not be used in another pond. Interactions with other wildlife, especially predators and scavengers, should also be limited. Dead fish should be quickly removed from culture ponds and their carcasses should be buried or incinerated to prevent scavengers from reintroducing the disease. If the disease occurs in the hatchery before the fish are transferred to ponds, farmers typically euthanize all fish in the affected trough and disinfect the equipment. There is relatively little investment at this point and farmers tend to take this drastic action rather than risking spread within the hatchery. The survivors of CCVD outbreaks are often reared to market without obvious negative effects, but these fish should be avoided in brood stock selection because they are likely to carry the virus and facilitate vertical transmission. After an epizootic has occurred, ponds should be depopulated, sterilized by drying and hydrated lime applied to wet areas before any restocking occurs (Camus, 2004).

8.6 Conclusions and Suggestions for Future Research

Channel catfish virus disease has caused sporadic high losses within channel catfish fingerling operations for >45 years and this virus is present subclinically in fish throughout the industry. Because CCVD outbreaks are associated with elevated water temperatures and high loading densities, the industry has optimized aquaculture practices to minimize the impact of the disease during the critical first summer of growth. Catfish production has evolved to use high-density systems. When combined with increasing temperatures and variable weather patterns, these densities are likely to make CCVD a greater threat. To minimize the impact of CCVD, it is critical to identify triggers for viral activation so that effective control measures can be developed. Research into the molecular basis of immune system regulation and effective viral recombination systems (Kunec *et al*., 2008) can elucidate the intricate host/pathogen relationships that are typical of herpesviruses. Furthermore, the establishment of virus-free fish stocks and use of effective biosecurity measures can help to achieve more successful prevention of CCVD. Methods to prevent vertical transmission should be evaluated, and these could be integrated into *in vitro* fertilization protocols for the production of hybrid catfish. The identification of the mechanisms responsible for the resistance of blue catfish to CCVD and the application of this information to optimize the genetics of hybrid stocks could further alleviate the deleterious impacts of CCVD within the catfish industry.

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9 Largemouth Bass Viral Disease

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9.1 Introduction

The first *Ranavirus* was isolated from leopard frogs, *Rana pipiens*, in the eastern USA (Granoff *et al*., 1965). Thirty years later, the first outbreak of largemouth bass viral disease was reported at Santee-Cooper Reservoir, South Carolina by Plumb *et al*. (1996), who isolated the virus concerned on fathead minnow, *Pimephales promelas* (FHM) cells inoculated with filtered homogenates from two infected adult largemouth bass, *Micropterus salmoides*, collected during the mortality event. Icosahedral virus particles (enveloped virions about 174 nm in diameter) were transmitted to uninfected largemouth bass by experimental transmission by injection. The isolate was tentatively classified as belonging to the family *Iridoviridae* and largemouth bass virus (LMBV) proposed as its name (Plumb *et al*., 1996).

Previously, iridovirus infections in fish were limited to *Lymphocystis disease virus 1* (LCDV-1), the type species of the genus *Lymphocystivirus* (Weissenberg, 1965; Wolf, 1988). Mao *et al*. (1997) showed that six fish iridoviruses were more closely related to *Ranavirus* than to LCDV; ranaviruses had originally been thought to only infect amphibians (Hedrick *et al*., 1992). These new fish iridoviruses caused systemic disease with high morbidity and mortality in fishes in Australia, Japan and Europe (Langdon *et al*., 1986, 1988; Ahne *et al*., 1989; Inouye *et al*., 1992; Hedrick and McDowell, 1995; Go *et al*., 2006).

Between August 1997 and November 1998, the Southeastern Cooperative Fish Disease Laboratory (Auburn University, Alabama) investigated LMBV outbreaks and surveyed 78 locations in eight US states. Virus was isolated from largemouth bass collected from six reservoirs on four different river systems (Plumb *et al*., 1999). The gross pathological signs noted in fish with LMBV were enlarged swim bladders (Fig. 9.1) and erythematous gas glands (Plumb *et al*., 1996, 1999; Hanson *et al*., 2001a,b). Sequence analysis showed that the virus from these surveys was identical to the LMBV found earlier at Santee-Cooper Reservoir in South Carolina.

The growth of LMBV in five fish cell lines had an optimum replication temperature of 30°C (Piaskoski *et al*., 1999). Bluegill fry-2 (BF-2) and FHM cells performed best, demonstrating an early onset cytopathic effect (CPE), rapid viral replication, and high titres of LMBV. In the initial study, clinical signs or mortality were not seen in adult largemouth bass experimentally infected with LMBV (Plumb *et al*., 1996), but juvenile fish were susceptible and sustained up to 60% mortality (Plumb and Zilberg, 1999b). Similarly, treated juvenile striped bass, *Morone saxatilis*, suffered 63% mortality.

Mao *et al*. (1999) confirmed that LMBV belonged to the genus *Ranavirus* and showed that it was similar to viruses from the doctor fish, *Labroides dimidatus* (doctor fish virus, DFV) and guppies, *Poecilia reticulata* (guppy virus 6, GV6) (Hedrick and McDowell, 1995). A retrospective investigation by Grizzle *et al*. (2002) genetically compared a 1991 iridovirus from a clinically normal largemouth bass from Lake Weir, Florida, with the 1995 Santee-Cooper isolate. The Lake Weir isolate was associated with sporadic fish kills in that lake. Restriction fragment length polymorphism (RFLP) analysis and determination of the DNA sequence of a portion of the major capsid protein (MCP) gene of the two isolates showed that they were identical. The authors noted that it was unknown whether

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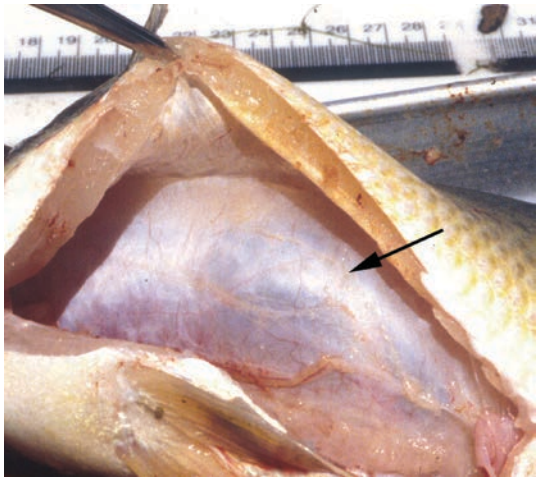

Fig. 9.1. Largemouth bass infected with largemouth bass virus (LMBV) showing an over-inflated swim bladder (arrow). Photo original courtesy of John Grizzle, Auburn University.

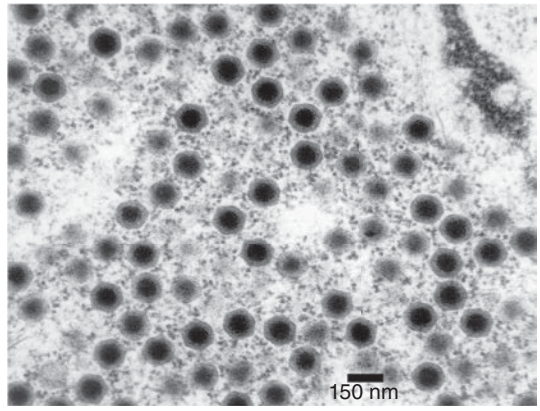

Fig. 9.2. Transmission electron microscope image of LMBV in cell culture. Photo courtesy of Andrew Goodwin; original from the Collection of John Plumb, Auburn University.

LMBV was a newly introduced pathogen spreading throughout the Southeastern USA, or whether it was a resident virus that was found because of increased testing. LMBV continued to be found throughout the eastern USA (Grizzle and Brunner, 2003). Many infected fish were clinically healthy, so infection was not always fatal (Plumb *et al*., 1999; Hanson *et al*., 2001b).

9.1.1 Description of LMBV

The family *Iridoviridae* consists of five genera of double-stranded DNA-viruses – *Iridovirus* and *Chloriridovirus* – infecting invertebrates, and *Ranavirus*, *Megalocytivirus* and *Lymphocystivirus*, which infect poikilotherms. Both *Ranavirus* and *Megalocytivirus* are important pathogens of marine and freshwater fishes (MacLachlan and Dubovi, 2011). Ranaviruses are genetically diverse, infecting bony fishes, amphibians and reptiles (Chinchar, 2002). LMBV was only one of many new ranaviruses identified during mortalities of fish during the mid-1980s and 1990s (Williams *et al*., 2005). Its present accepted name, as designated by the International Committee on Taxonomy of Viruses (ICTV) and after its original site of isolation, is *Santee-Cooper ranavirus*.

Electron microscopy of LMBV-infected FHM cells (Fig. 9.2) revealed cytoplasmic, icosahedral virions that averaged 132 nm from facet to facet and 145 nm from corner to corner (Plumb *et al*., 1996), within the *Iridoviridae* range of 120–200 nm diameter (MacLachlan and Dubovi, 2011). Infectious virions are either non-enveloped or enveloped, depending upon whether they are released from the cell by lysis or budded from the plasma membrane. *Ranavirus* infections are accompanied by marked CPE and cultured cells infected with ranaviruses undergo apoptosis (Chinchar *et al*., 2005).

9.1.2 Mode of transmission

The transmission of LMBV can occur when susceptible fish are immersed in water with the viral agent (Plumb and Zilberg, 1999b) or fed with LMBV-infected prey (Woodland *et al*., 2002a). Five of 24 young largemouth bass gavaged (force-fed) with LMBV-infected guppies tested positive, though none showed clinical signs of disease. Vertical transmission has not been demonstrated and there is no evidence of it occurring in hatchery fish (Woodland *et al*., 2002b). The virus is sometimes present in the cutaneous mucus of infected experimental fish (Woodland *et al*., 2002a), which could allow direct transmission. Several warm-water fishes such as smallmouth bass, *Micropterus dolomieu*, chain pickerel, *Esox niger*, bluegill, *Lepomis macrochirus*, and redear sunfish, *Lepomis microlophus*, can be infected but without clinical signs (Grizzle *et al*., 2003). Humans may spread LMBV unknowingly because the virus survives in water,

such as in the live wells of fishing boats or tanks on stocking trucks (Grizzle and Brunner, 2003). Johnson and Brunner (2014) suggested that microbial and zooplankton communities may rapidly inactivate ranaviruses in pond water and thereby minimize environmental transmission. Host behaviour, density and contact rates also play critical roles in shaping transmission dynamics.

9.1.3 Geographical distribution

Since the first reports of the virus (Plumb *et al*., 1996, 1999), LMBV has been documented throughout the eastern USA. In 1998, LMBV-related mortality was found in the Sardis Reservoir, Mississippi (Hanson *et al*., 2001b) and, in 1999, in wild fish populations in Tunica Cutoff and Lake Ferguson, also in Mississippi, as well as in waters in Texas and Louisiana. These new outbreaks suggested that LMBV was spreading or that conditions had become more optimal for LMBV-induced disease. Over 1400 largemouth bass were evaluated for LMBV from 17 different fisheries in Mississippi over 2 years; there was an increase in the prevalence of LMBV in seven fisheries in the second year at the same time that other fisheries saw a decrease (Hanson *et al*., 2001a). The most obvious result of the study was that LMBV was prevalent in Mississippi, occurring at detectable levels in 12 of 17 of largemouth bass populations (Hanson *et al*., 2001a).

The exotic origin of LMBV was suggested by the pattern of fish kills and viral isolations in North America, where it was first found in Lake Weir, Florida, in 1991 (Grizzle *et al*., 2002), with subsequent isolations in states to the north and west. In 2000, a survey of Texas waters in which 2876 adult largemouth bass were tested, detected LMBV in 15 of 50 reservoirs and eight of 13 major river basins (Southard *et al*., 2009). The prevalence of LMBV detected ranged from 1.7 to 13.3% in infected reservoirs. The virus also was found during a long-term mortality study of smallmouth bass brood stock (Southard *et al*., 2009). In 2002, LMBV-infected largemouth bass were found in Lake Champlain, Vermont. The virus was also found in largemouth bass from Lake St. Clair, which lies between Ontario (Canada) and Michigan (the USA), in 2003, and in the Bay of Quinte, Lake Ontario, Canada, in 2000, although no large fish kills were reported in the Great Lakes basin (US EPA and Environment Canada, 2009). Surveys of 37 water bodies in New York (2004 and 2005) verified the presence of LMBV in 23 of 283 largemouth bass and five of eight smallmouth bass when assayed using quantitative PCR (Groocock *et al*., 2008). Age, sex and season were not significantly associated with LMBV prevalence. In the summer of 2006, a 3-month-long LMBV-induced die-off at a 22 ha Arkansas private impoundment demonstrated that 7% of the estimated population of largemouth bass collected were LMBV positive (Neal *et al*., 2009).

To date, LMBV has been detected in at least 17 fish species from 30 states. Attempts have been made to detect LMBV in states west of the current known range (Fig. 9.3), but infected fish were found only in Arizona in 2010 (Silverwood and McMahon, 2012). The first report of LMBV in the invasive northern snakehead, *Channa argus*, involved collections from tidal tributaries within Chesapeake Bay, which is bordered by Maryland and Virginia (Iwanowicz *et al*., 2013). Centrarchids are part of the northern snakehead diet, so exposure to LMBV-infected prey was a likely source of infection. LMBV was detected with bacterial pathogens in large-scale fish kills of smallmouth bass in the Susquehanna and Potomac River watersheds. The role of LMBV in these fish kills is unclear (Blazer *et al*., 2010).

Ranaviruses were isolated from largemouth bass cultured in Guangdong Province, China, that died with skin and muscle ulcerations, as well as a swollen spleen and kidneys (Deng *et al*., 2011). Investigators were able to transmit the infection by intramuscular injection of the isolate into healthy bass and mandarinfish, *Siniperca chuatsi*. Amplification, sequencing and phylogenetic analysis of the MCP gene revealed that the virus was identical to DFV and closely related to LMBV (Deng *et al*., 2011). Also, using virus isolation, electron microscopy, PCR, MCP sequencing (99.9% identity) and transmission studies, George *et al*. (2015) confirmed that LMBV was the cause of mortalities of koi, *Cyprinus carpio*, in fish farms on the south-east coast of India.

Scientists have reported disease outbreaks attributed to other iridoviruses, which may suggest an increase in prevalence of LMBV. However, it was not clear whether this was due to an actual increase of iridovirus disease, or a result of the collective ability to detect iridovirus infections in more vertebrates (Williams *et al*., 2005). The first detections of an iridovirus in largemouth bass outside the USA were reported from Taiwan, where mortality rarely

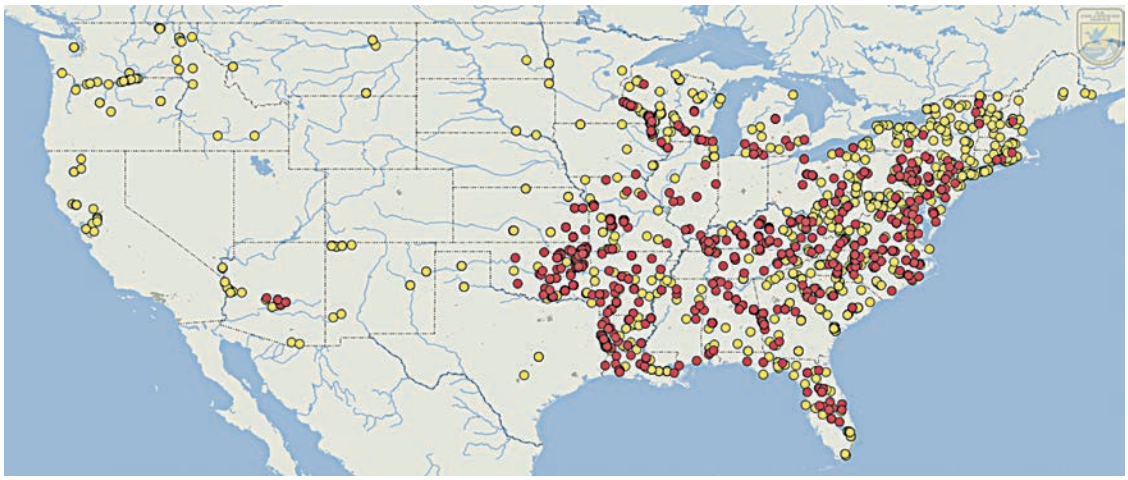

Fig. 9.3. National Wild Fish Health Survey (NWFHS) of the distribution of largemouth bass virus (LMBV) in the USA as on 2 November 2016. The red circles indicate LMBV detected and the yellow circles are largemouth bass (LMB) samples that tested negative. From US Fish and Wildlife Service (2015).

exceeded 30–50%. Sequence comparisons of the MCP initially showed they were closely related to grouper iridovirus in Taiwan (TGIV), but different from LMBV (Chao *et al*., 2002). Subsequent research designated this virus a *Megalocytivirus* (Chao *et al*., 2004; Huang *et al*., 2011).

9.1.4 Impacts of LMBV on fish populations

In 1999, 15 state hatcheries in ten states in the south-eastern USA were surveyed for LMBV. The virus was found in largemouth bass brood stock at five hatcheries in Florida, Louisiana, Tennessee and Texas. There were differences in the management practices at these hatcheries, so it was impossible to discriminate the factors that affected transmission. Juvenile largemouth bass were infected at one Texas location (Woodland *et al*., 2002b), but the vertical transmission of LMBV from infected brood fish was not established. The discovery of LMBV in juvenile fish in a hatchery does indicate that fish stocking contributes to virus spread. Signs of LMBV disease were not seen at any hatchery during the survey.

Another impact study occurred through repeated sampling at Sardis Reservoir, Mississippi, after a die-off of about 3000 adult largemouth bass when the surface waters were at temperatures of 29–32°C. Sampling on five separate occasions (at 1, 2, 4, 7 and 13 months) during the following year revealed that 53, 57, 42, 57 and 32%, respectively, of the 171 bass were positive for LMBV. The authors did not view a reduction of 3000 adult largemouth bass in the Sardis Reservoir population to have a major impact, even with evidence of LMBV persistence (Hanson *et al*., 2001b). Annual fishing losses in the lake exceed 30,000 largemouth bass per year. The authors suggested that further evaluations were needed to determine the impact that LMBV has on largemouth bass populations. No correlation was found with gender, size, handling injuries or the presence of skin lesions, although 97% of the 36 fish with swim bladder lesions were positive for LMBV by viral culture. LMBV was isolated from juvenile largemouth bass, confirming that the infection was not limited to adults. Hanson *et al*. (2001b) did not find LMBV infection in sympatric fish species, including bluegill, white crappie, *Pomoxis annularis*, white bass, *Morone chrysops*, and gizzard shad, *Dorosoma cepedianum*, 4–7 months after the outbreak.

Grizzle and Brunner (2003) emphasized that LMBV was associated with 24 largemouth bass die-offs, but sometimes the virus was found with no clinical signs. As decreases in bass populations after die-offs had not been documented, additional research was needed to determine whether LMBV had reduced the number of trophy-sized largemouth bass. Different geographical isolates of LMBV may exhibit major differences in pathogenicity and virulence (Goldberg *et al*., 2003). The mortality rate from LMBV infection and viral titre

in experimental survivors differed according to the origin of the isolate. The median survival times of bass injected with the three different isolates were 11.0, 7.5 and 4.5 days. The most virulent viral strain also replicated to the highest level in fish (Goldberg *et al*., 2003). The authors suggested that strain variation could explain clinical differences in responses of bass populations to LMBV.

9.2 Diagnosis

The gross lesions induced by *Ranavirus* are not unique to this virus. The visualization of 120–300 nm non-enveloped icosahedral particles within the cytoplasm of infected cells establishes probable infection by an iridovirus. Procedures for the isolation of LMBV are outlined in the American Fisheries Society (AFS) Fish Health Section (FHS) *FHS Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens* (AFS-FHS, 2014). The organs used for LMBV testing are the swim bladder, trunk kidney and spleen. Beck *et al*. (2006) showed that in the first few days after an immersion exposure, the gills and swim bladder would probably contain LMBV, and that the swim bladder and trunk kidney would have higher titres of virus. Pooled organ samples are recommended because a single organ may not always have detectable levels of LMBV (Beck *et al*., 2006). Currently, LMBV is isolated in cell culture and is then confirmed using PCR. Several fish cell lines promote the replication of LMBV (Piaskoski *et al*., 1999; McClenahan *et al*., 2005b). The FHM and BF-2 cell lines were optimal for LMBV isolation from both cell-culture fluids and homogenized organ samples. Frozen tissues from dead or moribund fish that were held at –10°C were stable if the fish were fresh when frozen or assayed within 5-months (Plumb and Zilberg, 1999a). Largemouth bass virus CPE in BF-2 cells began with pyknosis, rounding of the cells across the monolayer, followed by cell lysis and detachment. LMBV-inoculated FHM cells with CPE had foci of infection that became plaques devoid of cells (Fig. 9.4). High viral titres were reached in 24 h or less with BF-2 or FHM cells. A new cell line derived from the ovary of largemouth bass was recently developed and characterized (Getchell *et al*., 2014), though LMBV titres were tenfold lower in this than in BF-2 cells.

Cytoplasmic inclusion bodies are not visible without staining (AFS-FHS, 2014). Hanson *et al*. (2001b) evaluated the gills, spleen and swim bladder for the virus using cell culture. Gill tissue was rarely infected, whereas the highest frequency of viral isolation was from the swim bladder. The use of both the swim bladder and the spleen increased a positive diagnosis in 10% more fish than culturing of the swim bladder alone. Orally exposed largemouth bass that became infected with LMBV had varying viral titres in cutaneous mucus, swim bladder, head kidney, trunk kidney, spleen, gonads and intestine (Woodland *et al*., 2002a). The swim bladder produced the highest titres at $10^{5.5–9.5}$ $TCID_{50}$ (50% tissue culture infectious dose)/g, but lesions therein were not observed.

Mao *et al*. (1997, 1999) found that both PCR and reverse transcription PCR (RT-PCR) successfully

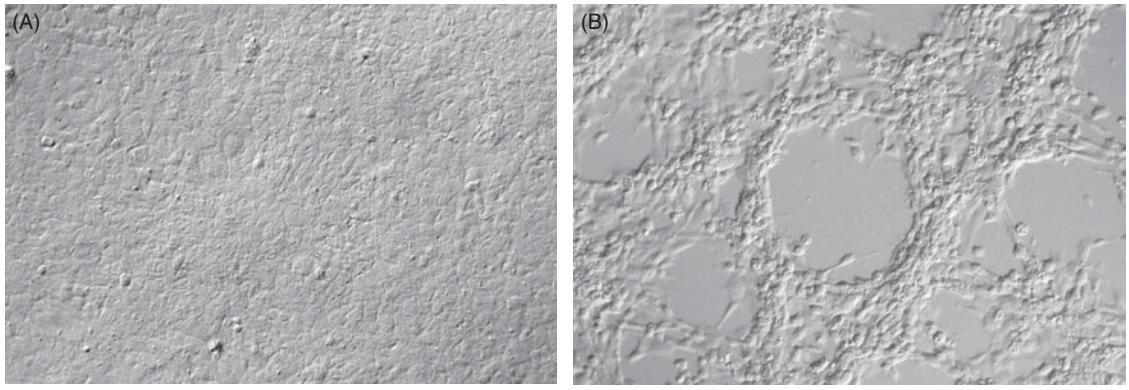

Fig. 9.4. (A) Control fathead minnow cells (FHM) and (B) largemouth bass virus (LMBV)-induced plaque formation on FHM cells. Photo original courtesy of John Grizzle, Auburn University.

amplified virus-specific nucleic acid and demonstrated that those piscine iridoviruses examined belonged within the genus *Ranavirus*. Electrophoretic analysis of proteins from virus-infected cells and RFLP analysis of viral DNA clarified the relatedness of these isolates. Later studies used PCR to confirm LMBV infection (Grizzle *et al*., 2002; Grizzle and Brunner, 2003). When there was not enough LMBV for cell culture, PCR could be used because of its greater sensitivity (Grizzle *et al*., 2003; McClenahan *et al*., 2005b). The PCR method can be simplified by adding diluted culture fluid from a presumptively positive cell culture to the PCR mixture, rather than using extracted DNA for the PCR template (McClenahan *et al*., 2005a). The specific LMBV288F-535R primer pair developed by Grizzle *et al*. (2003) amplified a 248 bp DNA fragment of LMBV from Santee-Cooper Reservoir, but did not amplify DNA from four other ranaviruses (*Frog virus 3*, FV3; DFV; GV6; *Epizootic haematopoietic necrosis virus*, EHNV) or from red sea bream iridovirus. This primer pair amplified the correct size fragment from 30 viral isolates that were presumptively identified as LMBV based on viral isolation and other methods. Later, Ohlemeyer *et al*. (2011) cloned and sequenced the complete MCP gene of DFV, GV6 and LMBV. The DFV and GV6 sequences were identical, and the LMBV sequence was 99.21% related. These authors then developed a Santee-Cooper-specific PCR that would not amplify related grouper iridoviruses. They concluded that these three viruses might not belong to the *Ranavirus* genus (Hyatt *et al*., 2000; Whittington *et al*., 2010).

Real-time PCR (qPCR) techniques to detect a fragment of the LMBV MCP were developed by Goldberg *et al*. (2003) and Getchell *et al*. (2007). The qPCR assay offered significant advantages for monitoring pathogen prevalence in fish populations, including high throughput capability and reduced contamination issues. Comparison of the qPCR assay with the plaque assay confirmed the extended linear range of the qPCR when dilutions of LMBV were tested and showed that the real-time qPCR assay was approximately 100 times more sensitive than the plaque assay when infected cell culture fluids were tested. Another qPCR assay developed to detect many fish ranaviruses was not tested against LMBV, but worked well with DFV and GV6 (Holopainen *et al*., 2011).

Serological tests are not sufficient to identify closely related ranaviruses due to extensive cross-reactivity (Hedrick *et al*., 1992; Marsh *et al*., 2002).

In situ hybridization targeted at MCP gene sequences was successfully employed to identify an iridovirus, although the tissues of the Malabar grouper, *Epinephelus malabaricus*, were fixed in formalin (Huang *et al*., 2004). PCR and RFLP were used to differentiate between species of *Ranavirus* (Marsh *et al*., 2002). Most recently, amplified fragment length polymorphism (AFLP) and qPCR were applied to detect and differentiate between geographical isolates of LMBV (Goldberg *et al*., 2003). Miller *et al*. (2015) do an excellent job in suggesting which diagnostic tools should be used to achieve specific investigational results. Ideally, multiple samples should be submitted and multiple tests performed.

9.3 Pathology

An enlarged swim bladder and an erythematous gas gland are gross pathological signs (Plumb *et al*., 1996) of LMBV infection. Zilberg *et al*. (2000) described the lesions in several juvenile largemouth bass injected intraperitoneally (IP) with LMBV. Clinical signs and external lesions included inflammation at the site of injection, distended abdomen, corkscrew swimming, lateral recumbency and lethargy. Internal lesions included focally pale livers, bright red spleens and reddened intestinal caecae. Microscopic lesions included acute fibrinous peritonitis and exudate present in the ventral aspect of the swim bladder. In another study (Hanson *et al*., 2001b), LMBV infections appeared to involve only the swim bladder and resulted in the accumulation of a yellow wax-like material in the lumen of the bladder (Fig. 9.5). The material consisted of erythrocytes, cellular debris and eosinophils in a fibrin clot. Grant *et al*. (2003) reported internal gross pathology such as exudative polyserositis, pneumocystitis and colour changes to various visceral organs, particularly the liver. Clinical signs observed in moribund infected juvenile koi included skin darkening, loss of scales, vertical hanging, uncoordinated swimming, turning upside down, lateral rotation, intermittent surfacing, settling at the bottom on their sides and death (George *et al*., 2015).

9.4 Pathophysiology

Fishing tournaments targeting largemouth bass occur in 48 out of 50 US states (Schramm *et al*., 1991). The specific effects of recreational fishing and environmental stressors, and their impact on

Fig. 9.5. Yellow exudate in the swim bladder of a largemouth bass infected with largemouth bass virus (LMBV). Photo original courtesy of Andrew Goodwin, US Fish and Wildlife Service.

the physiology of largemouth bass, may be associated with LMBV outbreaks (Schramm *et al*., 2006). The interaction of human-induced and environmental stressors with viral infection may precipitate fish kills. These physiological changes may contribute to increased pathogenicity of LMBV. LMBV-associated fish kills have been shown to typically occur during the hot summer months (Plumb *et al*., 1996; Hanson *et al*., 2001b). Environmental stressors affecting physiology during the summer months include elevated water temperature, low dissolved oxygen content and increased angling pressure (Grant *et al*., 2003). These may impede the immune response and permit increased viral replication. The identification of specific factors that initiate LMBV fish kills is important for managing the disease. Temperatures outside the normal thermal ranges have immunosuppressive effects in fish (Bly and Clem, 1992). Pickering and Pottinger (1989) also demonstrated that low chronic stress could lower disease resistance.

9.4.1 Effects on the endocrine system and osmoregulation

Catch-and-release angling is associated with physiological changes that affect homeostasis (Gustaveson *et al*., 1991; Suski *et al*., 2003; Cooke *et al*., 2004) and could result in immunosuppression (Anderson, 1990). Gustaveson *et al*. (1991) determined that the length of time a largemouth bass was played prior to landing the fish correlated with increases in blood cortisol and plasma lactate. Stress hormone production did not occur in largemouth bass hooked and played for 1–5 min in cold water (11–13°C), was moderate at 16–20°C and was severe in fish angled for 5 min at 28–30°C. Suski *et al*. (2003) showed the plasma cortisol and glucose concentrations and plasma osmolarity in tournament-caught bass were significantly greater than those in control groups. They suggested that factors such as live well confinement, handling and air exposure during weighing in might play an important role in the metabolic disturbances.

9.4.2 Effects on growth

Large changes in the metabolic status of largemouth bass sampled following fishing tournament weighing in included major reductions in the muscle energy stores and large increases in lactate concentrations (Suski *et al*., 2003). These may make largemouth bass more susceptible to LMBV. The Texas Parks and Wildlife Department staff measured bass growth parameters at two Texas impoundments where LMBV-attributed die-offs had occurred (Bister *et al*., 2006). No drop in mean length at age 3, relative weight or angling success at either reservoir was seen, whereas at five Alabama reservoirs, Maceina and Grizzle (2006) noted slower largemouth bass growth and lower relative weights associated with LMBV infections. This occurred at the same time as the decline in memorable length bass recorded in tournament and electrofishing catches. Beck *et al*. (2006) suggested that LMBV-induced hyperbuoyancy might cause exhaustion as the fish tried to maintain submergence.

9.4.3 Disease mechanism and bioenergetic cost(s) of LMBV

Grant *et al*. (2003) showed that largemouth bass infected with LMBV by IP injection at 30°C had higher mortality rates than bass injected and held at 25°C. They measured a higher viral load using qPCR in moribund and dead fish versus bass that survived the trial, and suggested that the higher temperature was probably more optimum for viral replication and more stressful for the host. There was a significant association between viral load and the presence of internal lesions. However, a similar challenge with the same source of largemouth bass but

with high LMBV titre did not find the same association (Goldberg *et al*., 2003). Juvenile largemouth bass kept in high-density captivity and infected with LMBV experienced higher mortality, increased viral loads and reduced body conditions compared with LMBV-inoculated bass raised at a lower density (Inendino *et al*., 2005). The conclusion was that 'Strategies that maximize the quality of the physical environment in which fish live while simultaneously minimizing sociobehavioural stress should most effectively increase health and productivity'.

Insights into the molecular mechanisms of pathogenesis were recently determined by studying the cell signalling events involved in LMBV-caused cell death (Huang *et al*., 2014). LMBV infections of epithelioma papulosum cyprini (EPC) cells produced apoptosis mediated by both intrinsic and extrinsic pathways involving caspase-8 and caspase-9. Phosphatidylinositol 3-kinase (PI3K) and extracellular-signal-regulated kinase (ERK) signalling pathways were involved in LMBV replication as well as infection-induced apoptosis. Identifying these mechanisms will contribute to the development of potential therapeutic targets for all iridovirus infections (Reshi *et al*., 2016).

9.5 Protective and Control Strategies

Oh *et al*. (2015) showed that fish vaccinated with a live red sea bream iridovirus following poly(I:C) (polyinosinic–polycytidylic acid) administration were protected, although DNA vaccines were less protective. No commercial vaccines are available to prevent LMBV.

Catch-and-release angling practices done by placing uninfected and infected largemouth bass in the same live well or holding tank facilitated LMBV transmission (Grant *et al*., 2005). The rapid uptake (1 h) of LMBV, combined with tank mates shedding virus, could mean that even a short exposure time could infect other fish in a live well (Beck *et al*., 2006). Compartmentalization of these tanks or decreasing their usage could reduce transmission, and lowering the temperature in live wells would be beneficial as higher temperatures would facilitate viral replication (Grant *et al*., 2003). Schramm *et al*. (2006) recommended that tournament-caught bass should not be retained or be confined with fish from infected populations. *Pseudomonas fluorescens* biofilms protected LMBV against hypochlorite and iodophor but not alcohol-based disinfectants (Nath *et al*., 2010). The virus was not detected in biofilms or water from ponds that had contained LMBV-positive fish 2 months earlier.

Concerns about the impacts of LMBV by fisheries managers, anglers and the sportfishing industry prompted the Bass Anglers Sportsman Society to arrange a multi-agency, multi-sector collaboration that facilitated research and guided action to control LMBV (Terre *et al*., 2008). Recommendations from workshops remind anglers and boaters to take the following steps to help prevent the spread of the virus:

1. Clean boats, trailers and other fishing equipment thoroughly between fishing trips.
2. Do not move fish or fish parts from one body of water to another, and do not release live bait into any water body.
3. Handle bass as gently as possible if you intend to release them and release them as quickly as possible.
4. Refrain from hauling the fish for long periods in live wells if you intend to release them.
5. Minimize the targeting of largemouth bass during the period from mid-July to mid-August, especially during exceptionally hot weather conditions.
6. Report dead or dying adult largemouth bass fish to natural resource agency offices.
7. Volunteer to help agencies collect bass for LMBV monitoring.
8. Educate other anglers about LMBV.

9.6 Conclusions and Suggestions for Future Research

9.6.1 Identifying gaps in our knowledge

The lack of a complete genome sequence for LMBV limits advances that will have an impact on control efforts of LMBV. Understanding how phenotypic and genotypic variation in LMBV affects virulence is important for understanding the biology, evolution and control of this disease. However, Goldberg *et al*. (2003) concluded that factors other than the inherent virulence of the pathogen, such as environmental and host-related factors, must contribute significantly to the clinical manifestations of LMBV infection in the field.

9.6.2 Suggestions for future studies

Reviewing the past research efforts on FV3 and the present work on other ranaviruses, as well as that on iridoviruses from other genera such as *Megalocytivirus*,

suggests that researchers will identify viral genes that play important roles in virulence and ultimately facilitate the creation of effective anti-ranavirus vaccines. Understanding how these viruses evade the antiviral immune responses of lower vertebrates will, it is hoped, be fruitful (Jancovich *et al*., 2015).

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10 Koi Herpesvirus Disease

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10.1 Introduction

Koi herpesvirus disease (KHVD) is a herpesvirus infection (Hedrick *et al*., 2000) that induces a lethal acute viraemia that is highly contagious in common carp (*Cyprinus carpio*) and varieties of *C. carpio* such as koi carp and ghost carp (koi × common carp) (Haenen *et al*., 2004). The causative agent is classified as *Cyprinid herpesvirus 3* (CyHV-3), a member of the family *Alloherpesviridae* and one of ten alloherpesviruses that infect fishes (Boutier *et al*., 2015a).

The transmission of CyHV-3 is horizontal and can occur directly or indirectly. Uchii *et al*. (2014) suggested that CyHV-3 in recovered fish reactivates periodically when the water temperature increases and transmits to naive fish when they are in close contact, such as at spawning. Direct transmission occurs by skin-to-skin contact between infected and naive carp, and through the cannibalistic and necrophagous behaviour of carp. Several vectors may facilitate the indirect transmission of CyHV-3. These include faeces, aquatic sediments, plankton and aquatic invertebrates (Boutier *et al*., 2015a). Contaminated water represents the major abiotic transmission medium as it can contain virulent virus excreted in urine and shed via faeces, gills and skin mucus. Infectious water is a highly efficient mode of transmission (Boutier *et al*., 2015b), although in the absence of hosts, CyHV-3 rapidly inactivates in water (Boutier *et al*., 2015a). There is currently no published evidence for vertical transmission.

Following the first reports of KHVD in Germany in 1997, and in Israel and the USA in 1998, the geographical range of the disease became extensive. Worldwide trade in koi carp is generally responsible for the spread of CyHV-3 and the disease now occurs or has been reported in fish imported into at least 30 countries (OIE, 2012; Boutier *et al*., 2015a). In Asia, the first outbreak of KHVD with mass mortalities of cultured koi carp occurred in Indonesia in 2002. In 2003, it was reported in Japan following mass mortalities of cage-cultured common carp (Haenen *et al*., 2004). CyHV-3 has since been detected in Taiwan, China, South Korea, Singapore, Malaysia and Thailand (Boutier *et al*., 2015a). In Africa, KHVD has only been reported in South Africa (Haenen *et al*., 2004).

In North America, the first KHVD outbreaks involved koi dealers in 1998 and 1999 (Haenen *et al*., 2004), but subsequently CyHV-3 caused mass mortalities of wild common carp in the USA and Canada (Boutier *et al*., 2015a). In Europe, the disease has been recorded in 18 countries, with widespread mass mortalities reported in carp in Germany, Poland and the UK (OIE, 2012; Boutier *et al*., 2015a).

CyHV-3 causes a highly virulent and contagious disease that induces massive mortalities and major economic losses in common and koi carp. The disease has been listed as notifiable by the World Organisation for Animal Health (OIE) since 2007 and is seasonal, occurring mostly at water temperatures between 17 and 28°C. Mortality occurs within 6–22 days postinfection (dpi), peaking between 8 and 12 dpi (Ilouze *et al*., 2006). Naturally occurring CyHV-3 infections have only been recorded from common carp and varieties of this species such as koi (OIE, 2012). However, *Carassius* spp. (crucian carp) hybridized with common carp may be susceptible to KHVD. Mortality reported in experimental infections has varied from 91 to 100% in crucian carp × koi and 35 to 42% in goldfish (*Carassius*

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auratus) × koi hybrids (Bergmann *et al*., 2010a), to only 5% in goldfish × common carp hybrids (Hedrick *et al*., 2006).

Goldfish are susceptible to CyHV-3 infection (OIE, 2012), but they do not develop disease. There is also increasing evidence that other cyprinid and non-cyprinid species (e.g. grass carp, *Ctenopharyngodon idella*; catfish, Siluriformes) are potential carriers that transmit the infection to carp (Boutier *et al*., 2015a).

From 1998 to 2000, KHVD spread to 90% of Israeli carp farms at an estimated cost of US$3 million a year. In Indonesia, between April and November 2002 the disease spread from East Java to four other major islands and the losses exceeded US$15 million by 2003 (Haenen *et al*., 2004). Undoubtedly, CyHV-3 had been spread globally before regulators became aware of the disease and when detection methods were unavailable. CyHV-3 DNA was detected in archived histological specimens collected during unexplained mass mortalities of carp in the UK in 1996 (Haenen *et al*., 2004) and in South Korea in 1998 (Lee *et al*., 2012). In Israel, piscivorous birds are suspected to spread CyHV-3 among farms (Ilouze *et al*., 2011). Other routes include the mixing of fish in the same tanks at koi shows, selling infected fish below the market price and the release of infected fish into public waters (Boutier *et al*., 2015a).

10.2 Diagnostics

The diagnosis of KHVD in clinically affected fish uses a range of tests, but few are fully validated. The chapter on KHVD in the *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2012) advises that the diagnosis of KHVD should rely on a combination of tests, including clinical examination as well as viral detection. The final diagnosis of KHVD must rely on the direct detection of viral DNA or the isolation and identification of CyHV-3 using immunological and molecular techniques.

10.2.1 Behavioural changes

The most evident behavioural sign of KHVD is lethargy. Fish will separate from the shoal and lie at the bottom of the tank or pond or hang in a head-down position. They may also gasp at the surface near the water inlet and other aerated areas or at the sides of the pond (OIE, 2012). Some fish may experience loss of equilibrium and disorientation but, at the same time, may become hyperactive (Hedrick *et al*., 2000).

10.2.2 External gross pathology

There are no pathognomonic gross lesions of KHVD. The most consistent gross pathology is pale, irregular patches on the skin, associated with excess mucus secretion, and also the underproduction of mucus, resulting in patches of skin with a sandpaper-like texture (Haenen *et al*., 2004; Fig. 10.1A). The skin may also show hyperaemia, haemorrhages and ulceration (Boutier *et al*., 2015a; Fig. 10.1B). As the disease progresses, fish may experience focal or extensive loss of skin epithelium. In less acutely affected carp, commonly reported clinical signs include anorexia and enophthalmia (eyes sunken into their sockets) (Boutier *et al*., 2015a; Fig. 10.1C). The gross pathology in the gills is the most consistent feature in clinical KHVD. The pathology varies from pale necrotic patches to extensive discoloration (bleaching) with severe necrosis and inflammation (OIE, 2012, Fig. 10.2).

10.2.3 Internal gross pathology

There may be accumulation of abdominal fluid and abdominal adhesions, and organs may be enlarged, darker and/or mottled, but these lesions are not pathognomonic of KHVD (Boutier *et al*., 2015a).

The appearance of gross lesions may also be complicated by heavy ectoparasite infections in diseased fish, particularly in the common carp. Eight parasite genera (e.g. *Gyrodactylus* sp.) are commonly associated with KHVD-affected carp; secondary infections with gill monogean parasites and infections with a range of bacteria (e.g. *Flavobacterium* sp.) have also been reported (OIE, 2012).

10.2.4 Sampling

Young carp (<1 year) are generally more susceptible to clinical KHVD and should be selected for sampling. Common carp, or varieties such as koi or ghost carp, are most susceptible to the disease, followed by any common carp × *Carassius* spp. hybrids (OIE, 2012). Moribund or freshly dead fish with clinical signs are suitable for most of the immunological and/or molecular tests that can be used. Tissues from decomposed carp may only be suitable for testing using polymerase chain reaction (PCR)-based methods. Samples from apparently

Fig. 10.1. External gross pathology of fish with koi herpesvirus disease (KHVD): (A) irregular patches on the skin mainly associated with the underproduction of mucus where patches of skin have a sandpaper-like texture; (B) hyperaemia and haemorrhages on the skin of a common carp; (C) koi displaying enophthalmia.

healthy carp, in a suspect population, are most reliably tested using PCR-based assays (OIE, 2012).

Organ samples should be collected immediately after the carp has been selected (OIE, 2012). Whole fish may be submitted for testing packed in ice and tissues preserved in viral transport medium or in 80–100% ethanol. Samples preserved in alcohol or those received frozen are only suitable for PCR-based tests.

Pooled samples should be avoided or restricted to a maximum of two fish per pool. The *Report from Meeting on Sampling and Diagnostic Procedures for the Surveillance and Confirmation of KHV Disease* held in Copenhagen in 2014 (Olesen *et al*., 2014) advises that, in acute cases, the tissues of five fish can be pooled. Recommended tissues include gill, kidney and spleen (OIE, 2012), as these contain the greatest DNA concentrations (Gilad *et al*., 2004).

10.2.5 Direct immunodiagnostic methods

A method for the direct detection of CyHV-3 from kidney imprints using an indirect fluorescent antibody test (IFAT) is available (OIE, 2012). Immunoperoxidase staining has also been used to detect the CyHV-3 antigen, but it is prone to producing false-positive staining (Pikarsky *et al*., 2004).

10.2.6 Detection using PCR-based assays

PCR-based assays are generally the most sensitive and reliable methods to detect CyHV-3 in tissues. The OIE (2012) recommends one assay that incorporates a primer set targeting the thymidine kinase (TK) gene (Bercovier *et al*., 2005) and another assay that is an improvement of the Gray SpH primer set protocol (Yuasa *et al*., 2005).

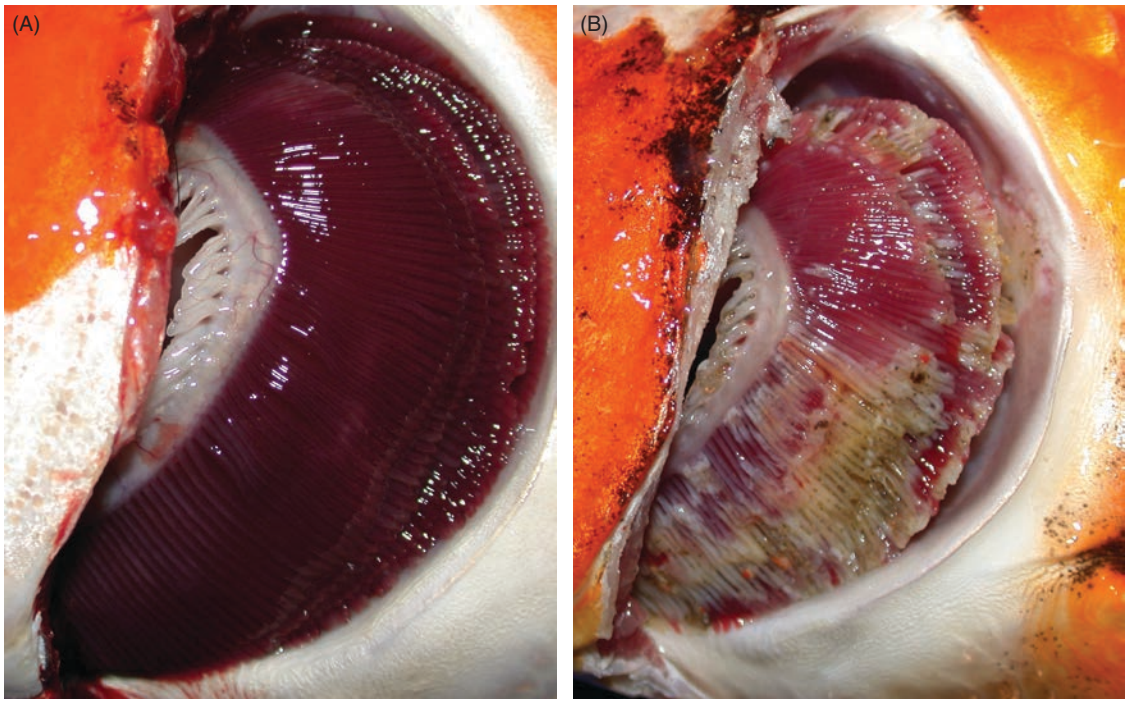

Fig. 10.2. External gross pathology of carp gill with the operculum removed: (A) normal healthy gill; (B) gill of fish with koi herpesvirus disease (KHVD) displaying inflammation and necrosis.

Alternatively, many diagnosticians favour quantitative (real-time) PCR (qPCR) assays over conventional PCR. The most common qPCR for the detection of CyHV-3 is the Gilad Taqman real-time PCR assay (Gilad *et al*., 2004) that detects and can quantitatively assess very low copy numbers of target nucleic acid sequences (Boutier *et al*., 2015a). Real-time PCR avoids much of the contamination risk inherent in conventional PCR (OIE, 2012); it also targets shorter DNA sequences and is more likely to detect degraded viral DNA in decomposing tissues.

Loop-mediated isothermal amplification (LAMP) is a rapid single-step PCR assay that is widely adapted for pond-side diagnosis because it does not require a thermal cycler. LAMP of the TK gene has been developed to detect CyHV-3 and is equal to or more sensitive than conventional PCR (Yoshino *et al*., 2009). An assay incorporating DNA hybridization technology and antigen–antibody reactions in combination with LAMP has also been developed and had improved sensitivity and specificity (Soliman and El-Matbouli, 2010). LAMP has potential for the non-destructive testing of clinically affected fish, but the OIE does not elaborate upon LAMP assays for KHVD because none have been submitted for assessment and registration.

The most sensitive PCR method is the Gilad Taqman assay mentioned above (OIE, 2012). In a comparison of PCR-based assays Bergmann *et al*. (2010b) showed that conventional PCR assays which include a second round with nested primers were as sensitive as real-time assays. However, nested PCR is more susceptible to cross-contamination by PCR products from previous tests, so producing false positives.

10.2.7 Histopathology

The histopathology of the disease is non-specific and variable. Alterations are found in the gills, skin, kidneys, heart, spleen, liver, gut and brain in KHVD-affected fish (OIE, 2012; Boutier *et al*., 2015a). Evidence of herpesvirus infection may be most readily observed in the skin, gills and kidney, as described in Section 10.3 Pathology.

10.2.8 Electron microscopy

The examination of tissues from clinically infected carp for viral particles using transmission electron microscopy (TEM) is not reliable unless the fish has a heavy infection (OIE, 2012).

10.2.9 Virus isolation in cell culture

The common carp brain (CCB) and koi fin (KF)-1 cell lines are recommended for the isolation of CyHV-3, but the former is more susceptible (OIE, 2012; J. Savage, UK, personal communication). Boutier *et al*. (2015a) list other cell lines that are susceptible to CyHV-3. Before clinical signs appear, viral levels are higher in gill than in kidney tissues (Gilad *et al*., 2004; Yuasa *et al*., 2012), and the virus is most reliably isolated from gill tissue. However, virus isolation in cell culture is not as reliable or sensitive as PCR-based methods for detecting CyHV-3 DNA (OIE, 2012).

10.2.10 Other diagnostic assays, including non-lethal methods

In situ hybridization (ISH) and IFAT have been used to detect and identify CyHV-3 in fish leucocytes (Boutier *et al*., 2015a). Although these methods have not been fully compared with other techniques, they are non-lethal and may facilitate diagnosis (OIE, 2012). Non-lethal samples such as blood, gill swabs, gill biopsy and mucus scrapes are also suitable substrates for diagnosis (Olesen *et al*., 2014). Other non-lethal immunodiagnostic methods include an ELISA developed to detect CyHV-3 antigen in fish faeces (Dishon *et al*., 2005). In addition, a lateral flow device that detects CyHV-3 glycoprotein (the FASTest Koi HV kit) in a 15 min pond-side test and works best with gill swabs (Vrancken *et al*., 2013).

10.3 Pathology

The gills and skin of CyHV-3-infected fish exhibit prominent clinical signs of the disease, which is reflected in the histopathology. Hyperplasia and hypertrophy are common in the gills (Boutier *et al*., 2015a; Fig. 10.3A). Pikarsky *et al*. (2004) observed pathological changes in the gills of experimentally infected fish at 2 dpi, including the loss of lamellae and a mixed inflammatory infiltrate in some filaments. Hyperplasia, severe inflammation and congestion of the central venous sinus became more pronounced at 6 dpi. There was subepithelial inflammation and congestion of blood vessels of the gill rakers, accompanied by reduction in their height, and sloughing of the surface epithelium; haemorrhages were sometimes observed in the lamellar capillaries. Enlarged branchial epithelial cells had nuclear swelling accompanied by margination of the chromatin and pale diffuse eosinophilic inclusions – the 'signet ring' appearance, also termed intranuclear inclusion bodies (Fig. 10.3B). Such cells usually contain CyHV-3 when observed using TEM. Inclusion bodies have also been observed in the heart, kidney (Fig. 10.3C), spleen, liver, intestine, stomach, brain and fin epidermis. The secondary lamellae are often fused with the hyperplastic branchial epithelium, and necrosis is often seen, particularly at the tips (Boutier *et al*., 2015a; Miwa *et al*., 2015).

Infiltration of cells under the epidermis of the fin is observed from 1 dpi onwards and epidermal integrity is disrupted from 2 dpi onwards. Loss of epidermis is also noted from 3 dpi onwards and the epidermis of the head is often sloughed. The number of goblet cells is reduced by 50% in infected fish, and they appear thin, which suggests that mucus is released and not replenished (Adamek *et al*., 2013). At later stages, erosion of the skin epidermis is frequently observed and is often the severest pathological change observed (Adamek *et al*., 2013; Miwa *et al*., 2015).

The kidneys exhibit notable pathology, starting with a peritubular inflammatory infiltrate from 2 dpi, followed at 6 dpi by a heavy interstitial inflammatory infiltrate, with congestion of the blood vessels. At or beyond 8 dpi there is hyperplasia or degeneration of the tubular epithelium and intraepithelial lymphocytes are present. Necrotic cells are observed; in severe disease haematopoietic cells are necrotic. Chronic glomerulitis, periglomerular fibrosis and interstitial nephritis with the loss of haemopoietic cells are observed (Boutier *et al*., 2015a; Miwa *et al*., 2015).

The liver exhibits mild inflammatory infiltrates, mainly in the parenchyma, with focal necrosis in some fish. Proliferation in the columnar epithelial cells of the bile duct may occur (Pikarsky *et al*., 2004; Cheng *et al*., 2011).

Hyperplasia in the epithelium lining the gastric gland occludes the lumen of the stomach and there is hyperplasia of the intestinal villi. Intestinal epithelial cells are sloughed into the lumen (El Din, 2011).

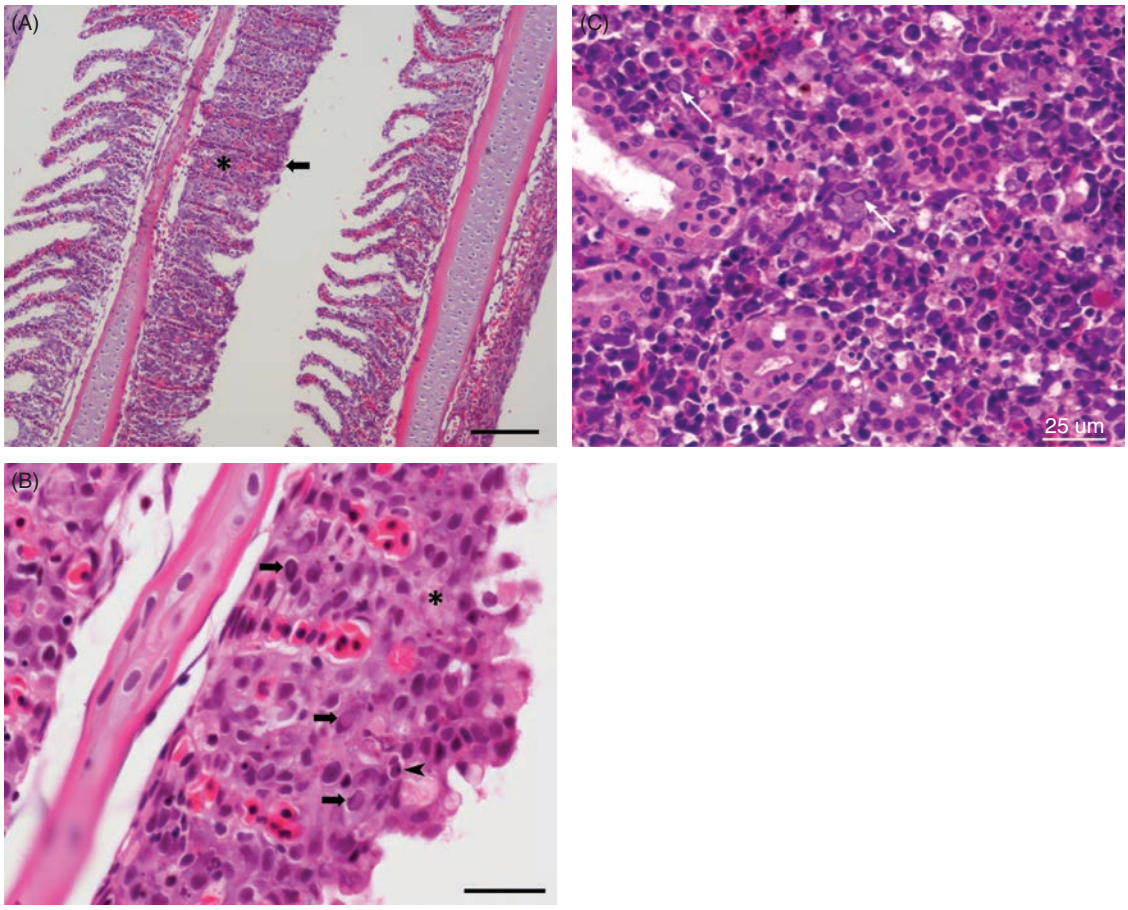

Fig. 10.3. Microscopic lesions in tissue sections (stained with haemotoxylin and eosin) from common carp with koi herpesvirus disease (KHVD); (A) Hypertrophy and hyperplasia of the branchial epithelial cells (∗) and fusion of secondary lamellae (arrow) of the gill; (B) necrosis and inflammation (∗), apoptosis (arrowhead) and intranuclear inclusions (arrows) in the branchial epithelium; (C) interstitial haematopoietic cell necrosis and nuclei with inclusions (arrows) in the kidney. Scale bars: (A) 100 µm; (B) 20 µm; (C) 25 µm.

The parenchyma of the spleen contains foci of necrosis, and there is necrosis of pancreatic acinar cells in the spleen and kidney (Hedrick *et al*., 2000; Cheng *et al*., 2011).

In the brain, there is focal meningeal and parameningeal inflammation and the congestion of capillaries and small veins (Boutier *et al*., 2015a).

Many myocardial cells exhibit nuclear degeneration and myofibrils are dilated or coagulated with the disappearance of cross-striations. Later in the course of the disease, macrophages and lymphocytes infiltrate the myocardium and necrosis of the heart muscle is observed (Miyazaki *et al*., 2008; Cheng *et al*., 2011).

10.4 Pathophysiology

The major portal of entry for CyHV-3 in carp has been found to be the skin, not the gills and intestines as initially proposed (Costes *et al*., 2009). However, gill lesions are observed early in infection (Hedrick *et al*., 2000; Pikarsky *et al*., 2004), and viral DNA is detected in the gills and intestines as early as 1 dpi (Gilad *et al*., 2004). Costes *et al*. (2009) used a luciferase (LUC)-expressing recombinant CyHV-3 to induce KHVD in common carp that was indistinguishable from disease induced by the parental wild type strain. Furthermore, imaging of naturally infected carp using this LUC-expressing

recombinant revealed that CyHV-3 entered the fish via the skin and not the gills. Early viral replication occurred in the skin epithelium, principally in the fins (Fig. 10.4). CyHV-3 RNA expression was shown in the skin 12 h postinfection (hpi) (Adamek *et al*., 2013), and viral DNA was detected in infected cells of fin epithelium by 2 dpi (Miwa *et al*., 2015). Immersion infection of carp with an attenuated CyHV-3 also demonstrated dispersion from the skin to other organs. (Boutier *et al*., 2015a). Using similar techniques, Fournier *et al*. (2012) showed that the pharyngeal periodontal mucosa was a major portal of entry after oral infection (Fig. 10.4). What is more, the spread of the virus to other organs and progress of clinical disease was comparable to that of infection via immersion.

Following infection, CyHV-3 spreads rapidly to other organs that represent secondary sites of replication. The tropism of CyHV-3 for white blood cells may explain the rapid spread of the virus via the blood (Boutier *et al*., 2015a). Viral DNA can be detected in the blood, gill, liver, spleen, kidney, intestine and brain tissue at 1 dpi (Gilad *et al*., 2004; Pikarsky *et al*., 2004). In acutely infected freshly dead carp, CyHV-3 DNA copy numbers range from 10^9 to $10^{11}/10^6$ host cells in gill, intestine and kidney tissue (Gilad *et al*., 2004). These osmoregulatory organs undergo marked pathological changes during the course of the disease, and Gilad *et al*. (2004) suggested that the loss of osmoregulatory function contributes to death. Negenborn *et al*. (2015) showed that electrolyte levels, mainly sodium ions in the urine, and a concomitant decrease in serum electrolyte levels occurred during experimental infection. Changes in electrolyte levels were paralleled by severe pathology in the kidney and gills, providing further evidence that severe osmoregulatory dysfunction could cause death. In experimental CyHV-3 infections, Miwa *et al*. (2015) reported extensive damage to the skin epidermis at 5–8 dpi. Serum osmolality was also very low just before death and suggested that hypo-osmotic shock from skin damage was a likely cause of death.

10.4.1 Innate immune response

Investigators have suggested a strong and rapid innate immune response to CyHV-3 infection, as evidenced by the early upregulation of complement-associated and C-reactive proteins (Pionnier *et al*., 2014). Interferon (IFN) plays an important mediation role and studies of the response in the skin and intestine revealed activation of IFN Class I pathways (Adamek *et al*., 2013; Syakuri *et al*., 2013). However, unlike spring viraemia of carp virus (SVCV), CyHV-3 can inhibit the IFN type-I pathway and inhibit the activity of stimulated macrophages and the proliferative response of lymphocytes (Boutier *et al*., 2015a). Furthermore, CyHV-3 does not induce apoptosis and stimulation of the apoptosis intrinsic pathway is delayed (Miest *et al*., 2015). Genes encoding claudins (the tight-junction proteins), mucin and beta defensin antimicrobial peptides are downregulated during CyHV-3 infection. The disruption of these important components of the skin mucosal barrier contribute to the disintegration of the skin (Adamek *et al*., 2013).

10.4.2 Adaptive immune response and immune evasion

Carp produce a strong, temperature-dependent, protective antibody against CyHV-3. Antibody response to the virus is slow at 12–14°C compared with a rapid response at 31°C. At temperatures permissive for viral infection, antibodies can be detected between 7 and 14 dpi, with peaks between 20 and 40 dpi, and remain detectable for at least 65 weeks (Perelberg *et al*., 2008; St-Hilaire *et al*., 2009).

In carp infected via immersion, an attenuated CyHV-3 recombinant virus was detectable in the skin mucosa and induced a strong protective mucosal immune response against wild type CyHV-3 (Boutier *et al*., 2015b). This may be related to the stimulation of B cells secreting IgT, an immunoglobulin isotype that is involved in mucosal immunity in teleosts (Boutier *et al*., 2015a).

In silico, *in vitro* and *in vivo* studies have suggested that CyHV-3 may express proteins involved in immune evasion (see Boutier *et al*., 2015a) and that this might explain the acute and dramatic clinical signs associated with KHVD.

10.4.3 Latent infection

The survivors of KHVD outbreaks in wild and cultured common carp are persistently infected with CyHV-3 (Baumer *et al*., 2013; Uchii *et al*., 2014), although latency has not been demonstrated conclusively for the virus (Boutier *et al*., 2015a). Seasonal reactivation is proposed to explain how CyHV-3 persists in convalescent populations of

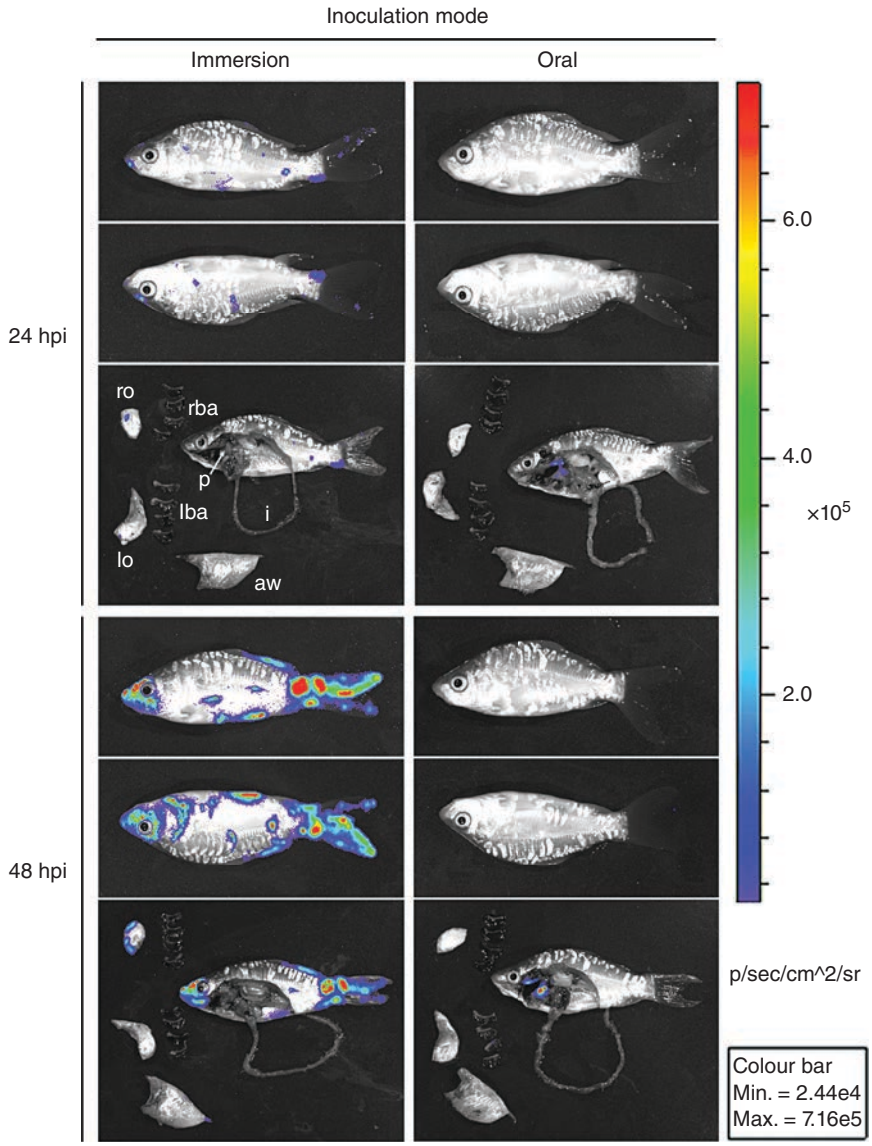

Fig. 10.4. The portals of entry of CyHV-3 in carp analysed by *in vivo* bioluminescent imaging. Two groups of fish (mean weight 10 g) were infected with a recombinant CyHV-3 strain expressing luciferase as a reporter gene, either by bathing them in water containing the virus (immersion, left column) or by feeding them with food pellets contaminated with the virus (oral, right column). At the indicated times (h) postinfection (hpi), six fish per group were analysed by a bioluminescence *in vivo* imaging system (IVIS). Each fish was analysed lying on its right and left sides. The internal signal was analysed after euthanasia and dissection. Dissected fish and isolated organs were analysed for *ex vivo* bioluminescence. One representative fish is shown for each time point and inoculation mode. Images collected over the course of the experiment were normalized using an identical pseudocolour scale ranging from violet (least intense) to red (most intense) using Living Image 3.2 software. Key: aw, abdominal wall; i, intestine; lba, left branchial arches; lo, left operculum; p, pharynx; rba, right branchial arches; ro, right operculum. Units: Min. and Max., standardized minimum and maximum threshold value for photon flux/; p/sec/cm^2/sr = photons/s/cm^2/steradian. Reproduced with permission from Fournier *et al*. (2012); original publisher BioMed Central.

carp. RNA expression of virus replication-related genes was detected in the brain of seropositive fish, while other fish expressed only latency-related genes (Uchii *et al*., 2014); this suggests that reactivation is a transient phenomenon within a population. The reactivation of CyHV-3 may be triggered by temperature stress (Eide *et al*., 2011) and netting stress (Bergmann and Kempter, 2011). Viral DNA was detected in white blood cells (WBCs), in the absence of clinical signs and detectable infectious virus particles, among koi carp that have been previously exposed to CyHV-3 and in wild common carp with no previous history of KHVD (Eide *et al*., 2011; Xu *et al*., 2013). The main WBC type involved in persistence was the IgM+ B cell, in which roughly 20× more DNA copies are found than in the remaining WBC fraction (Reed *et al*., 2014). The virus has been found in various tissues of long-term infected fish, and especially in the brain. The nervous system may represent an additional site of latency, as in other herpesviruses (Boutier *et al*., 2015a).

10.5 Prevention and Control

10.5.1 Resistant strains of carp

Strains of carp differ in their susceptibility to CyHV-3. In cross-breeding experiments, some wild strains of carp, such as the Sassan and Amur strains, confer resistance to KHVD when crossed with domestic strains (Shapira *et al*., 2005; Dixon *et al*., 2009; Piackova *et al*., 2013). The domestic Israeli strain Dor-70 has also shown high resistance to infection (Zak *et al*., 2007). In contrast, in Japan, indigenous common carp were more severely affected by KHVD than domesticated Eurasian common carp and koi (Ito *et al*., 2014). The analysis of disease resistance at the molecular level has identified links with the polymorphism of genes involved in the immune response, including the MHC (major histocompatibility complex) class II B genes (Rakus *et al*., 2012) and the carp IL-10 (interleukin 10) gene (Kongchum *et al*., 2011). The results from these studies have not been conclusive but do indicate that there are genetic markers for resistance that could enhance selective breeding.

10.5.2 Vaccination

Early attempts to vaccinate carp involved cohabiting them with CyHV-3-infected fish for 3–5 days at 22–23°C and then transferring them to ponds at 30°C for 30 days (Ronen *et al*., 2003). The fish were more resistant to the virus following challenge with CyHV-3. This method reduced the mortality in CyHV-3 endemic areas within Israel (H. Bercovier personal communication to Dixon, 2008) but the disadvantages included high costs and disease recurrence, the treated fish were reservoirs of infection and mortality remained significant.

CyHV-3 attenuated by serial passage in cell culture and injected intraperitoneally (IP) into carp conferred complete protection when they were cohabited with CyHV-3-infected fish (Ronen *et al*., 2003). The administration of attenuated virus by 10 min bath immersion induced protection, but the efficacy declined with time and was ineffective after 4 h in the water (Perelberg *et al*., 2005). The attenuated virus was irradiated with ultraviolet (UV) light to induce mutations that might prevent reversion to virulence. UV-treated virus protected carp against CyHV-3 infection, but no data were presented to confirm that the virus would remain avirulent. Research has continued into developing an attenuated vaccine (Perelberg *et al*., 2008; O'Connor *et al*., 2014; Weber *et al*., 2014). Recently, the efficacy and safety of this vaccine were examined in the USA (Weber *et al*., 2014) and it was found to be safe and efficacious, particularly in fish weighing >87g. Vaccinated koi carp (weight not stated) had 36% mortality compared with 100% mortality in non-vaccinated control fish when challenged at 13 months postvaccination (O'Connor *et al*., 2014).

An attenuated vaccine (Cavoy, manufactured by KoVax Ltd), is available in Israel and was authorized in 2012 for use in the USA as an immersion vaccine for carp weighing >100 g. However, the vaccine was withdrawn from sale after a year (Boutier *et al*., 2015b). The latter authors produced a recombinant CyHV-3 lacking ORF56 and ORF57, which replicated at lower levels and spread less efficiently within the host compared to the parental virus. Common carp were vaccinated with the recombinant virus via immersion and there were no mortalities 20 days later. After cohabitation challenge with carp infected with the parental virus, 80% survival was reported in the vaccinated groups compared with 0% survival among mock vaccinates. There was low-level transmission of recombinant virus when vaccinated fish were cohabited with sentinel fish, but none by water alone.

Yasumoto *et al.* (2006) inactivated CyHV-3 with formalin and entrapped it within liposomes. The CyHV-3-liposomes were sprayed on to dry-pelleted feed which was fed to common carp for 3 days, with normal pelleted feed fed thereafter. CyHV-3 virus was then administered to the gills of experimental fish, resulting in 23% mortality in two groups of vaccinated fish and 66 and >80% mortality in two groups of control fish.

Research groups are trying to develop DNA vaccines or recombinant vaccines that have no residual virulence or even the potential to revert to virulence. Prototype DNA vaccines based on the CyHV-3 glycoprotein gene (Nuryati *et al.*, 2010) and the ORF81 and ORF25 genes (Boutier *et al.*, 2015a) have provided varying degrees of protection to carp following injection, but none have been field tested. Vaccines based on recombinant viruses containing deletions in the TK, ribonucleotide reductase or deoxyuridine triphosphatase genes (Fuchs *et al.*, 2011) have given inconsistent results in immersion vaccination trials. Recently, genetically engineered *Lactobacillus plantarum* expressing both CyHV-3 ORF81 protein and SVCV glycoprotein was incorporated into a pelleted feed to produce an oral vaccine (Cui *et al.*, 2015). Under experimental conditions, there was 47% mortality in vaccinated common carp and 85 and 93% mortality in control fish following challenge with CyHV-3. The vaccine also protected koi carp against SVCV (see Chapter 7).

10.5.3 Management and biosecurity strategies

In the absence of proven commercial vaccines or therapeutic agents, management practices and biosecurity strategies are the main ways to prevent the spread of CyHV-3. These strategies range from national or international legislation and/or standards to good management practices at the farm level. The latter include on-site quarantine of new stocks of fish, the disinfection of equipment and use of footbaths, and reducing stress and other diseases, etc. New fish brought on to a site should be quarantined for a minimum of 4 weeks to 2 months before mixing with susceptible species, but the procedures undertaken and their impact will depend on the scale of the aquaculture undertaking. The rates of contagion and mortality can be rapid and limit the use of management practices, though increasing the water temperature to above 26–28°C and reducing stocking densities may reduce mortalities (Gilad *et al.*, 2003; Ronen *et al.*, 2003; Sunarto *et al.*, 2005).

There has only been one study on the inactivation of CyHV-3 by chemical and physical treatments (Kasai *et al.*, 2005), and the results were not conclusive. The minimum concentrations of the chemicals to effect 100% plaque reduction after 30 s and 20 min, respectively, at 15°C were iodophor 200 mg/l (both time periods), sodium hypochlorite >400 and 200 mg/l, benzalkonium chloride 60 mg/l (both time periods) and ethyl alcohol 40 and 30%. An ultraviolet dose of 4×10^3 µWs/cm^2 gave 100% plaque reduction in a virus solution containing 1×10^5 pfu/ml (time of exposure not stated) and 1.6×10^4 pfu/ml. CyHV-3 was inactivated at temperatures above 50°C for 1 min.

Yoshida *et al.* (2013) produced a laboratory-scale effluent treatment system in which bacterial substances inhibitory to CyHV-3 were adsorbed on to a porous carrier contained within a column. Samples of effluent water seeded with CyHV-3 taken before and after passage through the column were injected into carp and resulted in >80 and 0% mortality, respectively. The authors are working to engineer an operational system that could be used to treat aquaculture effluent water.

A majority of countries have enacted legislation to control the introduction and spread of diseases, including fish diseases. This is currently the most important way to prevent and control KHVD. Such legislation is often strengthened by international agreements. The OIE has produced the *International Aquatic Animal Health Code* (OIE, 2015), which outlines approaches to biosecurity that can be applied nationally and locally on production sites. Håstein *et al.* (2008) provided an overview of approaches to implementing biosecurity strategies, and Oidtmann *et al.* (2011) have reviewed international standards relating to movements of fish and fish products.

10.6 Conclusions

KHVD occurs worldwide and is an OIE notifiable disease. The virus, CyHV-3, induces a highly contagious acute viraemia that is responsible for severe financial losses in the common carp and koi culture industries.

Although there are no pathognomonic gross lesions, the rapid onset and severity of external clinical signs and numbers of affected carp provide good

indications of KHVD disease. The likely cause of death is osmoregulatory dysfunction or osmotic shock. Rapid identification of the virus is possible using PCR-based methods, and pond-side, non-lethal tests have been developed. The virus induces a strong innate immune and protective antibody response in carp, but the availability of commercial vaccines is limited.

Survivors of KHVD are persistently infected with CyHV-3 and there is evidence for seasonal and stress-induced reactivation. More studies are needed to confirm and identify both the site of latency and the latency-associated viral gene transcripts that might be useful targets for virus surveillance. Also, research is needed to assess how temperature affects and possibly regulates the switch between lytic and latent infection.

Some strains of carp are resistant to KHVD. Continuation of research into the genetic basis for resistance/susceptibility to CyHV-3 may enhance selective breeding programmes for resistance to KHVD.

Good management and biosecurity practices are the main ways to prevent the spread of CyHV-3. The last 15 years has seen improvements in these practices in cold-water aquaculture industries worldwide, many as a result of the threat from KHVD.

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11 Viral Encephalopathy and Retinopathy

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11.1 Introduction

Viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN) is a severe neuropathological disease caused by RNA viruses of the genus *Betanodavirus* (Family: *Nodaviridae*). This infectious agent, detected in the late 1980s, spread worldwide, became endemic and came to represent a major limiting factor for mariculture in several countries. The disease has recently been included among the most significant viral pathogens of finfish, given the expanding host range and the lack of properly effective prophylactic measures (Rigos and Katharios, 2009; Walker and Winton, 2010; Shetty *et al*., 2012).

11.2 The Infectious Agents

The causative agent of the disease is a small (25–30 nm diameter), spherical, non-enveloped virion, with a bi-segmented genome made of two single-stranded positive-sense RNA molecules. The name *Nodaviridae* originates from the Japanese village of Nodamura, where the prototype virus was first isolated form mosquitos (*Culex tritaeniorhynchus*). In 1992, a different nodavirus was isolated from larvae of the striped jack (*Pseudocaranx dentex*), which accounts for the name of striped jack nervous necrosis virus (Mori *et al*., 1992). Subsequent molecular studies separated these viruses into two different genera: *Alphanodavirus* and *Betanodavirus*, which infect insects and fish, respectively (King *et al*., 2011). A third genus, Gammanodavirus, which has not yet been accepted by the International Committee on Taxonomy of Viruses (ICTV), was recently detected in prawns (*Macrobranchium rosenbergii*) in India (NaveenKumar *et al*., 2013).

The *Betanodavirus* genome is formed by two open reading frames (ORFs); the RNA1 segment (3.1 kb) encodes for the RNA-dependent RNA-polymerase (RdRp) and the RNA2 segment (1.4 kb) encodes for the viral capsid protein (King *et al*., 2011). The transcription of the RNA1 segment apparently occurs at the beginning of the viral cycle, whereas the expression and production of the capsid, as well as the increase of infective viral particles, occurs at a later stage (Lopez-Jimena *et al*., 2011). A further subgenomic transcript of 0.4 kb, called RNA3, is cleaved from the RNA1 molecule during active viral replication and encodes for the B1 and B2 proteins, which antagonize host cell RNA interference mechanisms (Iwamoto *et al*., 2005; Fenner *et al*., 2006a; Chen *et al*., 2009). The synthesis of RNA3 in cell cultures is much more abundant than the transcription of RNA1 at an earlier point in time after infection (Sommerset and Nerland, 2004). The B2 non-structural protein is detected only at an early stage of infection, both in infected cell cultures and, recently, in infected Atlantic halibut *(Hippoglossus hippoglossus)*, while the *Betanodavirus* capsid protein is also present in chronically infected fish (Mézeth *et al*., 2009).

According to Nishizawa *et al*. (1997), there are four species of betanodaviruses based on the phylogenetic analysis of the T4 variable region within the RNA2 segment: the striped jack nervous necrosis virus (SJNNV), the tiger puffer nervous necrosis virus

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(TPNNV), the barfin flounder nervous necrosis virus (BFNNV) and the redspotted grouper nervous necrosis virus (RGNNV). Intra- and inter-genotype reassortment among betanodaviruses have both been detected (Panzarin *et al*., 2012; He and Teng, 2015), and the two reassortant viruses are RGNNV/SJNNV and SJNNV/RGNNV (Toffolo *et al*., 2007; Olveira *et al*., 2009; Panzarin *et al*., 2012). These reassortant strains may have occurred due to the coexistence of two different viral species in the same host, presumably in wild fish, and may have resulted from a single reassortment event in the 1980s (Sakamoto *et al*., 2008; Lopez-Jimena *et al*., 2010; He and Teng, 2015). Other *Betanodavirus*, i.e. the Atlantic cod nervous necrosis virus (ACNNV), Atlantic halibut nervous necrosis virus (AHNV), turbot nodavirus (TNV) and many others, have been described, but they still have to be recognized by the ICTV (King *et al*., 2011).

When polyclonal antibodies are used, betanodaviruses are grouped into three distinct serotypes: serotype A (the SJNNV genotype), serotype B (the TPNNV genotype) and serotype C (the RGNNV and BFNNV genotypes) (Mori *et al*., 2003). The antigenic diversity between the RGNNV and the SJNNV genotypes had already been assumed (Skliris *et al*., 2001; Chi *et al*., 2003; Costa *et al*., 2007) and has recently been confirmed using reverse genetic viruses (Panzarin *et al*., 2016). The C-terminal protruding domain of the capsid protein seems to be responsible for the different immunoreactivity and may contain host-specific determinants (Iwamoto *et al*., 2004; Ito *et al*., 2008; Bandín and Dopazo, 2011; Souto *et al*., 2015a). The reassortant viruses mentioned above still remain in the same antigenic group as the RNA2 donor strain. The BFNNV genotype was recently allocated to the B serotype (Panzarin *et al*., 2016) in contrast to the previous report by Mori *et al*. (2003). Further studies are needed to confirm the immunoreactivity and the molecular determinant(s) of *Betanodavirus*.

Genetically different betanodaviruses behave differently in response to environmental temperatures: BFNNV and the TPNNV are considered to be 'cold water VNN' viruses, because they are the most psychrophilic, with optimal culture temperatures from 15 to 20°C. However, SJNNV replicates best at 25°C, while RGNNV has tremendous temperature tolerance (15–35°C) with an optimum temperature between 25 and 30°C (Iwamoto *et al*., 2000; Hata *et al*., 2010). The reassortants behave like the RNA1 donor strain, which proves that by codifying for the polymerase, the RNA1 gene regulates the temperature dependency of fish betanodaviruses (Panzarin *et al*., 2014).

11.2.1 Geographical distribution, host range and transmission routes

Since its first description in 1985, VNN appeared in the 1990s almost simultaneously in Asia, Australia and southern Europe. By the beginning of 2000, the disease had spread across North America and northern Europe, and VNN is now present almost worldwide, affecting practically all farmed species of marine fishes (Shetty *et al*., 2012).

The geographic distribution of different betanodaviruses reflects their temperature dependency. RGNNV is the most common VNN virus and has caused clinical disease worldwide (Ucko *et al*., 2004; Sakamoto *et al*., 2008; Chérif *et al*., 2009; Gomez *et al*., 2009; Panzarin *et al*., 2012; Ransangan and Manin, 2012; Shetty *et al*., 2012; Binesh and Jithendran, 2013). The SJNNV strain ranks second (Maeno *et al*., 2004; García-Rosado *et al*., 2007; Sakamoto *et al*., 2008), followed by the reassortant RGNNV/SJNNV, which is common in the Iberian Peninsula and the Mediterranean Sea (Olveira *et al*., 2009; Panzarin *et al*., 2012; Souto *et al*., 2015a). It is noteworthy that VNN outbreaks from reassortant strains have been described only in the Mediterranean Sea (He and Teng, 2015). BFNNV is found only in the cold waters of the northern Atlantic Ocean, the North Sea and the Sea of Japan (Nguyen *et al*., 1994; Grotmol *et al*., 2000; Nylund *et al*., 2008), whereas TPNNV is only found in Japan (Nishizawa *et al*., 1995; Furusawa *et al*., 2007).

VNN has been detected in 160 fish species belonging to 79 families and 24 orders (see Table 11.1). Among the susceptible species listed in Table 11.1, the most frequently reported belong to the families: Carangidae, Percichthydae, Serranidae, Sciaenidae, Pleuronectidae, Mugilidae, Sebastidae and Gadidae. Indeed, the most commonly and severely affected species are sea bass (*Dicentrarchus labrax* and *Lates calcarifer*), grouper (*Ephinephelus* spp.), flatfish (*Solea* spp., *Scophtalmus maximus*, *Paralichthys olivaceus*), striped jack (*Pseudocaranx dentex*, *Trachinotus* spp.) and drum (*Umbrina cirrosa*, *Argyrosomus regius*, *Scienops ocellatus*, *Atractoscion nobilis*).

An increasing number of outbreaks of VNN have recently been reported in freshwater fishes (Vendramin *et al*., 2012; Binesh, 2013; Pascoli

Table 11.1. Fish species that are susceptible to viral nervous necrosis (VNN).[a]

Order	Family	Species	Reference
Acipenseriformes	Acipenseridae	Russian sturgeon (*Acipenser gueldenstaedti*)[b]	Maltese and Bovo, 2007
Anguilliformes	Anguillidae	European eel (*Anguilla anguilla*)[b]	
	Muraenesocidae	Daggertooth pike conger (*Muraenesox cinereus*)[c]	Baeck *et al*., 2007
	Murenidae	Ribbon moray (*Rhinomuraena quaesita*)[c]	Gomez *et al*., 2006
Atheriniformes	Melanotaeniidae	Dwarf rainbowfish (*Melanotaenia praecox*)[b,d]	Furusawa *et al*., 2007
		Threadfin rainbowfish (*Iriatherina werneri*)[b,d]	
	Telmatherinidae	Celebes rainbowfish (*Marosatherina ladigesi*)[b,d]	
Batrachoidiformes	Batrachoididae	Lusitanian toadfish (*Halobatrachus didactylus*)[c]	Moreno *et al*., 2014
Beloniformes	Adrianichthyidae	Medaka (*Oryzias latipes*)[b,d]	Furusawa *et al*., 2007
	Belonidae	Garfish (*Belone belone*)[c]	Ciulli *et al*., 2006a
Beryciformes	Monocentridae	Pineconefish (*Monocentris japonica*)[c]	Gomez *et al*., 2006
	Trachichthyidae	Mediterranean slimehead (*Hoplostethus mediterraneus*)[c]	Giacopello *et al*., 2013
Characiformes	Serrasalmidae	Red piranha (*Pygocentrus nattereri*)[b]	Gomez *et al*., 2006
Clupeiformes	Clupeidae	European pilchard (*Sardina pilchardus*)[c]	Ciulli *et al*., 2006a
	Engraulidae	Japanese anchovy (*Engraulis japonicus*)[c]	Gomez *et al*., 2006
Cypriniformes	Cyprinidae	Goldfish (*Carassius auratus*)[b]	Binesh, 2013
		Zebrafish (*Danieo rerio*)[b]	Lu *et al*., 2008
Cyprinodontiformes	Poeciliidae	Guppy (*Poecilia reticulata*)[b]	Maltese and Bovo, 2007
Gadiformes	Gadidae	Atlantic cod (*Gadus morhua*)	Munday *et al*., 2002
		Pacific cod (*Gadus macrocephalus*)	
		Haddock (*Melanogrammus aeglefinus*)[c]	Maltese and Bovo, 2007
		European hake (*Merluccius merluccius*)[c]	Ciulli *et al*., 2006a
		Whiting (*Merlangius merlangus*)[c]	
		Poor cod (*Trisopterus minutus*)[c]	
	Macrouridae	Glasshead grenadier (*Hymenocephalus italicus*)[c]	Giacopello *et al*., 2013
		Spearnose grenadier (*Caelorinchus multispinulosus*)[c]	Baeck *et al*., 2007
Gonorynchiformes	Chanidae	Milkfish (*Chanos chanos*)	OIE, 2013
Heterodontiformes	Heterodontidae	Japanese bullhead shark (*Heterodontus japonicus*)[c]	Gomez *et al*., 2004
Lophiiformes	Lophiidae	Yellow goosefish (*Lophius litulon*)[c]	Baeck *et al*., 2007
Notacanthiformes	Notacanthidae	Shortfin spiny eel (*Notacanthus bonaparte*)[c]	Giacopello *et al*., 2013
Perciformes	Acanthuridae	Convict surgeonfish (*Acanthurus triostegus*)	OIE, 2013
		Yellow tang (*Zebrasoma flavescens*)[c]	Gomez *et al*., 2006
	Acropomatidae	Black throat seaperch (*Doederleinia berycoides*)[c]	Baeck *et al*., 2007
	Anabantidae	Climbing perch (*Anabas testudineus*)[b,d]	Furusawa *et al*., 2007
	Anarhichadidae	Wolf fish (*Anarchichas minor*)	OIE, 2013
	Apogonidae	Narrowstrip cardinalfish (*Apogon exostigma*)	OIE, 2013
		Indian perch (*Apogon lineatus*)[c]	Baeck *et al*., 2007
	Blenniidae	Freshwater blenny (*Salaria fluviatitis*)[b]	Vendramin *et al*., 2012
	Carangidae	Striped jack (*Pseudocaranx dentex*)	Munday *et al*., 2002
		Greater amberjack (*Seriola dumerili*)	
		Yellow-wax pompano (*Trachinotus falcatus*)	
		Snub nose pompano (*Trachinotus blochii*)	Maltese and Bovo, 2007
		Mackerel (*Trachurus* spp.)[c]	Ciulli *et al*., 2006a
		Japanese jack mackerel (*Trachurus japonicus*)[c]	Baeck *et al*., 2007
		Black scraper (*Trachurus modestus*)[c]	Gomez *et al*., 2004
		Japanese scad (*Decapterus maruadsi*)[c]	
		Lookdown (*Selene vomer*)[c]	Gomez *et al*., 2006
	Callionymidae	Moon dragonet (*Callionymus lunatus*)[c]	Baeck *et al*., 2007
	Centrarchidae	Largemouth bass (*Micropterus salmoides*)[b]	Bovo *et al*., 2011

Continued

Table 11.1. Continued.

Order	Family	Species	Reference
	Cichlidae	Tilapia (*Oreochromis niloticus*)[b]	OIE, 2013
		Angelfish (*Pterophyllum scalare*)[b,d]	Furusawa *et al*., 2007
		Blue streak hap (*Labidochromis caeruleus*)[b,d]	
		Golden mbuna (*Melanochromis auratus*)[b,d]	
		Kenyi cichlid (*Maylandia lombardoi*)[b,d]	
	Eleotridae	Sleepy cod (*Oxyeleotris lineolata*)	Munday *et al*., 2002
	Ephippidae	Orbicular batfish (*Platax orbicularis*)	OIE, 2013
	Epigonidae	Cardinal fish (*Epigonus telescopus*)[c]	Giacopello *et al*., 2013
	Gobidae	Black goby (*Gobius niger*)[c]	Ciulli *et al*., 2006a
	Kyphosidae	Stripey (*Microcanthus strigatus*)[c]	Gomez *et al*., 2004
	Lateolabracidae	Japanese sea bass (*Lateolabrax japonicus*)	Maltese and Bovo, 2007
	Latidae	Asian sea bass/barramundi (*Lates calcarifer*)	Munday *et al*., 2002
	Latridae	Striped trumpeter (*Latris lineata*)	
	Leiognathidae	Silver ponyfish (*Leiognathus nuchalis*)[c]	Baeck *et al*., 2007
	Lutjanidae	Crimson snapper (*Lutjanus erythropterus*)	Maltese and Bovo, 2007
		Mangrove red snapper (*Lutjanus argentimaculatus*)[d]	Maeno *et al*., 2007
	Malacanthidae	Horsehead tilefish (*Branchiostegus japonicus*)	OIE, 2013
	Moronidae	Striped bass × white bass (*Morone saxatilis* × *M. chrysops*)[b]	Bovo *et al*., 2011
		European sea bass (*Dicentrarchus labrax*)	Munday *et al*., 2002
	Mugilidae	Grey mullet (*Mugil cephalus*)	Maltese and Bovo, 2007
		Golden grey mullet (*Liza aurata*)	
		Red mullet (*Mullus barbatus*)	OIE, 2013
		Surmullet (*Mullus surmuletus*)[c]	Panzarin *et al*., 2012
		Thicklip grey mullet (*Chelon labrosus*)[c]	
		Sharpnose grey mullet (*Liza saliens*)	Zorriehzahra *et al*., 2014
		Thinlip grey mullet (*Liza ramada*)[c]	Ciulli *et al*., 2006a
	Oplegnathidae	Barred knifejaw (*Oplegnathus fasciatus*)	Munday *et al*., 2002
		Spotted knifejaw (*Oplegnathus punctatus*)	
	Osphronemidae	Honey gourami (*Trichogaster chuna*)[b,d]	Furusawa *et al*., 2007
		Three spot gourami (*Trichopodus trichopterus*)[b,d]	
		Pygmy gourami (*Trichopsis pumila*)[b,d]	
		Siamese fighting fish (*Betta splendens*),[b,d]	
	Percichthydae	Australian bass (*Macquaria novemaculeata*)	Moody *et al*., 2009
		Macquarie perch (*Macquaria australasica*)[d]	Munday *et al*., 2002
		Murray cod (*Maccullochella pelii*)[d]	
	Percidae	Pike-perch (*Sander lucioperca*)[b]	Bovo *et al*., 2011
	Polycentridae	Amazon leaffish (*Monocirrhus polyacanthus*)[b]	Gomez *et al*., 2006
	Pomacentridae	Sebae clownfish (*Amphiprion sebae*)	Binesh *et al*., 2013
		Neon damselfish (*Pomacentrus coelestis*)[c]	Gomez *et al*., 2004
		Threespot dascyllus (*Dascyllus trimaculatus*)[c]	Gomez *et al*., 2008b
	Rachycentridae	Cobia (*Rachycentron canadum*)	Maltese and Bovo, 2007
	Sciaenidae	Red drum (*Sciaenops ocellatus*)	
		Shi drum (*Umbrina cirrosa*)	
		White sea bass (*Atractoscion nobilis*)	Munday *et al*., 2002
		Meagre (*Argyrosomus regius*)	Thiéry *et al*., 2004
		White croaker (*Pennahia argentata*)[c]	Baeck *et al*., 2007
	Scombridae	Pacific bluefin tuna (*Thunnus orientalis*)	OIE, 2013
		Chub mackerel (*Scomber japonicus*)[c]	Baeck *et al*., 2007
	Sebastidae	White weakfish (*Sebastes oblongus*)	Maltese and Bovo, 2007
		Korean rockfish (*Sebastes schlegeli*)	Gomez *et al*., 2004
		Spotbelly rockfish (*Sebastes pachycephalus*)	
		Black rockfish (*Sebastes inermis*)[c]	
		False kelpfish (*Sebastiscus marmoratus*)[c]	

Continued

Table 11.1. Continued.

Order	Family	Species	Reference
	Serranidae	Redspotted grouper (*Epinephelus akaara*)	Munday *et al*., 2002
		Yellow grouper (*Epinephelus awoara*)	
		Sevenband grouper (*Epinephelus septemfasciatus*)	
		Blackspotted grouper (*Epinephelus fuscoguttatus*)	
		Brownspotted grouper (*Epinephelus malabaricus*)	
		Dusky grouper (*Epinephelus marginatus*)	
		Kelp grouper (*Epinephelus moara*)	
		Greasy grouper (*Epinephelus tauvina*)	
		Dragon grouper (*Epinephelus lanceolatus*)	
		Humpback grouper (*Chromileptes altivelis*)	
		White grouper (*Epinephelus aeneus*)	OIE, 2013
		Orangespotted grouper (*Epinephelus coioides*)	
		Golden grouper (*Epinephelus costae*)	Vendramin *et al*., 2013
		Spotted coral grouper (*Pletropomus maculatus*)	Pirarat *et al*., 2009a
	Sillaginidae	Japanese sillago (*Sillago japonica*)[c]	Baeck *et al*., 2007
	Sparidae	Gilthead sea bream (*Sparus aurata*)	Munday *et al*., 2002
		Common pandora (*Pagellus erythrinus*)[c]	Ciulli *et al*., 2006a
		Axillary sea bream (*Pagellus acarne*)[c]	
		Common sea bream (*Pagrus pagrus*)	García-Rosado *et al*., 2007
		Redbanded sea bream (*Pagrus auriga*)	
		Red sea bream (*Pagrus major*)[c]	Baeck *et al*., 2007
		White sea bream (*Diplodus sargus*)	Dalla Valle *et al*., 2005
		Black sea bream (*Spondyliosoma cantharus*)[c]	Moreno *et al*., 2014
		Bogue (*Boops boops*)[c]	Ciulli *et al*., 2006a
	Terapontidae	Silver perch (*Bidyanus bidyanus*)[d]	Munday *et al*., 2002
	Zanclidae	Moorish idol (*Zanclus cornutus*)[c]	Gomez *et al*., 2004
	Zoarcidae	Blotched eel pout (*Zoarces gilli*)[c]	Baeck *et al*., 2007
Pleuronectiformes	Cynoglossidae	(Half-smooth) Tongue sole (*Cynoglossus semilaevis*)	Li *et al*., 2014
		Robust tonguefish (*Cynoglossus robustus*)[c]	Baeck *et al*., 2007
	Paralichthyidae	Japanese flounder (*Paralichthys olivaceus*)	Munday *et al*., 2002
		Fivespot flounder (*Pseudorhombus pentophthalmus*)[c]	Baeck *et al*., 2007
	Pleuronectidae	Barfin flounder (*Verasper moseri*)	Munday *et al*., 2002
		Winter flounder (*Pleuronectes americanus*)	
		Atlantic halibut (*Hippoglossus hippoglossus*)	
		Marbled sole (*Pleuronectes yokohamae*)[c]	Baeck *et al*., 2007
	Scophthalmidae	Turbot (*Scophtalmus maximus*)	Munday *et al*., 2002
	Soleidae	Common (Dover) sole (*Solea solea*)	Maltese and Bovo, 2007
		Senegalese sole (*Solea senegalensis*)	Ito *et al*., 2008
Rajiformes	Rajidae	Ocellate spot skate (*Okamejei kenojei*)[c]	Baeck *et al*., 2007
Salmoniformes	Salmonidae	Sea trout (*Salmo trutta trutta*)[c]	Panzarin *et al*., 2012
		Atlantic salmon (*Salmo salar*)[d]	Korsnes *et al*., 2005
Scorpaeniformes	Platycephalidae	Bartail flathead (*Platycephalus indicus*)	Munday *et al*., 2002
	Scorpaenidae	Luna lion fish (*Pterois lunulata*)[c]	Gomez *et al*., 2004
	Synanceiidae	Devil stinger (*Inimicus japonicus*)	
	Triglidae	Spiny red gurnard (*Chelidonichthys spinosus*)[c]	Baeck *et al*., 2007
		Tub gurnard (*Chelidonichthys lucerna*)[c]	Ciulli *et al*., 2006a
Siluriformes	Siluridae	Chinese catfish (*Parasilurus asotus*)[b]	Maltese and Bovo, 2007
		Australian catfish (*Tandanus tandanus*)[b]	Munday *et al*., 2002
Syngnathiformes	Centriscidae	Razorfish (*Aeoliscus strigatus*)[c]	Gomez *et al*., 2006
Tetraodontiformes	Balistidae	Triggerfish (*Balistapus* spp.)[c]	Panzarin *et al*., 2010

Continued

Table 11.1. Continued.

Order	Family	Species	Reference
	Diodontide	Longspined porcupinefish (*Diodon holocanthus*)[c]	Gomez *et al*., 2004
	Monacanthidae	Korean black scraper (*Thamnaconus modestus*)[c]	
		Threadsail filefish (*Stephanolepis cirrhifer*)	Pirarat *et al*., 2009b
	Tetraodontidae	Tiger puffer (*Takifugu rubripes*)	Munday *et al*., 2002
		Grass puffer (*Takifugu niphobles*)[c]	Baeck *et al*., 2007
		Panther puffer (*Takifugu pardalis*)[c]	Gomez *et al*., 2004
		Moontail puffer (*Lagocephalus lunaris*)[c]	Baeck *et al*., 2007
Zeiformes	Zeidae	John dory (*Zeus faber*)[c]	Baeck *et al*., 2007

[a]Classification of fish based on www.fishbase.org
[b]Freshwater species
[c]*Betanodavirus* detected in wild asymptomatic fish.
[d]Experimentally infected.

et al., 2016). Betanodaviruses can cause disease and infect a great number of fish species, and this is independent of water salinity (Furusawa *et al*., 2007; Maeno *et al*., 2007; Bovo *et al*., 2011). The detection of *Betanodavirus* in wild fish with no clinical signs is also extensive (as seen by the large number of species included in this category listed in Table 11.1), which may account for its widespread nature. Finally, the increased death rate in wild fish (especially groupers, *Epinephelus* spp.) with nervous disorders accompanied by the detection of the virus in these fish, is a concern (Gomez *et al*., 2009; Vendramin *et al*., 2013; Haddad-Boubaker *et al*., 2014; Kara *et al*., 2014).

Transmission of the disease occurs horizontally through direct contact with infected fish, contaminated water and/or contaminated equipment. The disease can easily be experimentally reproduced by bath exposure as well as by intramuscular or intraperitoneal injection (Munday *et al*., 2002; Maltese and Bovo, 2007). Evidence of oral transmission through the ingestion of infected fish or contaminated feed has been suggested (Shetty *et al*., 2012). The virus has been detected in several marine invertebrates (Gomez *et al*., 2006, 2008a; Panzarin *et al*., 2012; Fichi *et al*., 2015). Indeed, the virus has a very high resistance to chemical and physical agents (i.e. heat, pH and disinfectant) and, therefore, it cannot only contaminate marine water, invertebrates and microorganisms, but also nets, pens, tanks and other equipment (Maltese and Bovo, 2007).

Wild and farmed fish that have survived the disease are the most likely source of infections in 'VNN-free' farms. Disease and viral shedding in infected fish with no clinical signs may reactivate several times after the onset of the disease due to stress or water temperature variations (Johansen *et al*., 2004; Rigos and Katharios, 2009; Lopez-Jimena *et al*., 2010; Souto *et al*., 2015b).

Vertical transmission had been described in the most susceptible fish species (Shetty *et al*., 2012) and the virus has been found in gonads and seminal fluids.

11.3 Diagnosis of the Infection

11.3.1 Clinical signs

Clinical signs of VNN infection include variations in skin colour, anorexia, lethargy, abnormal swimming behaviour and nervous signs caused by lesions in the brain and retina. Generally, younger fish are most susceptible to infection. In larvae/juveniles, the onset of disease can be hyperacute, and the only apparent sign is a sharp increase in mortality. In older animals, the onset of disease can be slower and the cumulative mortality lower. Infected fish have reduced growth, resulting in uneven weight/size (Vendramin *et al*., 2014); this represents an indirect but significant economic loss, which is often underestimated. Hyperinflation of the swim bladder is another common clinical sign (Maltese and Bovo, 2007; Pirarat *et al*., 2009a; Hellberg *et al*., 2010; Vendramin *et al*., 2013).

In European sea bass (*D. labrax*) clinical signs include anorexia, darkening of the skin and abnormal swimming behaviour, which is characterized by swirling, circular movements that alternate with periods of lethargy, abnormal bathymetry and anomalous vertical positions in a water column. Fish appear blind and can display hyperexcitability when disturbed (Péducasse *et al*., 1999;

Athanassopoulou *et al*., 2003). Traumatic lesions to the jaw, head, eyes and nose (Fig. 11.1) are a natural consequence of impaired swimming capacity (Shetty *et al*., 2012). Hyperinflation of the swim bladder also contributes to the abnormal swimming behaviour, causing the fish to sink or float (Lopez-Jimena *et al*., 2011). In sea bass larvae and juveniles, congestion of the brain (sometimes perceptible through the skull) or of the whole head can be observed, as well as a typical 'sickle position' due to muscular hypercontraction associated with high mortality (Bovo, 2010, personal communication). Mortality varies according to water temperature and age, and outbreaks in hatcheries can be devastating, with extremely high mortalities (80–100%). Older fish are generally less affected, even though severe losses have been described in mature animals (Munday *et al*., 2002; Chérif *et al*., 2009). In sea bass, the disease is caused almost exclusively by the RGNNV genotype, which occurs at temperatures >23–25°C, and mortality decreases when temperatures fall below 18–22°C (Bovo *et al*., 1999; Breuil *et al*., 2001; Chérif *et al*., 2009). European sea bass may also be infected with other VNN strains, and when this happens, the clinical signs are milder (Vendramin *et al*., 2014; Souto *et al*., 2015a).

Darkening of the skin, clustering of the fish at the surface of the water, and a cumulative mortality of 60–100% was observed in naturally infected Asian sea bass larvae (*Lates calcarifer*). Larvae also displayed anorexia, pale grey pigmentation of the body, loss of equilibrium and corkscrew-like swimming prior to death. The mortality rate at all ages is generally >50% (Maeno *et al*., 2004; Azad *et al*., 2005; Parameswaran *et al*., 2008).

Groupers are among the most VNN-susceptible fishes; the disease occurs at any age, in both farmed and wild fish, and is mainly caused by RGNNV. The typical signs of disease include loss of equilibrium, swimming in a corkscrew fashion and lethargy associated with abnormal response to stimulation. Hyperinflation of the swim bladder and corneal opacity are the most relevant clinical signs (Fig. 11.2). Spinal deformities and exophthalmos have also been reported (Sohn *et al*., 1998; Gomez *et al*., 2009; Pirarat *et al*., 2009a; Vendramin *et al*., 2013; Kara *et al*., 2014).

Clinical signs in flatfish (order Pleuronectiformes) are less obvious; they remain at the bottom of the tank and bend their bodies with the head and tail raised, sometimes upside down on the bottom. They may tremble or drop to the bottom of the tank like a 'falling leaf' (Maltese and Bovo, 2007). Skin discoloration has been reported in the Atlantic halibut (*H. hippoglossus*) (Grotmol *et al*., 1997). During a natural outbreak, the common Dover sole (*Solea solea*) appeared either darker or paler than usual and at times anorexic, with cumulative mortality that may reach 100% (Starkey *et al*., 2001). The Senegalese soil (*S. senegalensis*), may also have

Fig. 11.1. A juvenile farmed European sea bass (*Dicentrachus labrax*) with viral nervous necrosis (VNN) showing skin erosion and congestion of the head.

Fig. 11.2. A wild dusky grouper (*Epinephelus marginatus*) from the Mediterranean Sea showing clinical signs of viral nervous necrosis (VNN): head skin erosion, corneal opacity and panophthalmitis (inflammation of all coats of the eye, including intraocular structures).

skin ulcers (Olveira *et al*., 2008) and is extremely susceptible to RGNNV/SJNNV, with a mortality rate of 100% at 22°C, although at 16°C, mortality is reduced to 8%. VNN can also cause a persistent infection in the sole, and the disease can be reactivated by an increase of the water temperature to 22°C (Souto *et al*., 2015a,b).

Sciaenidae, such as the shi drum (*Umbrina cirrosa*) and red drum (*Sciaenops ocellatus*) are highly susceptible to the disease, especially juvenile fish (Bovo *et al*., 1999; Oh *et al*., 2002; Katharios and Tsigenopoulos, 2010). In addition to the classical clinical signs, white sea bass (*Atractoscion nobilis*) have marked hyperinflation of the swim bladder at 12–15°C (Curtis *et al*., 2003); in contrast, infected wild meagre (*Argyrososmus regius*) did not show any particular clinical signs (Lopez-Jimena *et al*., 2010).

Some cold-water fish, such as cod (*Gadus morua*) and the Atlantic halibut (*H. hippoglossus*), can be infected at 6–15°C and display lethargy, anorexia, darkening of the skin, nervous clinical signs, swim bladder inflammation and corneal opacity (Grotmol *et al*., 1997; Patel *et al*., 2007; Hellberg *et al*., 2010).

Sparidae are often resistant to clinical disease. For example, gilthead sea bream (*Sparus aurata*) reared in cohabitation with *Betanodavirus*-infected European sea bass never showed mortality or clinical signs (Castric *et al*., 2001; Aranguren *et al*., 2002; Ucko *et al*., 2004). However, sea bream are susceptible to experimental infection (intramuscular injection with RGNNV viruses), which can provoke mortality in juveniles or enable them to act as non-clinical contagious hosts for sea bass (Castric *et al*., 2001; Aranguren *et al*., 2002). Increased outbreaks of VER with high mortality in sea bream larvae have recently been observed (Beraldo *et al*., 2011; Toffan, 2015, unpublished results). The viruses isolated from sea bream are always RGNNV/SJNNV, which indicates a special adaptation of this reassortant to sea bream, as reported for the Senegalese sole (Souto *et al*., 2015a).

Clinical signs in naturally or experimentally infected freshwater fish species are similar to those in marine fishes.

11.3.2 Laboratory diagnosis

VNN isolation is generally in continuous cell cultures, and the most commonly used cell lines are SSN-1 cells from snakeheads (*Ophicephalus striatus*) (Frerichs *et al*., 1996) and their derived clone E-11 (Iwamoto *et al*., 2000). The high susceptibility of these two cell lines has been attributed to their persistent infection with the snakehead SnRV retrovirus (Lee *et al*., 2002; Nishizawa *et al*., 2008). Other continuous fish cell lines (for examples the GF-1 and other cell lines from the orange-spotted grouper *Epinephelus coioides*; the SISS and ASBB cell lines from *L. calcarifer*; and the SAF-1 cell line from *S. aurata*) have been developed and used successfully, but most of these are not available commercially; also, their diagnostic performances and applications are unknown (Hasoon *et al*., 2011; Sano *et al*., 2011).

Suitable incubation temperatures depend upon the VNN genotype (Iwamoto *et al*., 2000; Ciulli *et al*., 2006b; Hata *et al*., 2010; Panzarin *et al*., 2014). A typical cytopathic effect (CPE) is the darkening and contraction of the infected cells. The appearance of clusters of vacuoles in the cytoplasm of infected cells, cell rounding up and detachment from the monolayer, and evolution into extended necrotic foci are typical signs. The cultivated virus can be identified by serum neutralization with monoclonal or polyclonal antibodies (Mori *et al*., 2003; V. Panzarin, Italy, 2015, personal communication), fluorescein-conjugated antibodies (Péducasse *et al*., 1999; Thiéry *et al*., 1999; Castric *et al*., 2001; Mori *et al*., 2003), enzyme-linked immunosorbent assay (ELISA) (Fenner *et al*., 2006b) and by molecular biology techniques. ELISA can be used directly with central nervous system (CNS) tissue of the infected fish (Breuil *et al*., 2001; Nuñez-Ortiz *et al*., 2016), as can indirect fluorescent antibody testing (IFAT) (Nguyen *et al*., 1997; Totland *et al*., 1999; Curtis *et al*., 2001; Johansen *et al*., 2003).

The rapid diagnosis of VNN is now possible using reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (qRT-PCR). The F2-R3 primer set, targeting the T4 variable region of the RNA2 segment, has been widely used for diagnostic purposes (Nishizawa *et al*., 1994). Several other PCR-based protocols have been developed to increase the sensitivity and specificity of *Betanodavirus* diagnostic assays (Grotmol *et al*., 2000; Gomez *et al*., 2004; Dalla Valle *et al*., 2005; Cutrín *et al*., 2007). Validated qRT-PCR methods are also available for detecting all known VNN genotypes (Grove *et al*., 2006a; Panzarin *et al*., 2010; Hick *et al*., 2011; Hodneland *et al*., 2011; Baud *et al*., 2015).

Viral isolation, histopathology or immunostaining can be used for the diagnosis and confirmation of VNN, whereas a combination of at least two different molecular assays (i.e. RT-PCR protocols targeting different regions of the viral genome, or RT-PCR followed by sequencing) is advisable when the disease is identified for the first time (OIE, 2013).

Antibodies in experimentally immunized/infected fish can be detected using an ELISA test or a serum neutralization assay. ELISA is the most common test used (Breuil *et al*., 2000; Watanabe *et al*., 2000; Grove *et al*., 2006b; Scapigliati *et al*., 2010), while serum neutralization is often used to evaluate the humoral response of fish (Skliris and Richards, 1999; Tanaka *et al*., 2001; Kai *et al*., 2010).

Antibodies in vaccinated as well as in naturally exposed fish can be detected for over a year, although important intra-fish species variations exist (Breuil and Romestand, 1999; Breuil *et al*., 2000; Grove *et al*., 2006b; Kai *et al*., 2010). However, there was a significant drop in serum-neutralizing antibody titres in groupers 75 days postvaccination (Pakingking *et al*., 2010). In temperate fish, higher antibody titres are more frequent in the summer than in the winter, which indicates that serum samples should be collected in the summer (Breuil *et al*., 2000). Due to the lack of robust research, the detection of specific antibodies has not been considered as a routine screening method for assessing the viral status of fish (OIE, 2013).

11.4 Pathology and Pathophysiology

11.4.1 Gross and microscopic lesions

Congestion, abrasion and sometimes necrosis of the nose, jaws and head are clinical signs of VNN in *D. labrax*. Corneal opacity has been reported in several species, as well as skin erosions on the body and fins caused by an impaired swimming ability (Maltese and Bovo, 2007; Shetty *et al*., 2012). At necropsy, the hyperinflation of the swim bladder is common in almost all susceptible species, with the congestion of the CNS and meninges being the most relevant internal lesions (Fig 11.3).

The encephalitis in the CNS is characterized by multiple intracytoplasmic vacuolations; numerous empty areas of 5–10 µm diameter, clearly separated from the surrounding areas, are present in the grey matter of the olfactory bulb, telencephalon, diencephalon, mesencephalon, cerebellum, medulla oblongata, spinal cord and retina (Munday *et al*., 2002; Grove *et al*., 2003; Mladineo, 2003; Maltese and Bovo, 2007; Lopez-Jimena *et al*., 2011). Severity of the vacuolization depends on the fish species, age and stage of infection but, notably, it is a consistent (almost pathognomonic) finding in the case of VNN. Pyknosis, karyorrhexis, neuronal degeneration and inflammatory infiltration have been described in all the nervous tissues of infected fish. The olfactory bulb, the optic tectum of the mesencephalon, the granular and Purkinje cell layers of cerebellum and the motor neurons of the spinal cord are generally the most affected areas (Totland *et al*., 1999; Pirarat *et al*., 2009a,b). In the preclinical phase of the infection of Asian sea bass, darkly stained and actively dividing neuronal zones are present in the CNS and spinal cord without vacuolization. Vacuoles appear later, together with the nervous signs of the disease (Azad *et al*., 2006). Congestion of the blood vessels in the encephalic parenchyma and meninges is frequent and can evolve into mild or extensive haemorrhages in the brain (Mladineo, 2003; Korsnes *et al*., 2005; Pirarat *et al*., 2009b).

Fig. 11.3. European sea bass (*Dicentrachus labrax*) with viral nervous necrosis (VNN) showing congestion of the brain and meninges.

Immunohistochemistry (IHC) or *in situ* hybridization (ISH) can be used to detect viral antigens surrounding vacuoles in the CNS, optic tectum and cerebellum (Fig. 11.4), but also when vacuoles are not present (Grove *et al*., 2003; Mladineo, 2003; Pirarat *et al*., 2009a,b; Katharios and Tsigenopoulos, 2010; Lopez-Jimena *et al*., 2011). Transmission electron microscopy (TEM) generally shows crystalline arrays of membrane-bound nodaviruses in the cytoplasm of infected brain and retina cells

(Grotmol *et al*., 1997; Tanaka *et al*., 2004; Ucko *et al*., 2004).

Vacuolar lesions (confirmed using IHC) have been observed in the dendritic cell of the spinal cord, particularly in the cranial part (Grotmol *et al*., 1997). Spinal cord lesions can appear before or after the occurrence of encephalic lesions (Nguyen *et al*., 1996; Pirarat *et al*., 2009b).

The retina of infected fish may also have major lesions, which have marked histopathological changes, such as massive necrosis of small round cells and spongiotic vacuolization in the outer and inner nuclear layers, as well as in the ganglion cell layer (Fig. 11.5). In some cases, vacuoles are also present in the internal plexiform layer (Pirarat *et al*., 2009a; Katharios and Tsigenopoulos, 2010; Lopez-Jimena *et al*., 2011). The optic nerve may show various degrees of alteration from extensive vacuolization to no apparent lesions (Mladineo, 2003; Lopez-Jimena *et al*., 2011).

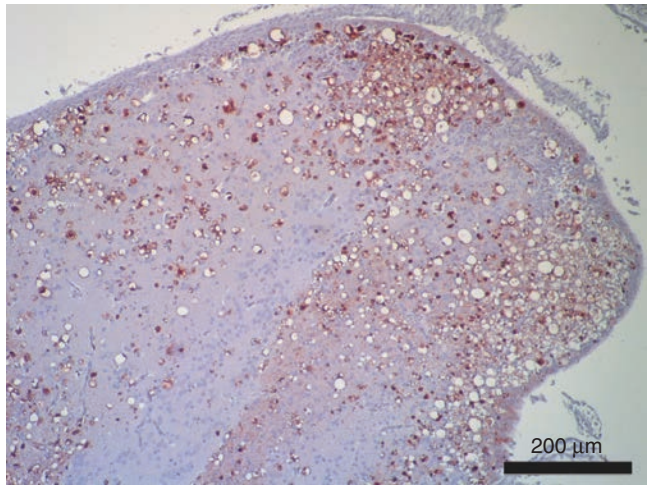

Fig. 11.4. Immunohistochemical detection of *Betanodavirus* in sea bass (*Dicentrachus labrax*) brain (10×) showing abundant immunoprecipitates and severe vacuolization of the olfactory lobe. Courtesy of Dr Pretto Tobia.

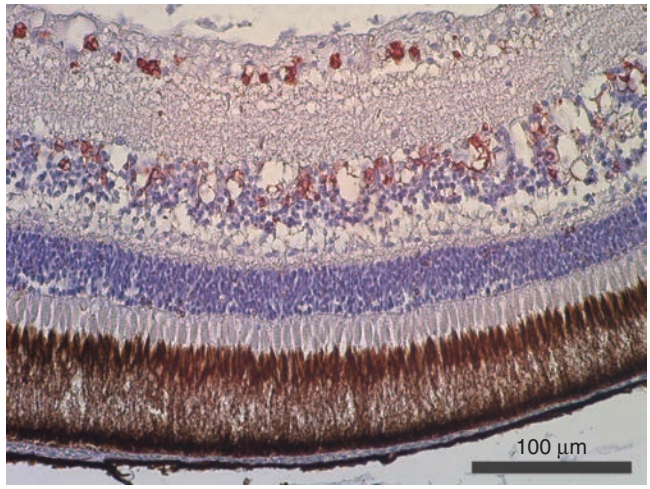

Fig. 11.5. Immunohistochemical detection of *Betanodavirus* in sea bass (*Dicentrachus labrax*) retina (25×). Immunoprecipitates and vacuolizations are evident in the internal nuclear layer and in the ganglion cell layer. Courtesy of Dr Pretto Tobia.

Viral particles were detected using IHC/ISH or PCR in non-nervous tissues such as the gills, fins, heart, anterior and posterior intestine, stomach, spleen, liver, kidney and gonads of several fish species (Grotmol *et al*., 1997; Nguyen *et al*., 1997; Johansen *et al*., 2003; Mladineo, 2003; Grove *et al*., 2006a; Korsnes *et al*., 2009; Lopez-Jimena *et al*., 2011; Mazelet *et al*., 2011). These organs are not generally believed to play an important role in the disease pathogenesis, unlike nervous tissues, which are the primary sites for viral replication. The digestive tract can also play a role in disease transmission (Nguyen *et al*., 1996; Totland *et al*., 1999). The strong presence of *Betanodavirus* antigen in the gastrointestinal cells of infected threadsail filefish (*Stephanolepis cirrhifer*) suggests that this may be a particularly active site for viral replication and spread (Pirarat *et al*., 2009b). Also, immunostaining in the reproductive tissues, such as the ovary, supports the vertical transmission of the disease (Nguyen *et al*., 1997; Azad *et al*., 2005, 2006).

Despite the recurrent and irreversible hyperinflation of the swim bladder in fish with clinical signs of VNN, this is not considered to be a target organ for nodaviruses because histopathological lesions have never been observed, except for a slight congestion or the presence of eosinophilic intranuclear inclusions (Pirarat *et al*., 2009a).

11.4.2 Pathogenesis

Despite several recent studies on the pathogenesis of VNN, the important viral mechanisms for the development of the disease remain unclear. The heat shock protein GHSC70 commonly expressed by the GF-1 cell line is a good VNN receptor candidate (Chang and Chi, 2015). Nevertheless, there may be other membrane proteins that act as viral receptors. The neurotropism of *Betanodavirus* is irrefutable, but the route to the nervous tissues is still a matter of discussion. Several tissues, such as the nasal cavity, the intestinal epithelium and intact skin, may be entry portals. Tanaka *et al*. (2004) infected sevenband groupers (*Epinephelus septemfasciatus*) intranasally and assumed that the virus first penetrated the nasal epithelium, reached the olfactory nerve and bulb, and finally invaded the olfactory lobe, where they replicated. This route was also suggested in naturally infected sevenband groupers, spotted coral groupers (*Pletropomus maculatus*) and European sea bass larvae in which pathological changes first developed in the olfactory lobes (Mladineo, 2003; Banu *et al*., 2004; Pirarat *et al*., 2009a).

The epithelial cells in the skin and the intestinal epithelium may also be possible entry portals (Totland *et al*., 1999; Grove *et al*., 2003). Positive IHC reactions in the skin and lateral line had led some authors to suggest that VNN can enter through the epithelial cells of the skin (Nguyen *et al*., 1997; Péducasse *et al*., 1999; Totland *et al*., 1999; Azad *et al*., 2006; Kuo *et al*., 2011), but this assumption needs further investigation.

Once in its host, the *Betanodavirus* multiplies and spreads to the target organs through a viraemic phase, as has been extensively demonstrated by detection of the viruses in the blood (Lu *et al*., 2008; Olveira *et al*., 2008; Korsnes *et al*., 2009; Lopez-Jimena *et al*., 2011). Recurrent endocardial lesions, probably due to viraemia, have also been reported in the Atlantic halibut (Grotmol *et al*., 1997). Another hypothesis is that the virus reaches the CNS directly by axonal transport through the cranial nerves (Grotmol *et al*., 1999; Húsgaro *et al*., 2001; Tanaka *et al*., 2004), where it then actively replicates into the target nervous organs; here, the manifestation of vacuolization is generally associated with the appearance of clinical signs (Azad *et al*., 2006).

Betanodavirus infection strongly induces an innate immunity (Lu *et al*., 2008; Scapigliati *et al*., 2010; Chen *et al*., 2014), and following this first, short-term, non-specific immune response, the development of specific circulating anti-VNN antibodies has been detected in different fishes. These induced antibodies neutralize the viral activity, but it is still debated whether they can overcome the haemato–encephalic (blood–brain) barrier to protect the CNS from damage and clear the infection. In fact, *Betanodavirus* tries to avoid the protective systems of the host by hiding in the nervous tissue, where the viruses generate latent infections (Chen *et al*., 2014). Clearly, the onset of the carrier status needs further investigation.

The transfer of maternal immunity has been described in the eggs and fry of groupers from vaccinated broodstock and has been hypothesized in the European sea bass (Breuil *et al*., 2001; Kai *et al*., 2010), but, again, further investigations are needed to understand its duration and efficacy.

11.5 Protective and Control Strategies

Some promising experimental vaccines against VNN range from inactivated whole viruses to

recombinant capsid proteins and virus-like particles (produced in different vector microorganisms), which are administered by intramuscular or intraperitoneal injection (Gomez-Casado *et al*., 2011; Shetty *et al*., 2012; Chen *et al*., 2014).

Good results have been obtained with a live vaccine in grouper cultured at low temperatures (17°C); however, due to the ability of *Betanodavirus* to cause a persistent infection, the vaccination cannot guarantee the clearance of latent infections (Nishizawa *et al*., 2012; Oh *et al*., 2013).

As indicated in Section 11.3.1, larval and juvenile fish are more susceptible to the disease, and these are the life stages when it is most impractical to inject a vaccine. To overcome this problem, a vaccine formulation for bath and oral administration has recently been tested, but the data that are available do not confirm the efficacy of either approach (Gomez-Casado *et al*., 2011; Nishizawa *et al*., 2012; Kai *et al*., 2014; Wi *et al*., 2015).

As no commercially available vaccines against *Betanodavirus* are available, the spread of the disease will be exponential in dense fish farming areas (e.g. the Mediterranean Basin, the Japanese and Taiwanese coasts and South-east Asia) and availability of non-carrier fish could be reduced, as already observed for grouper in Taiwan (Chen *et al*., 2014).

In the absence of an efficient vaccine, the best way to prevent the introduction and/or the spread of the disease in fish farms is to comply with strict biosecurity rules. Biosecurity must play a primary role in hatcheries, where the accidental introduction of VNN can devastate stocks. The most relevant safety measures include the disinfection of inlet water, the division of the hatchery into sectors with dedicated personnel and equipment, the periodic disinfection of tanks and equipment, periodic fallowing, quarantine and testing of newly introduced broodstock and control of frozen or live feeds (Munday *et al*., 2002; Maltese and Bovo, 2007; Shetty *et al*., 2012). Obviously, such measures are not applicable in open environments such as lagoons and sea cages. None the less, in open environments, biosecurity precautions (e.g. the fast and proper disposal of carcasses, reduction of stock densities, reduction of feeding rates, application of regular fallowing) and attentive management may help to reduce stress and minimize losses.

It is important to emphasize that betanodaviruses are extremely resistant to various environmental conditions (Arimoto *et al*., 1996; Munday *et al*., 2002; Liltved *et al*., 2006). They can tolerate extreme variations of pH and survive a 6 week storage at pH 3–7 and over 24 h at pH 2–11, as well as endure extreme temperature conditions for more than a month at 25°C and more than a year at 15°C (Frerichs, 2000). VNN inactivation is possible through a heat treatment at 60°C or higher for at least 30 min (Arimoto *et al*., 1996), which makes thermal inactivation inapplicable as a first choice cleaning method. Iodophors and hypochlorite solutions have the best virucidal effects and are fully effective at concentrations of 50 ppm for 10 min at 20°C (Munday *et al*., 2002), while formalin is less efficient because it requires a combination of concentration, temperature and exposure time that is difficult to apply in practice (Arimoto *et al*., 1996; Frerichs, 2000; Maltese and Bovo, 2007; Kai and Chi, 2008).

UV irradiation and ozone treatment are feasible disinfection measures (Arimoto *et al*., 1996; Frerichs, 2000). Particular attention should be paid to the disinfection of embryonated eggs. The application of ozone (4 mg O_3/l for 0.5 min) to eggs from experimentally infected halibut neutralized the virus with practically no impact on hatchability (Grotmol and Totland, 2000). Ozone was also successful in disinfecting haddock (*Melanogrammus aeglefinus*) eggs that were experimentally infected (Buchan *et al*., 2006), but the safe dosage of ozone varies among species (Grotmol *et al*., 2003). Nevertheless, ozonation may not always be efficacious for the complete inactivation of VNN viruses and discrepancies in results between studies are due to the lack of standard procedures to conduct inactivation experiments and to determine oxidant residues in seawater (Liltved *et al*., 2006). More trials under different environmental conditions are necessary to obtain a safe and efficient protocol that can be routinely applied in the field.

As there is vertical transmission of the virus in several fish species, the introduction of new broodstock into a disease-free farm always needs serious consideration. Some combination of serology and molecular biology techniques on ovary biopsies or on eggs themselves may provide criteria by which hatchery personnel can select virus-free broodstock (Mushiake *et al*., 1992; Breuil and Romestand, 1999; Watanabe *et al*., 2000; Mazelet *et al*., 2011).

The genetic selection of resistance genes is largely applied to fight viral infection in freshwater species (i.e. salmon), and not much research has been

conducted on this approach in *Betanodavirus*. An extremely high estimated within-strain heritability of survival in VNN-challenged Atlantic cod was observed, although the data structure was not optimal for obtaining conclusive results (Ødegård *et al*., 2010).

11.6 Conclusions and Suggestions for Future Research

At present, the major constraint to controlling VNN is the lack of immunizing strategies. There is an urgent need for safe and inexpensive commercial vaccines, especially against the most prevalent *Betanodavirus* genotypes (RGNNV and SJNNV).

The availability of reliable serology tests will be critical to shedding more light on the fish humoral response and evaluating the degree of protection elicited by any vaccine. It will also serve as a non–invasive diagnostic test to complement biomolecular protocols for confirming virus-free brood stocks. Standardized protocols for sampling, the certification of specific pathogen-free stocks and farm disinfections need to be developed, shared and applied.

More attention should be addressed to the interactions and exchanges of pathogens between farmed and wild fish populations in order to assess the risk of transmission of the infection from one environment to another, and to better understand the role of wild fish in ecology of *Betanodavirus*.

Finally, genetic selection programmes are needed to increase the natural resistance of different fish to the disease. Such a selection programme should be considered a synergistic tool to the use of vaccines to efficiently arrest the spread and impact of this disease.

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12 Iridoviral Diseases: Red Sea Bream Iridovirus and White Sturgeon Iridovirus

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12.1 Red Sea Bream Iridovirus

12.1.1 Introduction

The red sea bream iridovirus (RSIV) has a double-stranded DNA genome in an icosahedral virion capsid that is 200 nm in diameter. According to the International Committee on the Taxonomy of Viruses (ICTV), it is in the genus *Megalocytivirus* within the family *Iridoviridae*, although it has not yet been approved as a species within that genus (Jancovich *et al*., 2012).

Red sea bream iridoviral disease (RSIVD) was first detected causing mass mortality among cultured red sea bream (*Pagrus major*) in the summer of 1990 in Japan, (Inouye *et al*., 1992). The outbreak ceased in November when the water temperature fell below 20°C. The disease recurred in the next summer, not only in red sea bream, but also in other marine cultured species such as yellowtail or Japanese amberjack (*Seriola quinqueradiata*), greater amberjack (*S. dumerili*), sea bass (*Lateolabrax* sp.), striped jack (*Pseudocaranx dentex*) and barred knifejaw (*Oplegnathus fasciatus*) (Matsuoka *et al*., 1996). Since then, RSIVD has spread to more than 30 species of cultured marine fishes and has occurred every summer, causing substantial economic losses in mariculture within Japan (Kawakami and Nakajima, 2002). Similar viral diseases have been reported in many cultured fishes in East and South-east Asia since the 1990s, and the causative viruses are also megalocytiviruses (Kurita and Nakajima, 2012). These viruses include the Taiwan grouper iridovirus (TGIV) from a species of grouper (*Epinephelus* sp.) (Chou *et al*., 1998; Chao *et al*., 2004), ISKNV from the freshwater mandarin fish (*Siniperca chuatsi*) (He *et al*., 2000), rock bream iridovirus (RBIV) from the barred knifejaw (Jung and Oh, 2000) and turbot reddish body iridovirus (TRBIV) from the turbot (*Scophthalmus maximus*) (Shi *et al*., 2004). *Infectious spleen and kidney necrosis virus* (ISKNV) is the type species of *Megalocytivirus* (Jancovich *et al*., 2012).

These viruses are genetically classified into three groups according to the nucleotide sequence of the major capsid protein gene (Kurita and Nakajima, 2012). These are: the RSIV type, which are mainly reported in marine fishes (Inouye *et al*., 1992; Jung and Oh, 2000; Chen *et al*., 2003; Gibson-Kueh *et al*., 2004; Lü *et al*., 2005; Dong *et al*., 2010; Huang *et al*., 2011); the ISKNV type, which are reported in both marine and freshwater fish

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(He *et al*., 2000, 2002; Weng *et al*., 2002; Chao *et al*., 2004; Wang *et al*., 2007; Fu *et al*., 2011; Huang *et al*., 2011); and the TRBIV type, which are reported in flatfish (Shi *et al*., 2004; Kim *et al*., 2005; Do *et al*., 2005a; Oh *et al*., 2006). The RSIV-type viruses are widely distributed in East and South-east Asia, and all viruses in Japan are of the RSIV type. Viruses of the ISKNV type are from countries in South-east Asia, China and Taiwan, and reports of the TRBIV type are limited to China and South Korea (Kurita and Nakajima, 2012). Currently, RSIVD caused by the RSIV and ISKNV type viruses is listed by the World Organisation for Animal Health (OIE), but TRBIV is not included because the pathogenicity of the virus is still debatable (OIE, 2012).

An outbreak of RSIVD often spreads from one net pen to adjacent pens. Transmission is by cohabitation, which has been confirmed experimentally (He *et al*., 2002). However, RSIVD has not been reported in hatchery fish; hence, the risk of vertical transmission through eggs or sperm is low (Nakajima and Kurita, 2005). Once the disease has occurred, outbreaks recur every summer in the same area. This is in accord with the observation that the optimal *in vitro* growth temperature of RSIV is 25°C (Nakajima and Sorimachi, 1994). RSIV-infected fish can recover when they are reared at low water temperatures (<18°C) for more than 100 days, and survivors become resistant to RSIVD (Oh *et al*., 2014; Jung *et al*., 2015). In addition, RSIVD is most often reported among the yearlings of many species (Matsuoka *et al*., 1996; Kawakami and Nakajima, 2002). Annual outbreaks of RSIVD in fish newly introduced into net pens are probably caused by horizontal transmission from older recovered fish that still harbour the virus. Ito *et al*., (2013) found the viral genome in the spleen, kidneys, heart, gills, intestine and caudal fin of surviving Japanese amberjacks that were experimentally infected with RSIV. Around aquaculture areas, the viral genome was detected using PCR from many wild fishes (six orders, 18 families and 39 species) that had no clinical signs (Wang *et al*., 2007); this implies that such fish can also be virus carriers. Recently, Jin *et al*. (2014) suggested that some bivalves living around fish culture sites were potential vectors.

Megalocytiviruses are not host specific (Table 12.1); for example, they infect ornamental fishes such as the African lampeye (*Aplocheilichthys normani*) and dwarf gourami (*Colisa lalia*) in South-east Asia (Sudthongkong *et al*., 2002a, Jeong *et al*., 2008a, Weber *et al*., 2009; Kim *et al*., 2010; Sriwanayos *et al*., 2013; Nolan *et al*., 2015). The histopathology of these diseases is similar to those of RSIVD and the causative agents belong to the ISKNV type (Sudthongkong *et al*., 2002b; Weber *et al*., 2009; Kurita and Nakajima, 2012; Sriwanayos *et al*., 2013). In addition, a new *Megalocytivirus* to be formally known as the threespine stickleback iridovirus (TSIV) has been reported from the threespine stickleback (*Gasterosteus aculeatus*) (Waltzek *et al*., 2012).

12.1.2 Diagnosis

Infected fish are dark in colour, lethargic and often have conspicuous respiratory movements owing to severe anaemia. Slight exophthalmos (protrusion of the eyeballs) and haemorrhage from the skin are occasionally observed. Common signs include severe anaemia, petechia in the gills, an enlarged spleen, the presence of a haemorrhagic exudate in the pericardial cavity and pale internal organs (Inouye *et al*., 1992). In natural infections, the disease is usually chronic and cumulative mortality often reaches 20–60% in 1–2 months (Nakajima *et al*., 1999).

Abnormally enlarged cells, which are typical of RSIVD, can be confirmed by microscopic examinations of Giemsa-stained impression smears of the spleen. This is the simplest and most rapid method for presumptive diagnosis (Fig. 12.1), with the caution that enlarged cells are rarely found in some infected fishes.

Immunofluorescence staining using specific monoclonal antibody (M10) was developed for the diagnosis of RSIVD (Nakajima and Sorimachi, 1995; Nakajima *et al*., 1995). Although the TRBIV type has not been examined, M10 antibody reacts with many megalocytiviruses, including RSIV and ISKNV, but does not react with other iridoviruses, such as *Frog virus 3* (FV3), *Epizootic haematopoietic necrosis virus* (EHNV), European sheatfish virus (ESV) and Singapore grouper iridovirus (SGIV) (Nakajima and Sorimachi, 1995; Nakajima *et al*., 1998). The indirect fluorescent antibody test (IFAT) using M10 antibody is listed in the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2012) as a confirmatory diagnostic method for RSIVD and is widely used in Japan because it is rapid and reliable (Matsuoka *et al*., 1996; Kawakami and Nakajima, 2002).

Table 12.1. Fish host range of red sea bream iridovirus (RSIV) and other megalocytiviruses.[a]

Order	Family	No. species	Representative fish species	Virus	OIE[b]	References
Characiformes	Serrasalmidae	1	*Metynnis argenteus*	Unknown		Nolan *et al*., 2015
Cyprinodontiformes	Nothobranchiidae	1	*Aphyosemion gardneri*	Unknown		Nolan *et al*., 2015
	Poeciliidae	4	*Aplocheilichthys normani*	ISKNV, unknown		Paperna *et al*., 2001; Sudthongkong *et al*., 2002a; Nolan *et al*., 2015
Mugiliformes	Mugilidae	1	*Mugil cephalus*	ISKNV	+	Gibson-Kueh *et al*., 2004
Perciformes	Apogonidae	1	*Pterapogon kauderni*	ISKNV		Weber *et al*., 2009
	Belontiidae	2	*Trichogaster leeri*	ISKNV		Jeong *et al*., 2008a
	Carangidae	7	*Seriola quinqueradiata*	RSIV	+	Kawakami and Nakajima, 2002
	Centrarchidae	1	*Micropterus salmoides*	ISKNV	+	He *et al*., 2002
	Cichlidae	8	*Pterophyllum scalare*	Unknown		Armstrong and Ferguson, 1989; Rodger *et al*., 1997; Nolan *et al*., 2015
	Eleotridae	1	*Oxyeleotris marmoratus*	ISKNV		Wang *et al*., 2011
	Ephippidae	1	*Platax orbicularis*	ISKNV		Sriwanayos *et al*., 2013
	Haemulidae	2	*Parapristipoma trilineatum*	RSIV	+	Kawakami and Nakajima, 2002
	Helostomatidae	1	*Helostoma temminckii*	Unknown		Nolan *et al*., 2015
	Kyphosidae	1	*Girella punctata*	RSIV	+	Kawakami and Nakajima, 2002
	Lateolabracidae	2	*Lateolabrax japonicus*	RSIV	+	Kawakami and Nakajima, 2002; Do *et al*., 2005b
	Latidae	1	*Lates calcarifer*	RSIV, ISKNV	+	Huang *et al*., 2011
	Lethrinidae	2	*Lethrinus haematopterus*	RSIV	+	Kawakami and Nakajima, 2002
	Moronidae	1	*Morone saxatilis* × *M. chrysops*	RSIV	+	Kurita and Nakajima, 2012
	Oplegnathidae	2	*Oplegnathus fasciatus*	RSIV	+	Kawakami and Nakajima, 2002
	Osphronemidae	3	*Colisa lalia*	ISKNV, unknown		Paperna *et al*., 2001; Sudthongkong *et al*., 2002a; Kim *et al*., 2010,
	Percichthyidae	2	*Siniperca chuatsi*	ISKNV	+	He *et al*., 2000; Go *et al*., 2006
	Rachycentridae	1	*Rachycentron canadum*	RSIV	+	Kawakami and Nakajima, 2002
	Sciaenidae	2	*Larimichthys crocea*	RSIV, ISKNV	+	Weng *et al*., 2002; Chen *et al*., 2003
	Scombridae	3	*Thunnus thynnus*	RSIV	+	Kawakami and Nakajima, 2002
	Serranidae	10	*Epinephelus septemfasciatus*	RSIV, ISKNV	+	Kawakami and Nakajima, 2002; Sudthongkong *et al*., 2002b; Chao *et al*., 2004; Gibson-Kueh *et al*. 2004; Huang *et al*., 2011
	Sparidae	4	*Pagrus major*	RSIV	+	Inouye *et al*., 1992; Kawakami and Nakajima, 2002; Kurita and Nakajima, 2012
Pleuronectiformes	Paralichthyidae	1	*Paralichthys olivaceus*	RSIV, TRBIV	+	Kawakami and Nakajima, 2002; Do *et al*., 2005a
	Pleuronectidae	2	*Verasper variegatus*	RSIV, TRBIV		Kawakami and Nakajima, 2002; Won *et al*., 2013
	Scophthalmidae	1	*Scophthalmus maximus*	TRBIV		Shi *et al*., 2004
Scorpaeniformes	Sebastidae	2	*Sebastes schlegeli*	RSIV	+	Kim *et al*., 2002
Tetraodontiformes	Monacanthidae	1	*Stephanolepis cirrhifer*	RSIV		This chapter
	Tetraodontidae	1	*Takifugu rubripes*	RSIV	+	Kawakami and Nakajima, 2002

[a]ISKNV, *Infectious spleen and kidney necrosis virus*; RSIV, red sea bream iridovirus; TRBIV, turbot reddish body iridovirus.
[b]+ indicates listed in the OIE Manual (OIE, 2012).

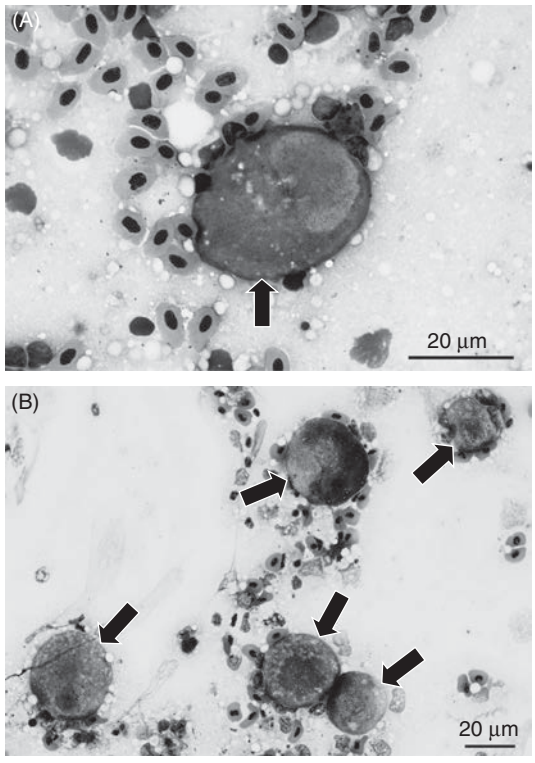

Fig. 12.1. Large, virus-infected cells in an impression smear of the spleen of red sea bream iridovirus (RSIV)-infected fish: (A) from the red sea bream (*Pagrus major*); (B) from the greater amberjack (*Seriola dumerili*). Arrowheads indicate presumptive virus-infected cells. Giemsa stain.

Several PCR primers have been developed (Kurita *et al*., 1998; Oshima *et al*., 1998; Jeong *et al*., 2004; Go *et al*., 2006; Rimmer *et al*., 2012), and the polymerase chain reaction (PCR) assay is used for diagnosis and surveys in wild fishes (Wang *et al*., 2007). Real-time PCR for the quantitative detection of RSIV is rapid, specific and sensitive (Caipang *et al*., 2003; Wang *et al*., 2006; Gias *et al*., 2011; Rimmer *et al*., 2012; Oh and Nishizawa, 2013).

RSIV was first isolated from splenic homogenates of diseased fish using RTG-2 (rainbow trout gonad), CHSE-214 (Chinook salmon embryo), FHM (fathead minnow), BF-2 (blue gill fry) and KRE-3 (kelp and red-spotted grouper embryo) cells (Inouye *et al*., 1992). The cytopathic effect (CPE) of RSIV is characterized by rounding and enlargement of the infected cells. However, the sensitivity of these cell lines is low and viral titres gradually decrease through serial passages (Nakajima and Sorimachi, 1994). The Grunt fin (GF) cell line is more sensitive to RSIV and it is often used for the isolation and propagation of the virus (Jung *et al*., 1997; Nakajima and Maeno, 1998; Ito *et al*., 2013). Nevertheless, complete CPE may not occur in RSIV-inoculated cells, even in GF cells, and it is difficult to recognize any CPE when the viral concentration is low (Ito *et al*., 2013). Several other fish cell lines are sensitive to RSIV and ISKNV, (Imajoh *et al*., 2007; Dong *et al*., 2008; Wen *et al*., 2008), but they are not available from the American Type Culture Collection (ATCC) or the European Collection of Authenticated Cell Cultures (ECACC), and are not otherwise publicly available.

12.1.3 Pathology

The most typical histopathological change is the presence of enlarged cells in the spleen, heart, kidneys, liver or gills. The cells are basophilically stained with haematoxylin in paraffin sections (Fig. 12.2), whereas they are often stained heterogeneously with May–Grunwald–Giemsa stain in impression smears (Fig. 12.1). A large nucleus-like structure is often seen in moderately enlarged cells. These abnormally enlarged cells are infected. They have been reported in RSIVD (Inouye *et al*., 1992; Jung *et al*., 1997; He *et al*., 2000; Jung and Oh, 2000) and other megalocytiviral infections (Sudthongkong *et al*., 2002a, Shi *et al*., 2004; Weber *et al*., 2009; Sriwanayos *et al*., 2013). The most severe lesions are usually in the spleen, where there are often a large number of the virus-infected enlarged cells, diffuse tissue necrosis and a decrease in melanomacrophage centres. Many abnormally enlarged cells also appear in the lymphoid tissues of the kidney interstitium and glomeruli, endocardium and central venous sinus, and beneath the epithelium of the gill filaments (Inouye *et al*., 1992). These enlarged cells stain positively using the IFAT (Nakajima *et al*., 1995) or by *in situ* hybridization (ISH) (Chao *et al*., 2004). In ISH, the hybridization signal appears first in the nucleus in an RSIV-infected cell, and the cytoplasm becomes positive in the later stages of infection (Chao *et al*., 2004).

In electron microscopy, icosahedral virions (approximately 200 nm in diameter) are found in the cytoplasm of enlarged cells. Each virion consists

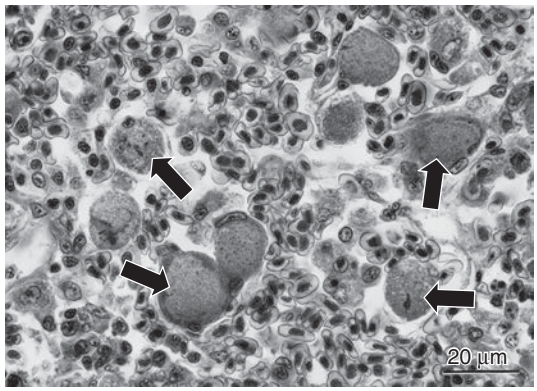

Fig. 12.2. The spleen of red sea bream (*Pagrus major*) with naturally occurring red sea bream iridovirus disease (RSIVD). Arrowheads indicate presumptive virus-infected cells. Haematoxylin and eosin stain.

of a central electron-dense core (120 nm) and an electron-translucent zone. In the virus-infected cells, enlargement of the cell and nuclear degeneration are observed before virion formation (Inouye *et al*., 1992).

12.1.4 Pathophysiology

RSIV has apparent tissue tropism to the spleen, which is where enlarged cells first appear (Chao *et al*., 2004) and the number of viral genomes first increases (Zhang *et al*., 2012; Ito *et al*., 2013), but enlarged cells are rarely observed in the brain, spinal cord and ganglia (Jung *et al*., 1997; Chao *et al*., 2004). Multiple organ failure may occur in later stages of the disease when large numbers of viral genomes are detected in the spleen, kidneys, heart, gills, fins and intestine (Ito *et al*., 2013). Nevertheless, the parenchyma of these organs, except for the spleen, appears intact. The virus-infected, enlarged cells are often in haematopoietic tissues such as the spleen and kidney, and throughout the blood vessels of various organs. These observations, together with the fact that virus-infected cells are not found among the cells composing tissue parenchyma, suggest that the targets of RSIV are haematopoietic cells. By damaging these cells, RSIV probably causes aplastic anaemia, which contributes to death. Further investigation is needed to identify the target cells of RSIV, and to clarify the cause of death.

12.1.5 Protective and control strategies

Vaccination against RSIVD is available but there is no chemotherapeutic agent. The vaccine for red sea bream has been sold in Japan since 1999 and is the first commercially available vaccine against a viral disease in marine fish. It consists of a formalin-inactivated culture supernatant of GF cells inoculated with RSIV. The effectiveness of the vaccine has been confirmed experimentally and in field trials (Nakajima *et al*., 1997, 1999). Currently, the vaccine is used in Japan for red sea bream, yellowtail, greater amberjack, striped jack, Malabar grouper (*Epinephelus malabaricus*), orange-spotted grouper (*E. coioides*), longtooth grouper (*E. bruneus*) and sevenband grouper (*E. septemfasciatus*). The frequency of outbreaks of RSIVD in Japan has dramatically decreased since the vaccine was approved. The cost of the vaccine increases the expense of fish production and, consequently, more economic recombinant subunit vaccines and DNA vaccines for RSIV have been studied, but they are not commercially available (Caipang *et al*., 2006; Kim *et al*., 2008; Shimmoto *et al*., 2010; Fu *et al*., 2012, 2014) and their efficacies are not satisfactory. Recently, another inactivated vaccine has been studied that was produced in a fish cell line (from mandarin fish) which yields more virus (Dong *et al*., 2013a,b). There is another problem in that for the vaccine currently sold in Japan, the efficacy in barred knifejaw and the spotted knifejaw (*O. punctatus*) is still inadequate. To overcome this problem, live vaccine has been suggested because virus-inoculated fish develop immunity when they are reared at low water temperatures (<18°C) (Oh *et al*., 2014; Jung *et al*., 2015).

Fasting for more than 10 days reduces RSIVD mortality (Tanaka *et al*., 2003). Even though the mechanism of this reduction is still unclear, fasting is often practised in fish farms after the onset of RSIVD, but it leads to considerable weight loss.

RSIVD most often occurs in yearling fish and fish seem to acquire some resistance as they grow larger (Matsuoka *et al*., 1996; Kawakami and Nakajima, 2002). Therefore, rapid growth of juveniles before the summer helps to avoid severe RSIVD.

12.1.6 Conclusions

RSIVD suddenly occurred in cultured red sea bream in a small area in Japan in 1990. It spread

rapidly to other areas and other fish species in subsequent years (Matsuoka *et al*., 1996). All of the viral isolates in Japan are of the RSIV type, suggesting that the virus is likely to have been introduced into Japan via imported fish. It currently occurs in coastal south-western Japan, and in East and South-east Asia, and its lack of host specificity leads to disease in many species whenever the water temperatures are conducive for the virus.

Megalocytiviruses have been reported in numerous fishes (Table 12.1), including ornamental fishes from South-east Asia (Sudthongkong *et al*., 2002a, Jeong *et al*., 2008a; Weber *et al*., 2009; Kim *et al*., 2010; Sriwanayos *et al*., 2013; Subramaniam *et al*., 2014; Nolan *et al*., 2015). A similar viral infection (characterized by the formation of basophilic enlarged cells) in ornamental fish was confirmed before RSIVD was found in Japan, though the virus was not identified (Armstrong and Ferguson, 1989). Therefore, the possibility of the transmission of megalocytivirus by ornamental fishes through international trade is worrisome (Jeong *et al*., 2008a). For example, an outbreak of megalocytivirus infection occurred in cultured Murray cod (*Maccullochella peelii peelii*) via the importation of dwarf gourami into Australia (Go *et al*., 2006; Go and Whittington, 2006). Also, the megalocytivirus from pearl gourami (*Trichogaster leeri*) was highly pathogenic to barred knifejaw (Jeong *et al*., 2008b).

12.2 White Sturgeon Iridovirus

12.2.1 Introduction

Over the last century, increased demand for caviar (traditionally from wild sturgeon in the Black and Caspian seas) and related habitat degradation (e.g. pollution and river damming) have resulted in the International Union for Conservation of Nature (IUCN) listing of several species, notably sturgeons and paddlefishes, as critically endangered (Birstein 1993; Pikitch *et al*., 2005). The development of global sturgeon aquaculture for enhancing the food and stock availability has sought to alleviate pressures on these diminished wild stock.

The emergence of a lethal viral disease of the integument, gills, and upper alimentary tract in cultured juvenile white sturgeon (*Acipenser transmontanus*), known as white sturgeon iridovirus (WSIV), has had a severe impact on commercial farms in the Pacific Northwest of North America since 1988 (Hedrick *et al*., 1990; LaPatra *et al*., 1994, 1999; Raverty *et al*., 2003; Drennan *et al*., 2006; Kwak *et al*., 2006). WSIV was first reported in juvenile white sturgeon reared on farms in northern and central California where it produced annual losses of up to 95% (Hedrick *et al*., 1990, 1992). Subsequently, WSIV was reported in cultivated white sturgeon from the lower Columbia River in Oregon and Washington, the Snake River in southern Idaho and the Kootenai River in northern Idaho (LaPatra *et al*., 1994, 1999). In 2001, white sturgeon fingerlings from an aquaculture facility in British Columbia, Canada, were infected, which are likely to have originated from brood stock obtained from the Fraser River drainage basin in that province (Raverty *et al*., 2003).

Experimental studies suggested that the horizontal transmission of WSIV can occur via contaminated water (Hedrick *et al*., 1990, 1992). Mortality in juvenile white sturgeon begins at 10 days and peaks between 15 to 30 days after viral exposure. Although adult white sturgeon appear to be resistant to clinical disease, persistently infected adults are believed to vertically transmit WSIV to their offspring. LaPatra *et al*. (1994) suggested that the sources of WSIV in epizootics among white sturgeon hatcheries in Idaho and Oregon were wild brood stock. The epizootiology of WSIV on a Californian sturgeon farm support vertical transmission to be more important than tank-to-tank transmission (Georgiadis *et al*., 2001). Finally, lake sturgeon (*A. fluvescens*) can be experimentally infected, but do not express clinical disease (Hedrick *et al*., 1992).

A similar lethal virus of captive juvenile pallid sturgeon (*Scaphirhynchus albus*) and the shovelnose sturgeon (*S. platorhynchus*), known as the Missouri River sturgeon iridovirus (MRSIV), has negatively affected stock enhancement programmes in hatcheries within the Missouri River Basin (Kurobe *et al*., 2010, 2011). Additional epizootics characterized by strikingly similar pathology have been reported in other cultured sturgeon species, including the Russian sturgeon (*A. gueldenstaedtii*) in northern Europe (Adkison *et al*., 1998), lake sturgeon in Manitoba, Canada (Clouthier *et al*., 2013) and shortnose sturgeon (*A. brevirostrum*) in New Brunswick, Canada (LaPatra *et al*., 2014).

WSIV has been tentatively classified as a member of the family *Iridoviridae* based on its histopathological and ultrastructural resemblance to iridoviruses (Hedrick *et al*., 1990; Jancovich *et al*., 2012).

However, the WSIV major capsid protein (MCP) lacks significant sequence homology to that of iridoviruses (Kwak *et al*., 2006). Preliminary phylogenetic analyses relying solely on the MCP support WSIV and the other sturgeon irido-like viruses as a new branch within the family *Mimiviridae* (Kwak *et al*., 2006; Kurobe *et al*., 2010; Clouthier *et al*., 2013, 2015; Waltzek *et al*., unpublished).

12.2.2 Diagnosis

Cultivated white sturgeon fry and fingerlings are highly susceptible to WSIV at water temperatures between 12 and 20°C (Hedrick *et al*., 1992; LaPatra *et al*., 1994; Watson *et al*., 1998b). Typical WSIV epizootics manifest as a chronic wasting syndrome in which anorexic juveniles linger at the bottom of the tank and gradually become emaciated, leading to impaired growth with up to 95% mortality (Hedrick *et al*., 1990; Raverty *et al*., 2003). WSIV is epitheliotropic, resulting in pathognomonic microscopic lesions in external tissues, including the integument, gills and upper alimentary tract (Hedrick *et al*., 1990; LaPatra *et al*., 1994; Watson *et al*., 1998a). Histological examination reveals characteristic hypertrophic epithelial cells that stain amphophilic to basophilic (Hedrick *et al*., 1990). An immunohistochemical assay employing a monoclonal antibody against WSIV can confirm the virus in tissue sections (OIE, 2003).

Viral isolation is challenging as WSIV grows slowly in white sturgeon spleen (WSS-2) and skin cells (WSSK-1) (Hedrick *et al*., 1992). Pooled gill and skin tissue homogenates are inoculated on to cell cultures, maintained at 20°C and examined for 30 days for CPE; a blind passage is performed with 15 additional days of observation if no CPE is observed (OIE, 2003). Although CPE may be detected as early as 1 week post inoculation, changes may not be observed for 2–3 weeks. Infected cells often occur singly or in small groups and are recognizable by their rounded, enlarged and refractile appearance (Hedrick *et al*., 1992; Watson *et al*., 1998a; Fig. 12.3). Infected cells eventually die and detach, but complete destruction of the monolayer only occurs in the most heavily infected cultures. Cultures with a suspicious CPE can be confirmed using a serum neutralization assay, indirect fluorescent antibody test or transmission electron microscopy (TEM) (Hedrick *et al*., 1990; OIE, 2003).

The development of a conventional PCR assay targeting the WSIV major capsid protein has provided a much needed specific, sensitive and rapid diagnostic tool (Kwak *et al*., 2006). The assay does not amplify other iridoviruses and detects down to 1 fg of target plasmid DNA. The sampling of tissue fragments of the pectoral fins is non-lethal and eliminates the need to sacrifice large numbers of valuable cultured or threatened populations of wild white sturgeon (Drennan *et al*., 2007a). Recently, a conventional PCR assay and two separate quantitative PCR assays targeting conserved regions of the MCP were designed to amplify all of the North American sturgeon nucleocytoplasmic large DNA viruses, including WSIV (Clouthier *et al*., 2015).

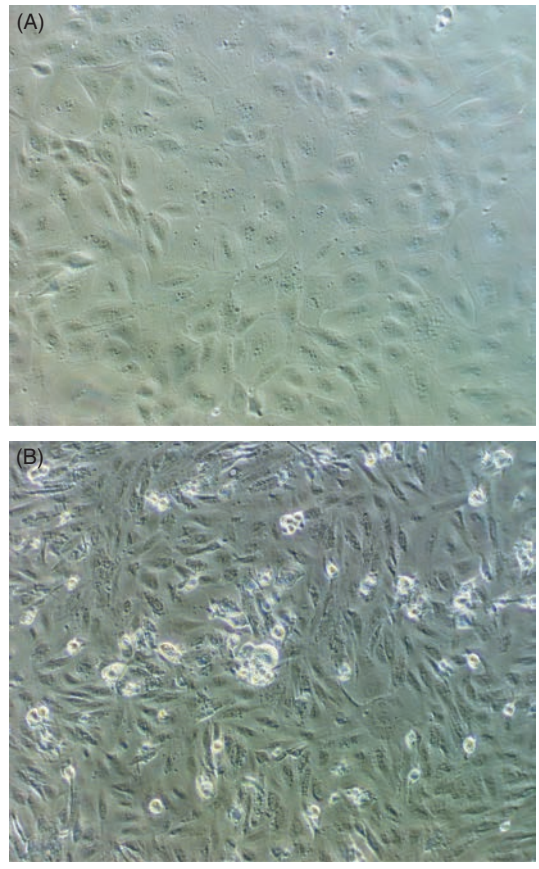

Fig. 12.3. White sturgeon (*Acipenser transmontanus*) spleen cells (WSS-2) infected with white sturgeon iridovirus (WSIV): (A) uninfected control; (B) infected cells scattered throughout the monolayer singly or in small groups and recognizable by their rounded, enlarged and refractile appearance. Images courtesy of Ronald P. Hedrick.

12.2.3 Pathology

The epitheliotropic nature of WSIV results in gross lesions on the skin and gills (Hedrick *et al*., 1990; LaPatra *et al*., 1994). The ventral scutes of infected fish are often red or haemorrhagic (Fig. 12.4A) and the gills may appear swollen and pale. Infected juveniles typically appear emaciated and stunted. Internally, a pale liver, an empty gastrointestinal tract and reduced fat stores may be observed.

Histopathological examination of the skin and gills typically reveals focal to diffuse epithelial hyperplasia with an associated enlargement of some epithelial cells that stain amphophilic to basophilic (Fig. 12.4.B). These hypertrophic epithelial cells are pathognomonic for WSIV infection and have been observed in the integument (skin and fins), olfactory organs (barbels and nares), oropharyngeal cavity (hard palate, tongue, lips, opercular flap), upper alimentary tract (oesophagus) and gills (Hedrick *et al*., 1990; Watson *et al*., 1998a). In advanced disease, both hyperplasia of the branchial epithelium and necrosis of the pillar cells lining the lamellar vascular channels leading to small haemorrhages may be observed (Hedrick *et al*., 1990). The histological or cytological evaluation of WSIV-positive fish may reveal secondary pathogens, including bacteria (*Flavobacterium* sp.), protists (*Trichodina* sp.) and oomycetes (*Saprolegnia* sp.) (Hedrick *et al*., 1990; Watson *et al*., 1998a).

TEM of infected tissues (e.g. skin or gills) or cultures can confirm the diagnosis. Aggregations of distinctive large and complex icosahedral-shaped virions can be observed around the viral assembly site within the cytoplasm of hypertrophic epithelial cells (Watson *et al*., 1998a). The average diameter of these virions is 262–273 nm between opposite sides

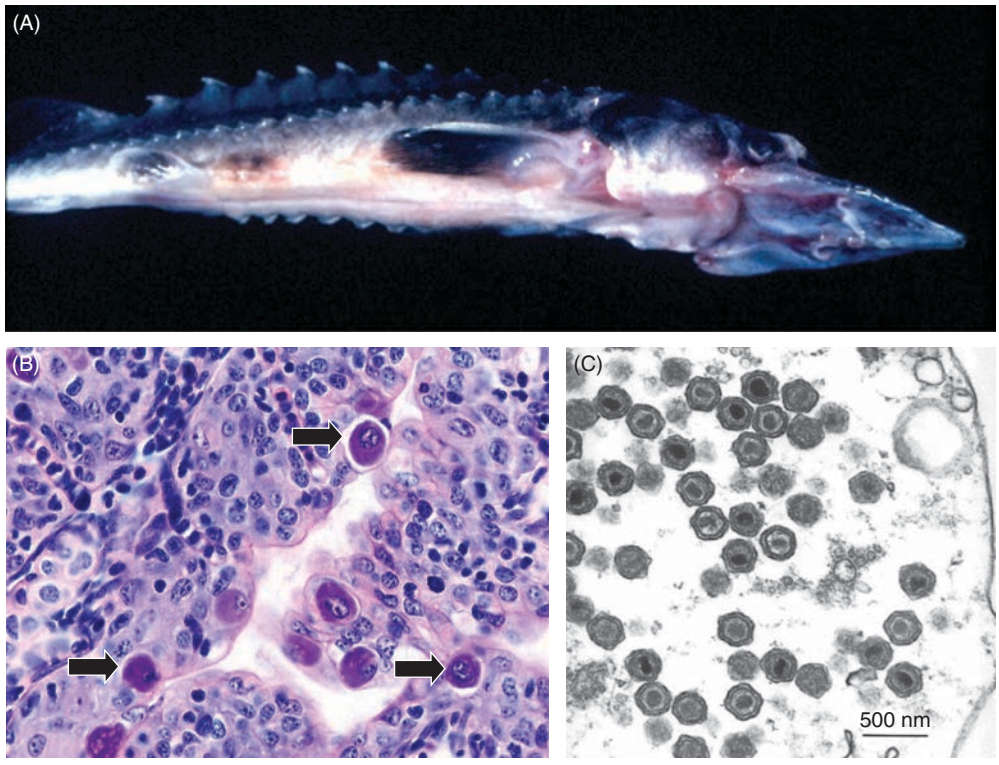

Fig. 12.4. Juvenile white sturgeon (*Acipenser transmontanus*) infected with white sturgeon iridovirus (WSIV): (A) small haemorrhages present along the ventral surfaces, including the oral region and lateral scutes; (B) WSIV-infected gill tissue section revealing numerous enlarged amphophilic cells (arrows), haematoxylin and eosin stain; (C) transmission electron photomicrograph illustrating the complex virions of WSIV as found directly from infected cells in the gill of white sturgeon. Images courtesy of Joseph M. Groff and Ronald P. Hedrick.

and 299–302 nm between opposite vertices (Hedrick *et al*., 1990; Watson *et al*., 1998a; Fig. 12.4C).

12.2.4 Pathophysiology

The pathophysiology of white sturgeon WSIV infection has been investigated (Watson *et al*., 1998a,b). Lesions in the integument and gills are believed to alter the respiratory and osmoregulatory capacities of infected sturgeons. Experimentally, infected juvenile white sturgeon had a decreased haematocrit and decreased plasma protein concentrations at higher water temperatures (Watson *et al*., 1998b). WSIV infection of the sensory epithelia (nares, barbels, lips, tongue) in juvenile white sturgeon is believed to result in anorexia, leading to a lethal wasting syndrome (Watson *et al*., 1998a). Watson *et al*. (1998b) found that juvenile white sturgeon experimentally infected with WSIV were unable to maintain normal hepatosomatic indices at lower water temperatures, which is indicative of food deprivation (Hung *et al*., 1997) and reduced dietary glucose (Fynn-Aikens *et al*., 1992). Finally, the WSIV induced disruption of mucosal barriers is hypothesized to predispose juvenile white sturgeon to secondary invaders (Watson *et al*., 1998b).

12.2.5 Protective and control strategies

Factors contributing to the occurrence or severity of WSIV epizootics under hatchery conditions include high stocking density, physical and procedural stressors, fluctuating or low water temperatures, and lapses in biosecurity (LaPatra *et al*., 1994, 1996; Watson *et al*., 1998b; Georgiadis *et al*., 2001; Drennan *et al*., 2005). Reducing stock densities and increasing the water flow reduced mortality (LaPatra *et al*., 1994). Experimental studies have demonstrated significantly higher cumulative mortality in groups held at higher versus lower stocking densities (LaPatra *et al*., 1996; Drennan *et al*., 2005). The physical and procedural stressors that can increase the frequency of WSIV outbreaks include handling, transportation and fluctuations in temperature (LaPatra *et al*., 1994, 1996). Stress can trigger disease onset in subclinical carrier fish, which subsequently spread the virus to other individuals (Georgiadis *et al*., 2001). An experimental infection study (Watson *et al*., 1998b) demonstrated that juvenile white sturgeon held at a lower water temperature (10°C) suffered significantly higher cumulative mortality and had more severe microscopic lesions than those held at a higher water temperature (23°C). Lower water temperatures promoted *in vitro* WSIV replication (Hedrick *et al*., 1992) and the virus may depress the immune response of sturgeon (Watson *et al*., 1998b). Although rearing juvenile white sturgeon at higher water temperatures reduces cumulative mortality, secondary bacterial (*Flavobacterium* sp.) infections are more problematic at these elevated temperatures (Watson *et al*., 1998b).

White sturgeon juveniles that survive a WSIV outbreak appear to be protected even following stressful events. The survivors develop individual or herd immunity, which protects them when they are re-exposed to WSIV (Georgiadis *et al*., 2001). Anti-WSIV serum and mucosal antibodies have been confirmed in survivors (Drennan *et al*., 2007b). Thus, production strategies involving the development of resistant brood stock or stocking with fish that have survived an outbreak have been proposed (Georgiadis *et al*., 2000, 2001). Additionally, farmers should practise stringent biosecurity, including quarantining new brood stock in a separate building and screening them for WSIV prior to spawning. The development of an economic WSIV vaccine and delivery system capable of stimulating mucosal immunity is needed to mitigate the effect of WSIV epizootics in sturgeon aquaculture.

12.2.6 Conclusions

In the last couple of decades, progress has been made: (i) in our understanding of WSIV pathology, pathophysiology and epizootiology; and (ii) in the availability of diagnostic tools for detecting WSIV. Physical and procedural stressors influence the occurrence and severity of epizootics. Through proper biosecurity and management practices, the effects of WSIV can be minimized; however, an economic vaccine would benefit the aquaculture of white sturgeon.

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13 Alphaviruses in Salmonids

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13.1 Introduction

Alphavirus is a genus of RNA viruses belonging to the family *Togaviridae*. Most known alphaviruses are mosquito borne and cause diseases in terrestrial hosts such as birds, rodents and larger mammals, including humans (Strauss and Strauss, 1994). Infections may lead to diverse symptoms, such as rashes, gastrointestinal problems, arthritis/muscular inflammation and encephalitis (Kuhn, 2007; Steele and Twenhafel, 2010). Salmon pancreas disease virus, which is commonly named Salmonid alphavirus and abbreviated SAV (Weston *et al*., 2002), is the only known alphavirus that has fish as a natural host (Powers *et al*., 2001). SAV is distantly related to other members of the genus, but it still causes pathology that may resemble some of that seen in mammals (McLoughlin and Graham, 2007; Biacchesi *et al*., 2016). The first isolation of SAV in cell culture was reported in 1995 from marine farmed Atlantic salmon (*Salmo salar*) in Ireland suffering from pancreas disease (PD) (Nelson *et al*., 1995). This occurred at the same time as the isolation of a similar virus from freshwater rainbow trout (*Oncorhynchus mykiss*) suffering from sleeping disease (SD) in France (Castric *et al*., 1997). Even though PD and SD are caused by the same virus species and are characterized by the same histopathology (Boucher and Baudin Laurencin, 1996; Weston *et al*., 2002), the two different names are still commonly used. This is probably for historical reasons, and because SD is mainly associated with the production of smaller sized (*c*. 0.3–2 kg at slaughter) rainbow trout in fresh water, whereas PD is associated with infections in the marine Atlantic salmon and rainbow trout industry.

The genome of SAV is an 11.9 kb large single-stranded RNA molecule in messenger sense, with two large open reading frames (Weston *et al*., 2002). These encode the four non-structural proteins (nsP1–4) that replicate the genome, and the structural proteins (capsid, E1, E2, E3 and 6K/TF). These latter proteins build an icosahedral-shaped virus particle that contains an inner capsid flanked by a membrane derived from the host (Villoing *et al*., 2000a). Embedded into this membrane are numerous glycoprotein spikes. The glycoprotein E2 constitutes the majority of the surface of the particle (Voss *et al*., 2010; Karlsen *et al*., 2015), and is also a main antigenic target for the immune system (Moriette *et al*., 2005).

SAVs from different areas of Europe show considerable genetic diversity, and six genetic subtypes, SAV1–6, have been described (Fringuelli *et al*., 2008; see Fig. 13.1). Subtypes 1, 2, 4, 5 and 6 have been found in Atlantic salmon and rainbow trout around the British Isles (Graham *et al*., 2012; Karlsen *et al*., 2014); SAV3 has only been described from Norway (Hodneland *et al*., 2005); and SAV2 appears to be more diverse than the other subtypes, and contains two distinct lineages, one that causes PD in marine farms in Scotland and Norway, and another that is the cause of SD epizootics in Continental Europe, with occasional outbreaks in Scotland and England (Graham *et al*., 2012; Hjortaas *et al*., 2013; Karlsen *et al*., 2014).

Although it remains unknown how SAV was initially introduced into farmed fish, a diverse wild reservoir of SAV may exist in or around the North Sea. Phylogenetic dating studies have shown that each of the subtypes must represent separate introduction events from this reservoir (Karlsen *et al*., 2014). Evidence for the presence of SAV in wild common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Platessa*

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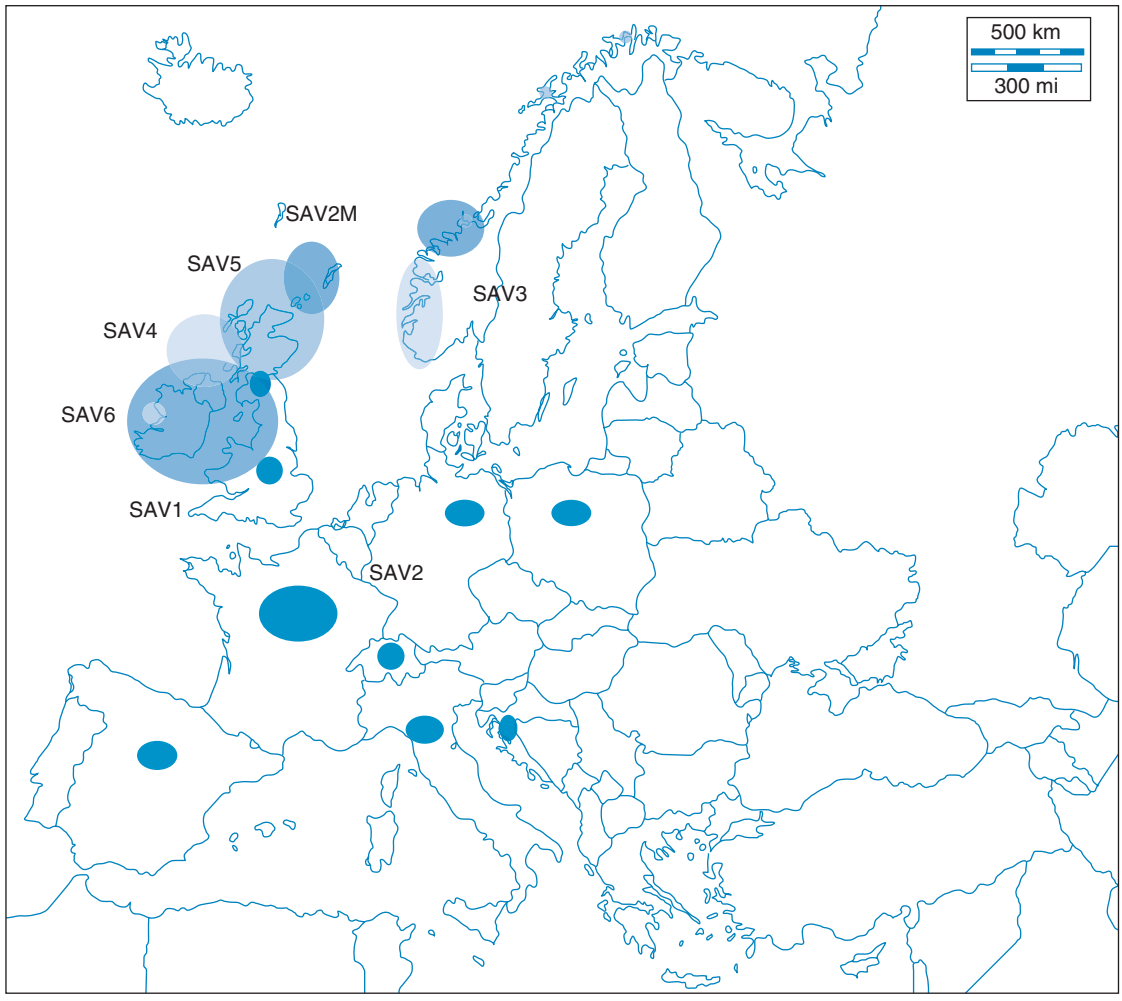

Fig. 13.1. Approximate distribution of salmonid alphavirus (SAV) subtypes in European salmonid farms. The geographical distribution is roughly indicated by the coloured spheres for each subtype. For subtype 2, the distributions of a freshwater form (SAV2) and a marine form (SAV2M) are included. Details of the distribution of freshwater SAV2 are poorly known, and the figure merely illustrates occurrences for this particular subtype at a country level. The map used as a basis for the figure was obtained from www.d-maps.com.

platessa) has been presented (Snow *et al*., 2010; Bruno *et al*., 2014; McCleary *et al*., 2014; Simons *et al*., 2016), and the prevalence of SAV in the common dab appears to be relatively high around the British Isles. It is still not clear whether these species of flatfish constitute the original wild reservoir of SAV, or if they have been infected as a result of spillover from salmon infections (Karlsen, 2015; Simons *et al*., 2016). Experimental transmission of the virus in brown trout (*Salmo trutta*) has shown that this species is also susceptible to SAV (Boucher *et al*., 1995).

Infection with SAV may develop into a high-titre viraemia (Desvignes *et al*., 2002) and fish shed considerable amounts of infectious virus particles into the water during the viraemic phase (Andersen *et al*., 2010; Andersen, 2012). Shedding to water is significantly reduced, or stops, when the host develops a strong antibody response and the viraemia is cleared. The shed virus particles infect nearby fish (McLoughlin *et al*., 1996). It is likely that marine SAVs persist in farmed populations of fish via horizontal transmission between farms in the same

current network (Viljugrein *et al*., 2009). The transport of fish and/or equipment between such networks can enhance transmission, and is the most probable explanation for occasional long-distance transmissions (Karlsen *et al*., 2014). Viral RNA has also been detected in salmon lice (*Lepeophtheirus salmonis*) that feed on infected fish, but active replication in the lice has not been demonstrated (Karlsen *et al*., 2006; Petterson *et al*., 2009), nor has it been possible to infect *L. salmonis* in the laboratory (Karlsen *et al*., 2015). The spread of SAV2/SD in the smaller sized rainbow trout industry is probably connected with trade practices that involve the transport of live fish or ova, and vertical transmission cannot be excluded (Castric *et al*., 2005; Borzym *et al*., 2014). The epizootiology of SD has been only poorly studied.

In Europe, the economic impact of losses due to SAV is significant (Aunsmo *et al*., 2010); when comparing pathogen-related losses in the Norwegian salmon industry, those from SAV are probably only second to those from sea lice infestations. Biomass lost through mortalities contributes to one part of the loss, but poor growth and a reduction in fillet quality are also major consequences of infection. In Norway, during 2007 it was estimated that SAV infections increased the production costs of Atlantic salmon by 6.0 Norwegian kroner (NOK) per kilo (from 25–30 NOK/kg). An outbreak at a 500,000 smolt production site was estimated to cost about 14.4 million NOK (Aunsmo *et al*., 2010). These numbers should not be taken as representative of SAV2 epizootics in the rainbow trout industry, where outbreaks affect smaller fish. Because outbreaks of SD are not reported on a regular basis, the cost due to SAV in rainbow trout is difficult to estimate.

13.2 Diagnosis

SAV can be detected either indirectly by observing effects on the host, or more directly by identifying virus-specific molecules such as the viral genome, viral proteins or the infectious virus particle within the host. A preliminary and indirect diagnosis can be based on gross clinical signs and histopathology (McLoughlin *et al*., 2002; Taksdal *et al*., 2007), but because other pathogens may produce similar signs, it is always appropriate to identify viral molecules to confirm SAV infection.

Clinical disease occurs during the whole year, but it is more common in marine farms when the water temperature is higher in the summer, probably reflecting that the virus has an optimum temperature of 10–15°C (Villoing *et al*., 2000a; Graham *et al*., 2008). The first clinical sign is usually a drop in appetite. Fish later aggregate towards the surface of the pen and swim sluggishly, sometimes with their dorsal fins above the water surface. This behaviour often begins in one net pen and spreads to others. Abundant yellowish faecal casts are frequently observed in the water during a clinical outbreak of the disease (McVicar, 1987; McLoughlin *et al*., 2002; Taksdal *et al*., 2007). Most SAV infections result in an increased rate of mortality.

A study of 23 Norwegian sites experiencing PD due to SAV3 in 2006–2007 suggested that the mean mortality was 6.9% during the outbreak, and that elevated mortality lasted an average of 2.8 months (Jansen *et al*., 2010). Mortalities due to SAV2 in Norway have been suggested to be lower (Jansen *et al*., 2015). Mortality rates from outbreaks around the British Isles have been reported to be very variable (Crockford *et al*., 1999), but it is difficult to exclude the contributions of other pathogens in extreme cases.

The gross pathology due to SAV includes a miscoloured liver, petechiae on the pyloric caeca and visceral fat, and ascites in the intraperitoneal cavity (Fig. 13.2). The intestine can be either empty or filled with yellowish faeces (McLoughlin and Graham, 2007). Damage in the fillet, melanization and miscoloured areas are other signs that are associated with ongoing or previous SAV infection (Taksdal *et al*., 2012).

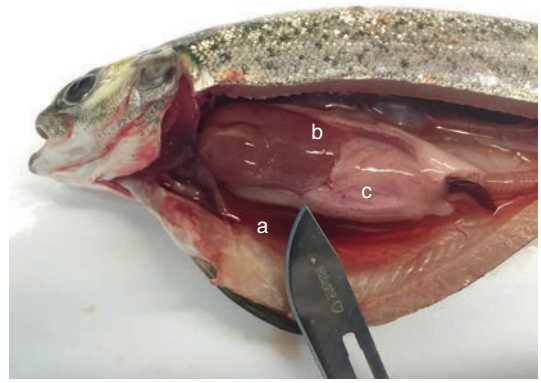

Fig. 13.2. Atlantic salmon parr suffering from pancreas disease (PD) after experimental infection by salmonid alphavirus (SAV). The typical gross pathology of clinical PD includes: (a) ascites; (b) a discoloured liver; and (c) petechiae on the pyloric caeca. Photo by Rolf Hetlelid Olsen/PHARMAQ AS.

In the late stage of outbreaks, fish with very low K-factor (low body weight relative to length) can be observed (McLoughlin and Graham, 2007).

SAV does not always manifest as an acute clinical disease. The infection may persist following an acute outbreak (Graham *et al*., 2010), and it can also occur without an acute phase and with few observable signs (Graham *et al*., 2006b; Jansen *et al*., 2010). Such subclinical infections probably transmit virus, and this underscores the importance of not relying on clinical signs, but rather detecting SAV in the farm with a more specific method (Graham *et al*., 2006b, 2010; Hodneland and Endresen, 2006).

A conclusive diagnosis of PD is usually based on histopathology and the detection of viral RNA. Both the gross pathology and the histopathology due to SAV bear similarities to those of other viral diseases of salmon, e.g. heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and infectious pancreatic necrosis (IPN) (McLoughlin and Graham, 2007). The combination of pancreatic lesions and pathology in the heart and skeletal muscle does separate PD from these conditions. SAV is easily detected in fish during an ongoing infection, and real-time RT-PCR is commonly used due to its superior sensitivity, specificity and ability to rapidly test many samples (Graham *et al*., 2006a; Hodneland and Endresen, 2006). Several commercial laboratories in Norway and the UK (e.g. Patogen Analyse, PHARMAQ Analytiq, Fish Vet Group) provide this service. Viral RNA can be detected in most, if not all, organs during viraemia, but the amount of viral RNA is usually higher in the heart (Andersen *et al*., 2007). The temporal duration of the PCR signal is also longer in this organ. PCR-based methods can be used to separate the different SAV subtypes (Hodneland and Endresen, 2006) and consequently these methods do provide more detailed information on epizootiology than most other detection methods.

Other methods of diagnosis include isolation of the virus in cell culture from serum or tissue homogenates during the viraemic phase, before an antibody response has been activated (Nelson *et al*., 1995; Castric *et al*., 1997; Desvignes *et al*., 2002). Most fish cell lines appear to be susceptible to the virus (Graham *et al*., 2008), but a cytopathic effect (CPE) can be weak or absent in some cases (Karlsen *et al*., 2006). Subsequent confirmation of *in vitro* SAV replication by specific molecular methods, e.g. the immunofluorescence antibody test (IFAT) (Todd *et al*., 2001; Moriette *et al*., 2005) is, therefore recommended. Viral infection can also be detected *in situ* using antibody-mediated assays such as immunohistochemistry (Villoing *et al*., 2000b) or hybridization to RNA probes (Cano *et al*., 2015). These methods are useful for studying tissue tropism and pathogenesis, but are less sensitive and more laborious than PCR. As a result, they are not routinely used for diagnosis. Late-stage or completely cleared infections can be detected using a neutralizing antibody assay. A virus neutralization test has been developed, and is used particularly in Ireland and the UK to screen fish that have had previous exposure to SAV (Graham *et al*., 2003).

13.3 Pathology

13.3.1 Mortality in laboratory challenges

Early experimental infection studies with SAV did not lead to increased mortality, and resembled the subclinical infections sometimes reported from the field (Boucher *et al*., 1995; Boucher and Baudin Laurencin, 1996; McLoughlin *et al*., 1996; Andersen *et al*., 2007; Christie *et al*., 2007). Later studies have, though, demonstrated significant mortalities accompanied by clinical signs after experimental infection (Boscher *et al*., 2006; Moriette *et al*., 2006; Karlsen *et al*., 2012; Xu *et al*., 2012; Hikke *et al*., 2014a). Experimental infections with SAV do therefore adequately span the range of clinical signs and observations that have been reported from the field. Clinical disease can be reproduced through all the infection routes that have been tested: intraperitoneal and intramuscular injections, bathing and cohabitation. If the average SAV3 field mortality of 6.9% calculated by Jansen *et al*. (2010) is representative, then one would expect only two dead fish in a group of 30. Hence, many of the experimental infections reported in the literature lacked the statistical power to detect a mortality rate of this small size.

Factors such as host status, challenge titre and virus strain affect the severity of the disease in laboratory experiments. It is, however, possible to induce mortality rates higher than even extreme field cases, especially in injection models using high doses of virulent strains (Karlsen *et al*., 2012).

13.3.2 Histopathological changes

The name pancreas disease comes from the initial description of the disease, which reported pancreatic

necrosis (Munro *et al*., 1984). It later became evident that myopathy in the heart, skeletal muscle and oesophagus were also significant features of the disease (Ferguson *et al*., 1986). These lesions develop in a sequential manner, with pancreatic necrosis followed by myopathy and inflammation of the heart (Boucher and Baudin Laurencin, 1994; McLoughlin *et al*., 1996; Weston *et al*., 2002). The lesions in skeletal muscle develop later.

The first histological observations in experimental infections are the necrosis of single cells in the heart (Fig. 13.3b) and pancreas (Fig. 13.4b) (see Mcloughlin and Graham, 2007). The necrotic myocardial cells develop a characteristic hyaline eosinophilic look with haematoxylin–eosin (HE) staining, and are often referred to as PD cells, and the process designated single cell necrosis (SCN). At this stage, there is little or no clinical sign of the disease. During clinical outbreaks in the field, the most common findings are severe inflammation in the ventricle of the heart (Fig. 13.3c) and necrosis and or atrophy of the pancreas (Figs 13.4b and 13.4c). Inflammation in the skeletal muscle may also be observed, especially in red muscle along the lateral side of the fish (Fig. 13.3d). Liver necrosis is often observed in fish with reduced capability to oxygenate the blood, and may be observed in fish with PD if the heart is severely affected (Fig. 13.4d).

The pancreas can fully regenerate in fish that survive the disease. Damage to the heart and skeletal muscle takes longer to heal and can reduce fillet quality (Taksdal *et al*., 2012).

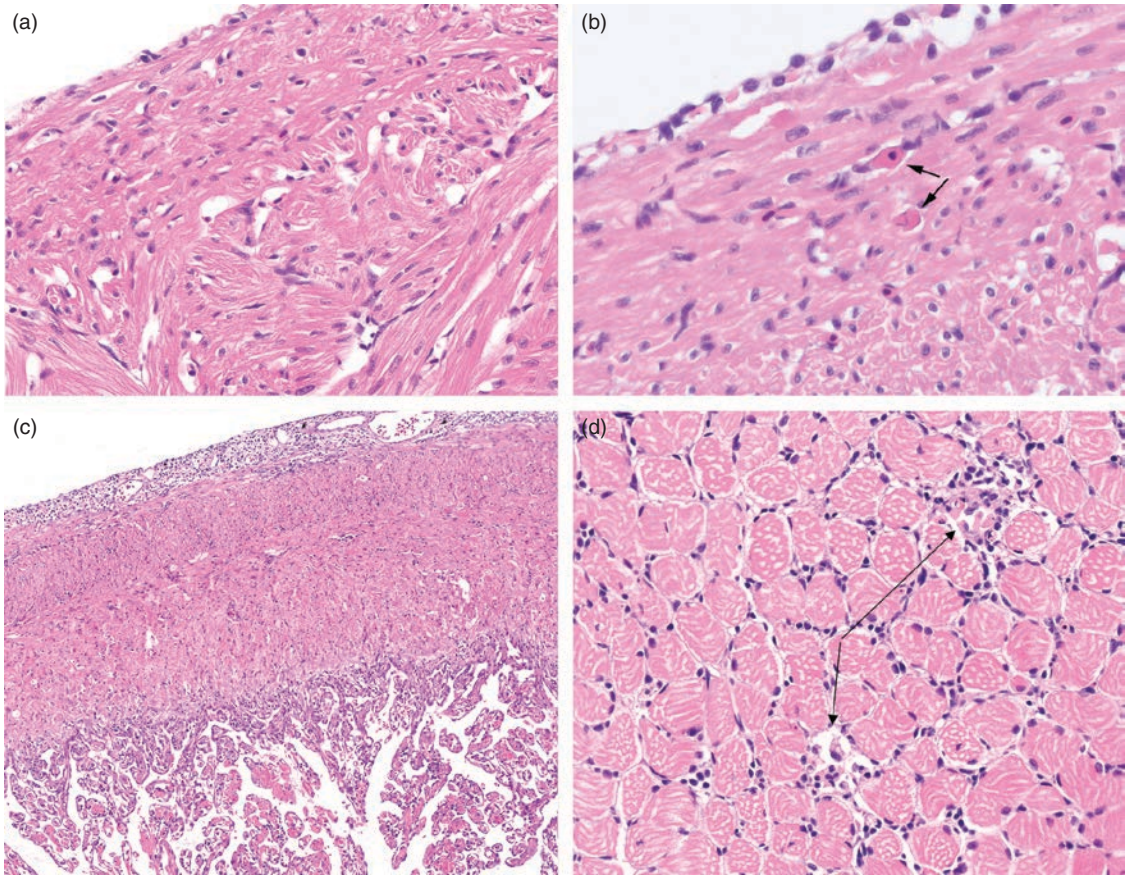

Fig. 13.3. Histological images from salmon using haematoxylin–eosin staining: (a) normal heart tissue; (b) ventricle in the early stage of a salmonid alphavirus (SAV) infection with single cell necrosis (arrows); (c) ventricle with severe inflammation from the clinical stage of a pancreas disease (PD)-outbreak; and (d) focal inflammation (arrows) in the red skeletal muscle of salmon with PD.

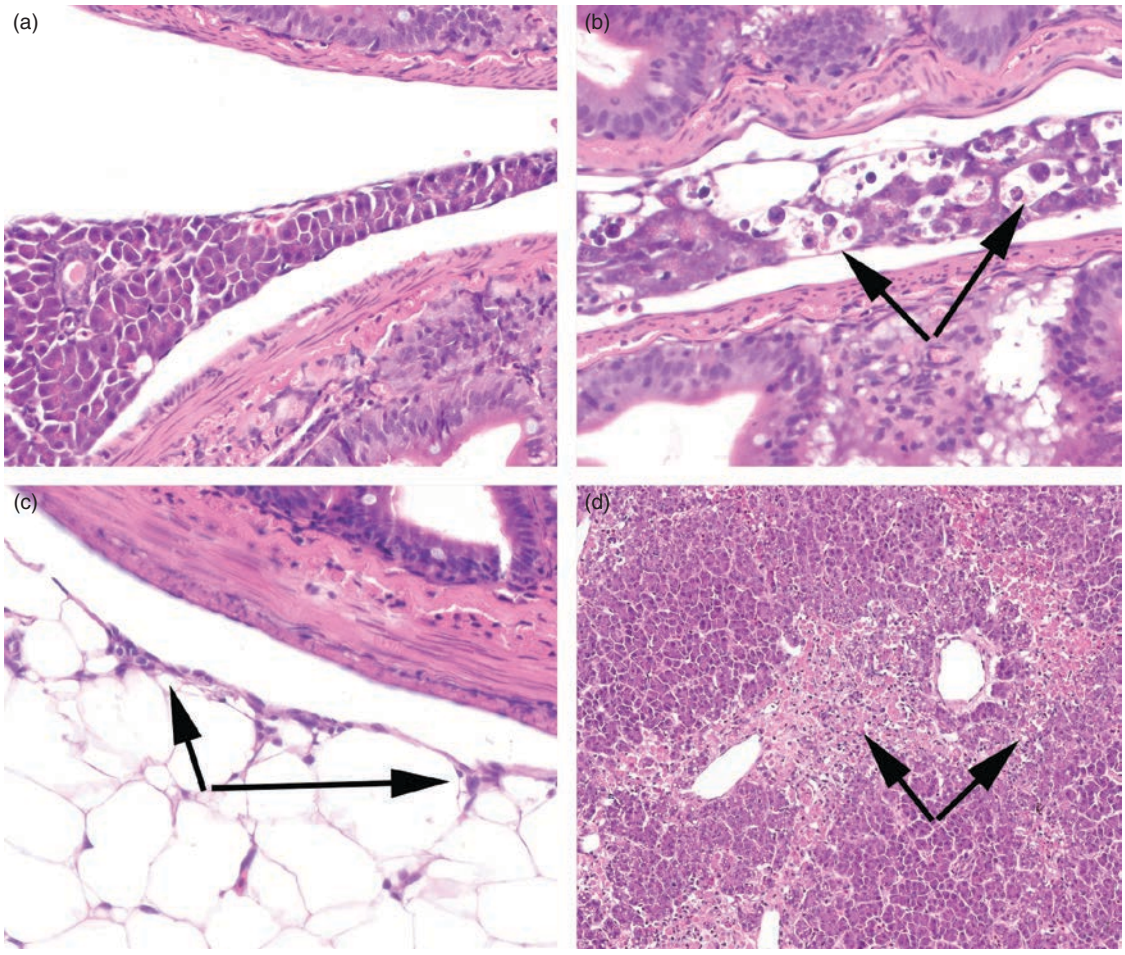

Fig. 13.4. Histological images from salmon using haematoxylin–eosin staining: (a) normal pancreatic tissue in between the small intestines; (b) necrosis of pancreatic cells in the early stages of pancreas disease (PD) (arrows); (c) pancreatic atrophy has led to the absence of pancreatic tissue in the clinical stage of PD (arrows); and (d) liver necrosis in a fish with heart failure due to salmonid alphavirus (SAV) infection (arrows).

13.3.3 Tissue tropism and replication *in vivo*

Alphaviruses in general are not very host cell specific. The replication apparatus of SAV is functional under diverse conditions, and in cells from fish, mammals, insects and crustaceans (Graham *et al*., 2008; Olsen *et al*., 2013; Hikke *et al*., 2014b). Although the choice of host cell can be influenced by interactions between the replication apparatus and the host proteins, tissue tropism is probably decided more by the structural proteins, in particular E2, which covers most of the surface of the viral particle (Kuhn, 2007; Voss *et al*., 2010; Karlsen *et al*., 2015). It is assumed, based on homology with other alphaviruses, that E2 is the receptor binding protein of SAV (Villoing *et al*., 2000a). While there is considerable genetic diversity in E2 in the different SAV subtypes (Fringuelli *et al*., 2008), they all seem to have qualitatively the same tissue tropism (Graham *et al*., 2011). It is possible, however, that they may differ somewhat in quantitative terms in their choice of target organ.

The route of entry of SAV into the host is unknown, but the virus transmits via water contact (McLoughlin *et al*., 1996). Cells in the intestine, gills and the skin are, therefore, plausible entry sites. Following the initial infection, SAV rapidly

amplifies in the host and spreads systemically to several organs through a high-titre viraemia (Desvignes *et al*., 2002; Andersen *et al*., 2007). Viral RNA can be detected in all blood-rich organs in this phase. Andersen *et al*. (2007) demonstrated that in the later stages of infection (up to 190 days post intraperitoneal injection), viral RNA can still be detected in the pseudobranch, gills, heart and kidney. It is not clear whether this persistence results in limited shedding of infectious particles, but laboratory experiments have linked the main shedding period to the viraemic phase of the infection (Andersen, 2012). Attempts to provoke the recurrence of a clinical outbreak in such carriers have not succeeded (Andersen *et al*., 2007). Active replication has been shown to occur in pancreatic tissue, the heart, skeletal muscle and leucocytes (Houghton, 1995; Villoing *et al*., 2000b; Moriette *et al*., 2005; Andersen, 2012; Cano *et al*., 2014). Recently, it was also demonstrated that muscle satellite cells are target cells for the virus in rainbow trout (Biacchesi *et al*., 2016). Several alphaviruses are associated with neurotrophic infections and neuropathology (Strauss and Strauss, 1994), but this seems not to be a significant feature of SAV infections in salmonids (McLoughlin and Graham, 2007).

13.3.4 Virulence differences among strains

The strains of SAV do not appear to differ qualitatively in the pathology they cause, but they do show significant differences in the quantity of tissue damage that they cause (Weston *et al*., 2002; Christie *et al*., 2007; Graham *et al*., 2011; Taksdal *et al*., 2015). Although it is not clear whether differences in virulence are subtype specific, or if larger variation exists within each subtype, an initial comparison between SAV2 and SAV3 suggested that SAV3 is generally more virulent than SAV2 (Taksdal *et al*., 2015).

SAV adapts to cell culture through mutations that lead to lower virulence in fish (Moriette *et al*., 2006; Merour *et al*., 2013; Petterson *et al*., 2015), which has been observed for the strains SAV2 and SAV3. The changes in virulence are caused by mutations in E2, and a single substitution is enough to attenuate a pathogenic SAV2 in rainbow trout (Merour *et al*., 2013). This demonstrates that even though SAV acts as a rather stable and predictable virus in its pathology, it has the capacity to rapidly change its characteristics. Substitutions in E2 have also been observed in field isolates of SAV3. One substitution, from proline to serine in E2 position 206 now dominates in contemporary strains of the SAV3 subtype, but it has been suggested that it could affect the rate at which the virus transmits rather than its virulence (Karlsen *et al*., 2015).

13.4 Pathophysiology

The main target organs of SAV (the exocrine pancreas, heart muscle and skeletal muscle) may be severely affected by the infection. Necrosis of pancreatic tissue probably reduces the ability to digest food, and this may be a reason behind the reduced growth and the production of abundant faecal casts that are often observed during outbreaks (McLoughlin and Graham, 2007). Another reason for reduced growth is the loss of appetite, which is a common general reaction of fish to severe infections.

Heart inflammation leads to reduced circulatory capacity and could explain some of the autopsy findings, such as discoloured liver, bleeding and ascites. Liver necrosis could be a consequence of the reduced capacity to oxygenate blood and, thus, possibly a secondary finding due to heart failure. Fish suffering from PD also commonly carry infections with heterologous pathogens in the gills, and this could reduce gas exchange (Nylund *et al*., 2011). Circulatory failure could be particularly dangerous for fish with such co-infections.

Discoloration of the fillet is probably a consequence of the inflammatory response to SAV. The red muscle is also sensitive to low oxygen levels. Accordingly, circulatory failure may explain why the red muscle often is more affected than the white anaerobic muscle.

An interesting aspect of SAV infection at the cellular level is the ability of the virus to modulate transcription and/or translation (Xu *et al*., 2010). Alphaviruses have several different strategies for achieving this, including the interactions of nsP2 and the capsid with cellular proteins. It has been shown that fish cells that express the SAV capsid cannot continue with cell division (Karlsen *et al*., 2010b). It is likely that many of these transcriptomic changes are transient, and it is not clear how this influences the organism.

13.5 Control Strategies

In Norway, the driving reservoir of both SAV3 and SAV2 appears to be farmed salmonids (Viljugrein

et al., 2009; Karlsen *et al*., 2014; Hjortaas *et al*., 2016). Mitigation strategies have, therefore, focused on this reservoir. It is likely that water contact between marine farms is one major route of transmission (Viljugrein *et al*., 2009). A second route of unknown importance is mechanical transmission via a vector, for example well-boats or other equipment that is shared between farms (Karlsen *et al*., 2014). The mitigation of SAV3 was intensified in Norway in 2007 when PD became a notifiable disease, and an endemic zone was defined on the west coast. Fish positive for SAV, or showing other signs of PD, are not allowed out of this zone. Sites within the zone are allowed to keep SAV-positive fish until slaughter, but the site has to be disinfected and fallowed before restocking. Infected fish outside the zone are immediately slaughtered and the farms fallowed to prevent further establishment of the disease. With the introduction of SAV2 north of the SAV3-endemic zone (Hjortaas *et al*., 2013), an SAV2-specific regulation was made to define an SAV2-endemic zone north of the SAV3-endemic zone. Marine farms that receive smolts from this zone must show freedom from SAV by PCR testing 2 and 4 months after transfer out of the zone. This regulation also specifies other actions, such as restrictions on the transport of SAV2-positive fish, and details for the disinfection of well-boats and net pens.

One strategy used by the industry to reduce economic losses due to PD is prescheduled harvesting. In this strategy, farmers take advantage of information generated using PCR-based monitoring of the infection status in the farm. As disease prevalence increases, it can be expected that a clinical outbreak is near and farmers may choose to slaughter fish to avoid the costs associated with mortality, reduced growth and poor fillet quality. It has been estimated that this strategy becomes cost-effective when the fish weigh >3.2 kg (Pettersen *et al*., 2015).

13.5.1 Vaccines

A monovalent inactivated water-in-oil formulated whole-virus vaccine based on the SAV1 type-strain F93-125 has been commercially available from MSD Animal Health in Ireland and in Norway since 2003 and in the UK since 2005. This vaccine has been used extensively in geographical regions where PD commonly appears. Although it has been reported to have a positive effect on some production parameters, its efficacy has not been sufficient to eliminate SAV from farmed fish populations, and vaccinated fish have continued to experience outbreaks of PD (Bang *et al*., 2012). Two multivalent vaccines based on F93-125 became available for sale in 2015 (also from MSD Animal Health).

Laboratory experiments, as well as commercial-scale field trials, have identified promising new vaccine candidates based on inactivated whole-virus technology (Karlsen *et al*., 2012), DNA plasmids (Hikke *et al*., 2014a; Simard and Horne, 2014), live attenuated strains (Moriette *et al*., 2006) and recombinantly expressed subunit antigens (Xu *et al*., 2012). Inactivated whole-virus vaccines have the benefit of following a relatively predictable regulatory pathway for approval. Their efficacies can be improved, for example through the addition of CpG oligonucleotides or poly(I:C) (polyinosinic–polycytidylic acid) to the formulation in order to activate a cellular immune response (Strandskog *et al*., 2011; Thim *et al*., 2012, 2014). Inactivated antigens also have an advantage over other vaccine technologies in that they are easier to include in multivalent vaccines, together with bacterial and viral antigens protecting against other diseases.

DNA vaccines expressing the structural proteins of SAV appear to give significant protection against infection provided that all of the glycoproteins are expressed together to ensure proper folding (Xu *et al*., 2012; Hikke *et al*., 2014a; Simard and Horne, 2014). This vaccine technology is already used to protect salmonids against infectious hematopoietic necrosis virus (IHNV) in Canada, but the registration process of DNA vaccines for food animals in Europe is less clear than that for inactivated whole-virus vaccines. Recently, however, a DNA vaccine against SAV was recommended to be granted marketing authorization in Europe.

An infection with SAV provides strong protection against subsequent infection (Lopez-Doriga *et al*., 2001). Live vaccines are, therefore, likely to provide very good efficacy, and an attenuated strain of SAV2 has been shown to protect rainbow trout fry against subsequent infections by the wild type virus (Moriette *et al*., 2006). The challenge with such technology is to ensure safe attenuation without loss of efficacy. The attenuated strain of SAV2 mentioned is attenuated through only one or two mutations (Merour *et al*., 2013). A safer strategy could be to delete parts of the genome, or to replace them with homologous parts from other alphaviruses. This has been done for alphaviruses

that are pathogenic to humans, and is likely to provide a stronger hurdle for reversion to virulence. Reverse genetics systems that can be used to construct such attenuated strains have been made for the SAV2 and SAV3 strains (Moriette *et al*., 2006; Karlsen *et al*., 2010a; Guo *et al*., 2014). In addition to regulatory requirements, several patents cover aspects of vaccine development against SAV, which may reduce the freedom to operate for pharmaceutical companies.

13.5.2 Other tools to mitigate SAV

Initiatives have been undertaken to breed salmon with improved resistance to SAV infection. This strategy has been successfully applied to improve the control of infectious pancreatic necrosis virus (IPNV), and it is likely that this has contributed significantly to the reduction of IPNV outbreaks in recent years. It appears that resistance towards PD also has a moderate-to-high heritability in Atlantic salmon, and a quantitative trait locus (QTL) affecting the resistance of the species to SAV infection was recently identified (Gonen *et al*., 2015). It is still rather too preliminary to conclude that introduction of this QTL into populations of farmed salmon will reduce PD to the same degree as breeding for improved resistance to IPN, but major producers of ova offer eggs that have been selected on the basis of PD resistance.

'Functional feeds' designed to have beneficial effects during viral infection are marketed by feed companies such as EWOS, Skretting and Biomar. These are meant to either reduce inflammatory responses or to be more easily digested (e.g. Alne *et al*., 2009; Martinez-Rubio *et al*., 2012). Although considerable amounts of these feeds are used by the industry, few published studies have addressed their effect against PD, so it remains unclear how effective they are.

Disinfectants commonly used in aquaculture (Virkon S, Virex, Halamid, FAM30 and Buffodine) have been reported to be generally effective in inactivating SAV, but Virkon S and Virex were found to perform better under conditions with increased organic load or varying temperature (Graham *et al*., 2007).

13.6 Conclusions and Future Perspectives

SAV is a major viral problem in European Atlantic salmon farming, and it also affects the rainbow trout industry. Infection causes pathology in the pancreas, heart and skeletal muscle, and may lead to reduced growth and fillet quality, as well as mortality. Several lines of work could help to improve mitigation of the disease. Improved preventive methods such as better vaccines or breeds of fish with improved resistance could potentially reduce transmission and improve control by challenge pressure within local transmission networks. In relation to this, it would be useful to document these networks better, and to monitor virus spread more closely using molecular techniques such as PCR and sequencing. This could greatly aid decision making on when and where to transfer new cohorts of fish.

One likely development in the future diagnostics of SAV and other RNA viruses is the increased focus on sequences, which contain a much richer amount of information than PCR signals. SAV evolves, like most RNA viruses, with a rather high rate of substitution (Karlsen *et al*., 2014). A parental strain of SAV in farm A can give rise to strains of SAV in farms B and C that will be separated by several substitutions in their genome sequences just a year after the initial transmission event (Karlsen, 2015). This phylogenetic signal can be very useful for tracking transmission chains and identifying more accurately the mechanisms behind transmission.

Conflict of Interest Statement

The authors are employees of PHARMAQ and PHARMAQ Analytiq, subsidiaries of Zoetis, companies with financial interests in fish vaccines and diagnostics. MK is a shareholder in Zoetis.

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14 *Aeromonas salmonicida* and *A. hydrophila*

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14.1 Introduction

Aeromonas belongs to the family *Aeromonadales* within the class *Gammaproteobacteria* (Colwell *et al*., 1986). Aeromonads occur in freshwater, estuarine and marine environments, invertebrates, vertebrates and soils (Janda and Abbott, 2010). The type species is the motile *A. hydrophila*, an animal pathogen; in contrast, the species *A. salmonicida*, a fish pathogen, is non-motile. Aeromonads induce furunculosis, atypical furunculosis, ulcerative diseases, motile *Aeromonas* septicaemia (MAS) and tail and fin rot in fishes (Cipriano and Austin, 2011). *A. hydrophila* and other motile species (e.g. *A. veronii* biovar. *sobria*, *A. bestiarum*, *A. dhakensis*) cause diseases in aquaculture and are potentially zoonotic pathogens (Rahman *et al*., 2002; Janda and Abbott, 2010; Austin and Austin, 2012a; Colston *et al*., 2014). The intraspecies classification of *A. salmonicida* and *A. hydrophila* needs reassessment (Martin-Carnahan and Joseph, 2005). Genomic sequencing of some aeromonads has revealed the potential for horizontal gene transfer, which enhances bacterial adjustment to different environments and hosts (Piotrowska and Popowska, 2015).

A. salmonicida is among the most important fish pathogens worldwide. It consists of four psychrophilic subspecies, *A. s. salmonicida*, *A. s. achromogenes*, *A. s. masoucida* and *A. s. smithia*, and the mesophilic subspecies *A. s. pectinolytica* (Pavan *et al*., 2000; Martin-Carnahan and Joseph, 2005). *A. s.* subsp. *salmonicida* is typical for the species and produces systemic furunculosis that affects many cold-water fishes. The disease had an economic impact on the farming of salmonids internationally until oil-adjuvanted furunculosis vaccines were marketed (Gudding and van Muiswinkel, 2013). Other atypical strains of *A. salmonicida* are heterogeneous both genetically and phenotypically (Austin *et al*., 1998).

Atypical *A. salmonicida* strains also cause significant problems in aquaculture (Gudmundsdottir, 1998; Wiklund and Dalsgaard, 1998). *A. salmonicida* subsp. *pectinolytica* is the only non-pathogenic subspecies (Austin and Austin, 2012b).Vaccination against atypical furunculosis is problematic and the disease currently threatens the farming of Atlantic cod (*Gadus morhua*); there is also a problem among farmed Arctic charr (*Salvelinus alpinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic wolffish (*Anarhichas lupus*), spotted wolffish (*Anarhichas minor*) and various 'cleaner fish' – wrasses (*Labridae*) and the lumpsucker (*Cyclopterus lumpus*) (Gulla *et al*., 2015b). Ulcerative diseases caused by atypical *A. salmonicida* occur in goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), flounder (*Platichthys flesus*) and many more species (Trust *et al*., 1980; Wiklund and Dalsgaard, 1998).

Infection by *A. salmonicida* is transmitted horizontally through contaminated water, surface-infected eggs, vertebrate and non-vertebrate carriers, and equipment and clothing; vertical transmission has not been confirmed (Nomura, 1993; Wiklund, 1995). *A. salmonicida* attaches to the gills and skin/mucus regions of fish (Ferguson *et al*., 1998). Except for New Zealand, atypical *A. salmonicida* infections occur worldwide. Typical *A. salmonicida*

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infections have not been reported within Australia, Chile and New Zealand.

The second species covered in this chapter, *A. hydrophila*, infects warm- and cool-blooded animals, including humans. It causes serious mortalities in farmed warm-water fishes (Janda and Abbott, 2010; Cipriano and Austin, 2011; Colston *et al.*, 2014). Highly virulent *A. hydrophila* of Asian origin has caused epizootics among channel catfish (*Ictalurus punctatus*) in the south-eastern USA since 2009 (Hossain *et al.*, 2014). *A. hydrophila* has two subspecies, *A. h. hydrophila* (Seshadri *et al.*, 2006) and *A. h. ranae* (Huys *et al.*, 2003); a third subspecies, *A. h. decolorationis*, has been proposed (Ren *et al.*, 2006). Furthermore, *A. hydrophila* belongs to three DNA hybridization groups (HGs) (Martin-Carnahan and Joseph, 2005). Most reports of *A. hydrophila* infections do not define the subspecies classification. The species is opportunistic and disease occurrence is commonly related to stress (e.g. high fish density, elevated temperature). Mortalities can also peak at low temperatures (Cipriano and Austin, 2011). Vaccination is problematic owing to strain variation and the nature of the different hosts (Austin and Austin, 2012b). *A. hydrophila* spreads horizontally through contaminated water, carrier fish, external parasites, equipment and clothing (Rusin *et al.*, 1997; Udeh, 2004; Austin and Austin, 2012a).

14.2 Diagnosis

Diagnosis is based on clinical signs, the history of the facility involved, and the isolation and identification of the bacterium concerned. Infections often result in septicaemias, in which the bacteria or bacterial products are found in numerous organs and with skin ulcerations, but mortalities also occur without any detectable pathology. Disease signs may include a darkened colour, lethargy, abnormal swimming behaviour, inappetance, pale gills, dermal ulcerations, fin and tail rot, erythrodermatitis, haemorrhages, septicaemia, bloating, and the protrusion or loss of scales. Bacterial identification is based on phenotypic characterization and molecular techniques (Martin-Carnahan and Joseph, 2005; Tenover, 2007; Austin and Austin, 2012a,b).

Aeromonads are Gram-negative diplobacilli 0.3–1.0 µm in diameter and 1.0–3.5 µm long. Coccoid forms occur and staining may be bipolar. They are cytochrome oxidase positive, facultative anaerobes that ferment glucose with or without the production of gas and are resistant to the vibriostatic agent O/129. The initial isolation is usually from the head kidney, skin lesions or gills. *A. salmonicida* is non-motile and does not grow at 37°C, whereas *A. hydrophila* is motile and does grow at this temperature (Martin-Carnahan and Joseph, 2005). The bacterium is cultured on tryptone soya agar (TSA) or brain heart infusion agar (BHIA), but many atypical *A. salmonicida* require blood agar (Cipriano and Bertolini, 1988; Wiklund, 1990; Gulla *et al.*, 2015b). The latter strains are very fastidious and are easily overgrown by other organisms. Therefore, other techniques have been developed to identify these, including enzyme-linked immunosorbent assay (ELISA), agglutination with specific antibodies (Adams and Thompson, 1990; Gilroy and Smith, 2003; Saleh *et al.*, 2011) and DNA sequence-based methods using 16S rRNA gene sequencing, PCR amplification and real-time quantitative PCR (qRT-PCR) techniques (Gustafson *et al.*, 1992; Byers *et al.*, 2002; Clarridge, 2004; Balcázar *et al.*, 2007; Beaz-Hidalgo *et al.*, 2013). The intraspecies grouping of *A. salmonicida* and *A. hydrophila* is complex and requires bioinformatic comparisons of genes or genomes (Colston *et al.*, 2014).

A. salmonicida has a protein surface A-layer protein (VapA) that gives the bacterium increased hydrophobicity and its autoagglutination characteristic (Chart *et al.*, 1984; Belland and Trust, 1985). The addition of Coomassie Brilliant Blue R-250 dye in media helps to identify A-layer positive isolates (Cipriano and Bertolini, 1988). Incubation at 15–20°C is recommended, because temperatures above 20°C may enhance loss of the A-layer (Moki *et al.*, 1995). Recent data indicate that *vapA* typing, based on sequence variation, separates *A. salmonicida* subspecies and recognizes undescribed subtypes (Gulla *et al.*, 2015a). The optimal growth temperature of *A. salmonicida* is 22–25°C and most strains do not grow at 37°C (Martin-Carnahan and Joseph, 2005). However, there is a motile biogroup that grows at 37°C and motility is induced by cultivation at 30–37°C (Mcintosh and Austin, 1991). Furthermore, some strains may be oxidase negative (Wiklund *et al.*, 1994). Typical *A. salmonicida* generally produce a water-soluble brown pigment when grown in tryptone media, but achromogenic strains do exist (Wiklund *et al.*, 1993). If atypical strains do produce brown pigment, the production of the pigment is slower. Schwenteit *et al.* (2011) reported that pigment production is regulated by quorum sensing (Fig. 14.1), and this may correlate with virulence.

A. hydrophila is motile and has a polar flagellum. It is a heterogeneous group whose members differ serologically and genotypically. Consequently, 16S rRNA gene sequences, in addition to phenotypic characterizations, are necessary for identification (MacInnes *et al.*, 1979; Valera and Esteve, 2002; Martin-Carnahan and Joseph, 2005).

14.3 Pathology

Furunculosis is a systemic disease caused by *A. salmonicida* subsp. *salmonicida* (McCarthy and Roberts, 1980), whereas atypical furunculosis is caused by other subspecies (Gudmundsdottir, 1998). Another form of disease caused by *Aeromonas* is skin ulcer disease, which is induced by some atypical *A. salmonicida* strains. Mortality is generally less severe in fish with the ulcerative disease than in systemic furunculosis (Austin and Austin, 2012b).

The systemic disease caused by *A. salmonicida* can be peracute, acute, subacute or chronic (McCarthy, 1975). Peracute disease is common in young fish that die without signs other than darkening and exophthalmos (protruding eyes). Fish that survive often develop lesions with hyperaemia and punctuate haemorrhages. Focal haemorrhages and gill congestion may be evident. Bacteria are found in the anterior kidney, gills, spleen and myocardium, where necrosis of the cardiac tissue may occur. The disease progresses rapidly, with minimal host response. Acute furunculosis is manifested by general septicaemia; mortality is high and fish may die without detectable pathology. Hyperaemia occurs in all serosal surfaces and the spleen is often enlarged and red. The kidneys become soft and friable or liquefied. The liver is pale with subcapsular haemorrhages, or the surface may appear mottled owing to focal necrosis with ascites in the coelomic cavity. Skin lesions may develop, either as haemorrhagic patches along the body or as typical furuncles.

Toxic septicaemia changes are more severe in the acute than in the subacute and chronic forms of the systemic disease. Toxic haemopoieitic necrosis, and myocardial and renal tubule degeneration are seen irrespective of where the bacteria localize. The subacute and chronic forms of furunculosis are more common in older fish, which often survive and recover. Infected fish have a darkened skin and loss of appetite. Lethargy and furuncles are common. As shown in Fig. 14.2, the ulcers induced by typical strains of *A. salmonicida* extend deeper into

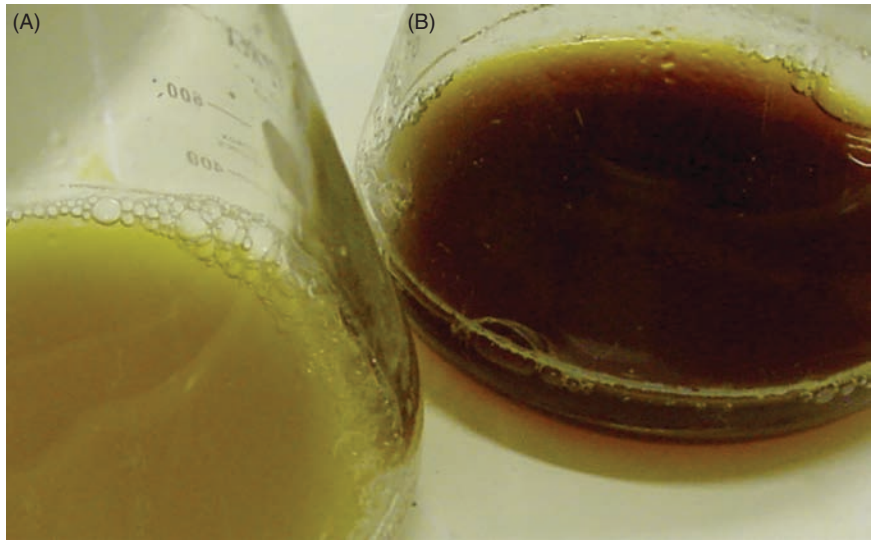

Fig. 14.1. Pigment production is quorum sensing regulated in *Aeromonas salmonicida.* The figures show cultures of *A. salmonicida* subsp. *achromogenes* strain Keldur 265-87 grown in brain heart infusion agar (BHIA) at 16°C for 190 h: (A) a Δ*asaI* mutant (quorum-sensing negative); and (B) the wild type quorum-sensing positive strain. Photo: Bjarnheidur K Gudmundsdottir.

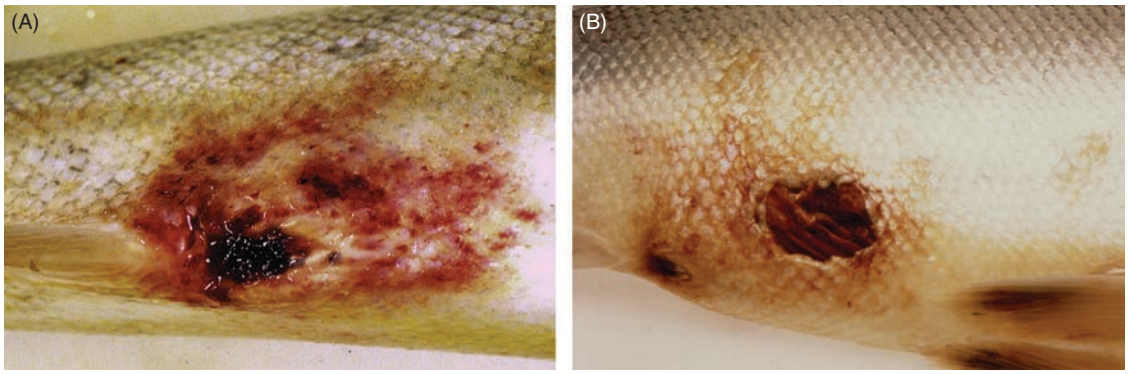

Fig. 14.2. Wild Atlantic salmon (*Salmo salar*; 2.5 kg average weight) from the river Ellidaár in Iceland suffering from chronic furunculosis induced by (A) *Aeromonas salmonicida* subsp. *salmonicida* and (B) *A. salmonicida* subsp. *achromogenes*, respectively. Photo: Ragnar Th. Sigurdsson.

the musculature, liquefactive necrosis is more prominent, and skin haemorrhages are more intensive than in fish infected with atypical strains. The gross pathology of subacute furunculosis is similar to the acute form but general visceral congestion and peritonitis are pronounced in the chronic form. The heart and spleen are frequently affected. Fish that survive epizootics become carriers that may spread the disease (McCarthy, 1975; Hellberg *et al*., 1996; Gudmundsdottir, 1998; Bjornsdottir *et al*., 2005).

The pathological features of Atlantic cod with systemic *A. salmonicida* subsp. *achromogenes* infection can be explained by distinct features of the cod immune system. The major histocompatibility complex class II (MHC class II) gene, which is fundamental for humoral response, is absent in cod (Star *et al*., 2011). Therefore, cod rely on cellular and innate responses, in which granuloma formation (Fig. 14.3) is typical (Magnadottir *et al*., 2002, 2013).

Ulcerative diseases induced by atypical *A. salmonicida* involve superficial pathology. The initial signs are small haemorrhages in skin that progress to multiple lesions commonly found on the flanks. Secondary infections by other bacteria are common. Fish can die without detectable bacteraemia, but bacteria are found systemically and within internal organs as the infection progresses (Mawdesley-Thomas, 1969; Bootsma *et al*., 1977). Wiklund and Bylund (1993) divided ulcer development into three stages in the flounder. First, there is weak skin haemorrhage; secondly, this develops into white lesions rimmed by haemorrhagic inflammatory tissue; finally, the ulcer manifests itself as eroded skin with exposed muscles. Other *A. salmonicida*-induced ulcer diseases include carp erythrodermatitis (Bootsma *et al*., 1977), ulcer disease of salmonids (Paterson *et al*., 1980; Bullock *et al*., 1983), goldfish ulcer disease (Elliott and Shotts, 1980) and miscellaneous expressions in minnows (*Phoxinus phoxinus*) (Hastein *et al*., 1978), Japanese eel (*Anguilla japonica*) (Kitao *et al*., 1984) and rockfish (*Sebastes schlegeli*) (Han *et al*., 2011).

The pathology of fish infected with *A. hydrophila* varies. Lesions may be restricted to the skin, but systemic infections also occur. Infections can result in acute, chronic and covert conditions (Cipriano and Austin, 2011). In acute MAS, fish may die before signs of the disease become visible. Signs may include exophthalmia, haemorrhage and necrosis of the skin, fins and oral cavity. Skin ulcers may develop in which necrosis extends into the muscle. Hepatic petechiation, enlargement of the spleen and swollen kidneys are common. The gills may haemorrhage and the scales protrude. Histopathologically, the skin and musculature may exhibit acute-to-chronic dermatitis and myositis with neutrophilic infiltrates. Alterations often involve necrosis in the kidney, liver, spleen and heart (Cipriano *et al*., 1984; Cipriano and Austin, 2011). Pathological signs in carp are most severe in the liver and kidneys. Degeneration of the epithelium of the intestine and heart, and haemorrhages in the interstitial tissues of organs are common (Stratev *et al*., 2015). Infections in the channel catfish can induce systemic and cutaneous disease (Ventura and Grizzle, 1988). In cutaneous infections,

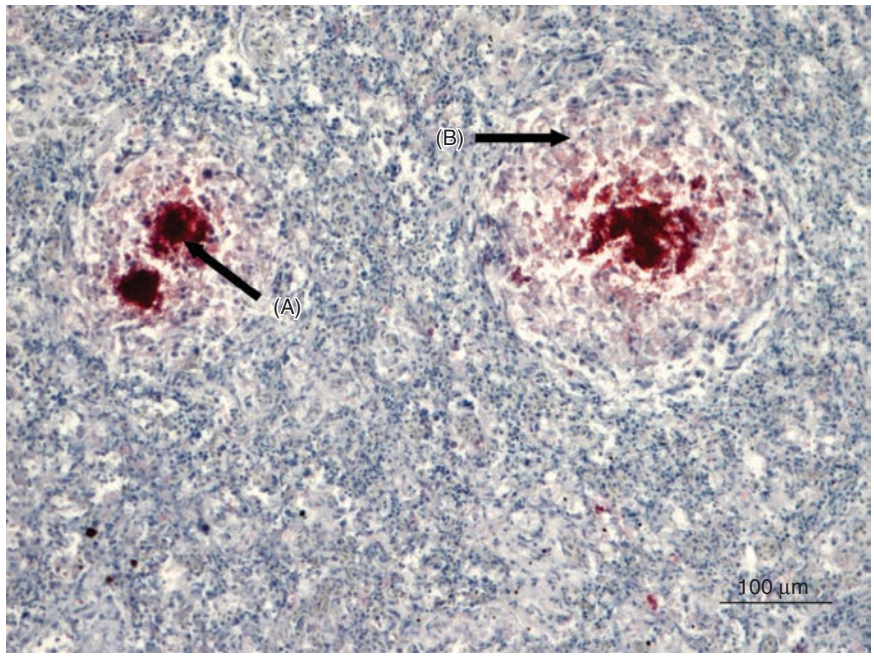

Fig. 14.3. Granulomas in the spleen of Atlantic cod (*Gadus morhua*) with atypical furunculosis immunostained with anti-*Aeromonas salmonicida* subsp. *achromogenes* antibodies. Granuloma centres consist of (A) bacterial colonies and cellular debris that are (B) surrounded by epitheloid cells. Haematoxylin staining with alkaline phosphatase staining of bound antibodies. Photo: Bergljot Magnadottir.

fish may have several types of concealed lesions that may be induced by bacterial toxins (Ventura and Grizzle, 1988).

14.4 Pathophysiology

14.4.1 Pathogenomics

The genomes of a few *Aeromonas* spp. are sequenced and available in the NCBI (US National Center for Biotechnology Information) database. Ten *A. salmonicida* genomes are available. The genome of the non-virulent A449 subspecies *salmonicida* is complete (Reith *et al*., 2008) and consists of a circular chromosome and five plasmids. Uncompleted genomes of five typical strains and four atypical strains are also available, including the *A. salmonicida* subspecies *pectinolytica* (Pavan *et al*., 2013), *masoucida* and *achromogenes* (Han *et al*., 2013), although the strain identified as *achromogenes* may be subsp. *salmonicida* (Colston *et al*., 2014). A microarray-based comparison of *A. salmonicida* virulence genes indicated subspecies variability (Nash *et al*., 2006).

The genome (a single chromosome) of the *A. hydrophila* type strain ATCC 7966^T was described by Seshadri *et al*. (2006). Analysis revealed that the strain has broad metabolic capabilities and numerous putative virulence genes and systems, which allows it to survive in diverse ecosystems. Reith *et al*. (2008) found and published a large number of dysfunctional genes in *A. salmonicida* subsp. *salmonicida* strain A449, which are restricted to this subspecies and include several putative virulence genes. The genome of A449 shows a distinctively high number of insertion sequence (IS) transposases. The majority of these transposases are within *A. salmonicida*-specific genes, suggesting that they may be used to exchange DNA through horizontal transfer or non-reproductive gene transfer (Studer *et al*., 2013). The accumulation of pseudogenes has decreased the ability of *A. salmonicida* subsp. *salmonicida* to produce several organelles and synthesize some metabolic enzymes. These events are key to speciation and reflect its host specificity for salmonids (Reith *et al*., 2008).

14.4.2 Virulence factors

The following is a summary of the virulence factors of *A. salmonicida* and *A. hydrophila*. For greater detail, see Tomás (2012), Beaz-Hidalgo and Figueras (2013) and Dallaire-Dufresne *et al.* (2014).

Surface adhesins

The crystalline surface layer of *Aeromonas* is the outermost cell envelope that is constructed from an auto-assembling single protein. In *A. salmonicida*, it is the A-layer, encoded by *vapA* (Chu *et al.*, 1991), and in *A. hydrophila* it is the S-layer, encoded by *ahsA* (Dooley and Trust, 1988). The A-layer is linked to lipopolysaccharide (LPS) and provides resistance to phagocytosis and protection against complement-mediated lysis; it also facilitates adhesion to host cells and immunoglobulins (Munn *et al.*, 1982; Phipps and Kay, 1988; Garduno *et al.*, 2000). Several other outer membrane proteins (OMPs) have been identified (Ebanks *et al.*, 2005), including complement-activating porin (Yu *et al.*, 2005).

The structure, biosynthesis and genomics of *A. salmonicida* and *A. hydrophila* LPS have been reviewed by Tomás (2012). The O-antigens of *A. salmonicida* are immunologically heterogeneous and the LPS core of each subspecies is unique (Wang *et al.*, 2007; Jimenez *et al.*, 2009). Merino *et al.* (2015) demonstrated that all strains of *A. salmonicida,* shared the same O-antigen biosynthesis gene cluster and that all subspecies, except for *pectinolytica*, produced an A-layer. Motile aeromonads, such as *A. hydrophila*, have a single polar flagellum (Canals *et al.*, 2007), but other strains also express lateral flagella in viscous environments. Both flagellum types are involved in adherence, invasion and biofilm formation (Kirov *et al.*, 2004). Non-motile *A. salmonicida* has disrupted gene sets for both flagella, which explains why they are not functional (Reith *et al.*, 2008). Genes encoding type I and three type IV pili systems are found in typical *A. salmonicida*, though no pili have been detected (Boyd *et al.*, 2008; Reith *et al.*, 2008). Type I pili in *A. salmonicida* aid adherence (Dacanay *et al.*, 2010) and type IV pili contribute to virulence (Masada *et al.*, 2002; Boyd *et al.*, 2008). Type IV pilus systems have also been identified in *A. hydrophila* (Seshadri *et al.*, 2006).

Secreted virulence components

Aeromonas produces and secretes toxins, lipases and peptidases (Pemberton *et al.*, 1997). Although typical and atypical *A. salmonicida* share most of their cell-associated factors, their secreted extracellular virulence factors vary (Gudmundsdottir *et al.*, 2003a; Nash *et al.*, 2006).

A. hydrophila produces both cytotoxic and cytotonic enterotoxins, such as aerolysin, Act, Ast and Alt (Seshadri *et al.*, 2006; Pang *et al.*, 2015). Aerolysin (AerA) in *A. salmonicida* and *A. hydrophila* acts as a pore-forming toxin and imparts the haemolytic and enterotoxic activities of *A. hydrophila* (Chakraborty *et al.*, 1987; Bucker *et al.*, 2011). Knockouts of three enterotoxins, Axt, Alt and Ast, were constructed in a clinical *A. hydrophila* diarrhoeal isolate (SSU) and each contributed to gastroenteritis by evoking fluid secretions in mice (Sha *et al.*, 2002). Several atypical *A. salmonicida* produce an aspzincin metalloendopeptidase (Gudmundsdottir *et al.*, 2003a), AsaP1, which is the major secreted virulence factor of subsp. *achromogenes*. AsaP1 is lethal, but a knockout of the peptidase gene impairs virulence (Arnadottir *et al.*, 2009). The typical *A. salmonicida* strain A449 does not produce a functional AsaP1 (Boyd *et al.*, 2008).

Several types of haemolysins and peptidases are associated with virulence, including a serine peptidase (AspA or P1) which causes muscle liquefaction and contributes to furuncle formation (Fyfe *et al.*, 1987). The peptidase also activates the lipase glycerophospholipid:cholesterol acyltransferase (GCAT). GCAT attacks membrane phospholipids, lyses fish erythrocytes (Ellis, 1991) and is produced by both *A. salmonicida* and *A. hydrophila* (Chacón *et al.*, 2002). Furthermore, GCAT complexes with LPS (GCAT-LPS), which is toxic for salmonids (Lee and Ellis, 1990). However, GCAT-deficient mutants are virulent (Vipond *et al.*, 1998).

Iron acquisition

The genome of *A. salmonicida* subsp. *salmonicida* has two complete gene clusters that encode siderophore synthesis and uptake. One encodes a putative catechol siderophore, anguibactin (or acinetobactin); the other encodes amonabactin, which is found in *A. hydrophila* (Stintzi and Raymond, 2000; Reith *et al.*, 2008). Recently, Balado *et al.* (2015) showed that several strains of subsp. *salmonicida* produce acinetobactin and amonabactin, but that some pathogenic strains only produced acinetobactin. They further concluded that acinetobactin type siderophores were limited to *A. salmonicida*, whereas amonabactin occurred throughout the

genus. So far, only typical *A. salmonicida* produce siderophores but some atypical strains have siderophore-independent systems that sequester iron (Hirst *et al*., 1991; Hirst and Ellis, 1996). Such independent mechanisms involve OMPs or receptors that specifically bind host proteins, such as haem, which then are internalized through a process dependent on TonB, a transport protein that interacts with OMPs. *A. salmonicida* and *A. hydrophila* have haem uptake genes (Ebanks *et al*., 2004; Najimi *et al*., 2008; Reith *et al*., 2008). The expression of many genes involved in iron acquisition, both siderophore dependent and independent, is regulated by the ferric uptake regulator (Fur) iron-binding repressor protein (Braun *et al*., 1998). When iron is limited, *A. salmonicida* exhibits iron-regulated outer membrane proteins, or IROMPs, including siderophores and haem receptors (Ebanks *et al*., 2004; Menanteau-Ledouble *et al*., 2014). Najimi *et al*. (2009) found significant genetic diversity of the iron-regulated genes in *A. salmonicida* subsp. *salmonicida*.

Secretion systems

Gram-negative bacteria have developed at least six secretion systems (T1SS–T6SS) which translocate proteins/virulence factors to host tissues (Costa *et al*., 2015). *A. salmonicida* has T2SS and T3SS. The genes encoding a non-functional T6SS are present in strain A449 (Reith *et al*., 2008; Vanden Bergh and Frey, 2014). The gene sets identified as the secretion system in subsp. *achromogenes* (ATCC 33659) are similar to the gene sets in typical strains (Nash *et al*., 2006). *A. hydrophila* possesses T2SS, T3SS and TSS6, although T3SS is not present in all strains (Seshadri *et al*., 2006; Pang *et al*., 2015).

The T2SS is a two-step system, which secretes toxins and peptidases (Nivaskumar and Francetic, 2014). *Aeromonas* strains utilize the T2SS to secrete a range of factors, including aerolysin and GCAT (Brumlik *et al*., 1997; Schoenhofen *et al*., 1998).

T3SS secretes and translocates effector proteins or toxins in a single step (Portaliou *et al*., 2015). Effector proteins disrupt the actin cytoskeleton, induce apoptosis, and prevent signal transduction and phagocytosis (Sierra *et al*., 2010; Tosi *et al*., 2013). Several *A. salmonicida* T3SS effector proteins have been described (Vanden Bergh and Frey, 2014) which are critical for pathogenicity (Burr *et al*., 2005; Dacanay *et al*., 2006). The genes encoding the T3SS structural and regulatory proteins in *A. salmonicida* are located on a large plasmid (pAsa5), which when incubated above 22°C leads to loss of functional T3SS and avirulence (Daher *et al*., 2011).

T6SS, like T3SS, delivers effector proteins directly into the host cytoplasm or competing bacteria (Cianfanelli *et al*., 2016). *A. hydrophila* possesses T6SS, which induces cytotoxicity and contributes to innate immune evasion (Suarez *et al*., 2010a,b; Sha *et al*., 2013).

Quorum sensing

The quorum sensing systems in *Aeromonas* are homologous to LuxI/R (Jangid *et al*., 2007), which is named AsaI/R and AhyI/R in *A. salmonicida* and *A. hydrophila*, respectively. The main autoinducer produced by both species is butanoyl-l-homoserine lactone, CH_4-AHL (Swift *et al*., 1997; Schwenteit *et al*., 2011; Tan *et al*., 2015). Biofilm development (Lynch *et al*., 2002) and the production of both serine protease and metalloprotease activities (Swift *et al*., 1999) are regulated by quorum sensing in *A. hydrophila*. The production of the AsaP1 toxic metalloprotease, cytotoxicity and brown pigment is regulated by quorum sensing in *A. salmonicida* subsp. *achromogenes* (Schwenteit *et al*., 2011). The virulence of both *A. salmonicida* and *A. hydrophila* to burbot (*Lota lota*) is quorum sensing regulated (Natrah *et al*., 2012).

14.5 Protective and Control Strategies

14.5.1 Sound animal husbandry practices and disinfection

Quality rearing water, the education of personnel, good hygiene and sound animal husbandry limits disease on fish farms. Fish should be inspected and screened regularly for known pathogens. Disinfection is advised within hatcheries using recirculated water. Ozonation, filtration and ultraviolet irradiation are effective against aeromonads (Bullock and Stuckey, 1977; Colberg and Lingg, 1978). Fallowing is effective when tanks and gear can be desiccated. Fish eggs should be disinfected to kill bacteria on egg surfaces (Bergh *et al*., 2001). When an aeromonad outbreak occurs, disinfection procedures may be necessary and isolation may be required before restocking (Torgersen and Hastein, 1995). Mainous *et al*. (2011) found that 1 min of

contact with a number of agents (2% glutaraldehyde; 50 or 70% ethanol; 1% benzyl-4-chlorophenol/phenylphenol; 1% potassium peroxymonosulfate + sodium chloride; 50, 100, 200 or 50,000 mg/l sodium hypochlorite; 1:256 *N*-alkyl dimethyl benzyl ammonium chloride; 50 or 100 mg/l povidone–iodine) were active against *A. salmonicida* and *A. hydrophila* at 5–22°C. Formalin (250 mg/l) was ineffective at these temperatures. Chlorination (0.1 mg Cl_2/l soft water) killed 99.9% of *A. salmonicida* within 30 s, but the Cl_2 concentration needs to be doubled in hard water (Wedermeyer and Nelson, 1977).

14.5.2 Antibiotics and inhibition of bacterial pathogenicity

The control of aeromonad infections relies on the prudent use of effective antibiotics (Cabello, 2006). Requirements vary between countries, and culturists must consult the regulating authority in their country when a treatment is needed. Tetracyclines (oxytetracycline), quinolones (oxolinic acid, flumequine and enrofloxacin) and phenicols (florfenicol) are commonly used. Amoxicillin and sulfonamides may also be administered (Rodgers and Furones, 2009; Cipriano and Austin, 2011).

Resistance to antibiotics drives the need for new drugs. The seaweeds *Gracilaria folifera* and *Sargassum longifolium* have antibacterial activities against *A. salmonicida* (Thanigaivel *et al*., 2015). Aniseed (*Pimpinella anisum*) is antagonistic to *A. salmonicida* and *A. hydrophila* (Parasa *et al*., 2012). The plant *Dorycnium pentaphyllum* produces antibacterial activity against *A. hydrophila,* but does not inhibit *A. salmonicida* (Turker and Yıldırım, 2015).

The inhibition or inactivation of quorum sensing signal molecules, termed quorum quenching, attenuates processes regulated by quorum sensing and can potentially control diseases (Defoirdt *et al*., 2011). *In vitro* studies have shown that quorum quenching can reduce the pathogenicity of *A. salmonicida* and *A. hydrophila* (Swift *et al*., 1999; Rasch *et al*., 2007; Schwenteit *et al*., 2011), but this requires further study.

14.5.3 Non-specific immunostimulation

Probiotics

Probiotics not only improve fish growth and health, but combat aeromonad infections. A fluorescent pseudomonad strain limits the growth of *A. salmonicida* by competing for free iron (Smith and Davey, 1993) and Gram-positive lactobacilli (LAB) reduce the mortality of rainbow trout (*Oncorhynchus mykiss*) challenged with *A. salmonicida* subsp. *salmonicida* (Nikoskelainen *et al*., 2001). *A. hydrophila, Carnobacterium* sp., *Vibrio fluvialis* and an unidentified bacterium in the intestines of salmonids and turbot are antagonistic to *A. salmonicida* infections of rainbow trout (Irianto and Austin, 2002), and feeding rainbow trout with dead probiotic cells restricted the development of furunculosis (Irianto and Austin, 2003). Probiotic *V. alginolyticus* may induce resistance to furunculosis in Atlantic salmon (*Salmo salar*) (Austin *et al*., 1995).

Vine *et al*. (2004) used the intestinal microbiota of clownfish (*Amphiprion percula*) to combat *A. hydrophila*. Feeding grass carp (*Ctenopharyngodon idellus*) for 28 days with probiotics based on *Shewanella xiamenensis* and *Aeromonas veronii*, reduced mortality following infection with *A. hydrophila* (Wu *et al*., 2015). Cellular products from *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Lactobacillus plantarum* also enhanced protection against *A. hydrophila* in rohu (*Labeo rohita*) (Giri *et al*., 2015b).

Prebiotics and other immunostimulants

Feeding rainbow trout with mannanoligosaccharide (MOS)-enriched feed (2.5 g MOS/kg) improved growth and enhanced protection against *A. salmonicida* (Rodriguez-Estrada *et al*., 2013), and feed enriched with beta-glucans can significantly enhance acute phase responses during *A. salmonicida* infection (Pionnier *et al*., 2013).

Yersinia ruckeri flagellin has been used as a non-adjuvanted subunit vaccine that evoked non-specific immunoprotection in rainbow trout against *A. salmonicida* (Scott *et al*., 2013). Immunostimulants of herbal origin have also gained increased attention. Ashagwanda (Indian ginseng, *Withania somnifera*) possesses several medicinal properties (Mirjalili *et al*., 2009) and rohu resisted *A. hydrophila* infection when fed with root powder of ashagwanda (Sharma *et al*., 2010). Feed containing garlic extract (0.5 or 1.0%) increased the resistance of rainbow trout to *A. salmonicida* infection, but higher doses were not effective and became detrimental to fish health (Breyer *et al*., 2015). Feeding rohu with guava (*Psidium guajava*) leaves promoted growth and increased protection against *A. hydrophila* (Giri *et al*., 2015a).

Heat shock protein (Hsp)-enriched bacteria may control diseases in aquaculture (Sung, 2014). Enhancing the Hsp70 response by repeated handling during winter increased the resistance of gibel carp (*Carassius auratus gibelio*) and channel catfish to *A. hydrophila* (Yang *et al*., 2015). Expression analysis of the genes encoding seven Hsp proteins during *A. hydrophila* infection in rohu has been reported (Das *et al*., 2015); Hsp70 was downregulated, but HspA5 was upregulated in the liver, spleen and anterior kidney, indicating its potential immune regulatory role. HspA8 and Hsp70 may also mediate immune responses against *A. hydrophila* in Prenant's schizothoracin (*Schizothorax prenanti*) (Li *et al*., 2015).

14.5.4 Vaccination

Vaccination has controlled furunculosis since the 1990s when bacterins of *A. salmonicida* subsp. *salmonicida* emulsified in oil adjuvant became commercially available. Currently, commercial vaccines contain inactivated bacterins and are most frequently licensed for salmonids. Parenteral furunculosis vaccines are used to vaccinate salmonids, turbot, koi carp (*Cyprinus carpio*) and several other species against typical and atypical furunculosis; they have varying success owing to the antigenic diversity among atypical *A. salmonicida* and differences in the immune responses of fishes. In some regions (e.g. Norway, the Faroe Islands and Iceland) vaccines against atypical furunculosis of salmonids and marine fishes, including the lumpsucker, are commercially available (Hastein *et al*., 2005; Gudmundsdottir and Bjornsdottir, 2007; Coscelli *et al*., 2015; see http://www.pharmaq.no/). Atlantic cod cannot be effectively vaccinated against pathogens with protective antigens that are T-cell dependent, and the lack of effective vaccines to protect cod against atypical furunculosis limits cod farming (FAO, 2014; Magnadottir, 2014).

There is significant correlation between circulating *A. salmonicida* antibodies and protection in Atlantic salmon (Romstad *et al*., 2013), rainbow trout (Villumsen *et al*., 2012), Arctic charr (Schwenteit *et al*., 2014), Atlantic halibut (Gudmundsdottir *et al*., 2003b), spotted wolffish (Ingilae *et al*., 2000) and turbot (Bjornsdottir *et al*., 2005; Coscelli *et al*., 2015). Long-lasting protection against waterborne *A. salmonicida* in rainbow trout was induced by bath vaccination in a formalin-inactivated bacterin (Villumsen and Raida, 2013) and by intraperitoneal (IP) injection with bacterins emulsified in an oil adjuvant (Villumsen *et al*., 2015). No elevation of circulating antibodies was detected among the immersion-vaccinated trout, but antibody levels correlated with protection in the IP-vaccinated fish.

The heterogeneity of *A. hydrophila* complicates the development of effective vaccines. There is no commercial vaccine, and current vaccines involve autogeneous preparations that are licensed within a restricted region (Yanong, 2008/9, rev. 2014). Vaccines based on native and recombinantly produced *A. hydrophila* proteins have induced immunoprotection. These include: an adhesin (Fang *et al*., 2004); LPS (Sun *et al*., 2012); OMPs (Khushiramani *et al*., 2012; Maiti *et al*., 2012; Sharma and Dixit, 2015); and haemolysin co-regulated protein (Hcp) (Wang *et al*., 2015).

Vaccines based on live attenuated *A. hydrophila* have been constructed and evoke good protection in homologous challenges (Liu and Bi, 2007; Pridgeon *et al*., 2013). Furthermore, the delivery of *A. hydrophila* antigens by live bacterial carriers (attenuated *Vibrio anguillarum*) has produced favourable vaccination results (Zhao *et al*., 2011). Tu *et al*. (2010) used empty cell envelopes (ghosts) of *A. hydrophila* as well as a vaccine based on killed bacteria to orally vaccinate carp. The protective mucosal and systemic immune responses evoked by the ghosts were superior.

14.6 Conclusions and Suggestions for Future Research

Currently, around 350 species of finfish are farmed worldwide (FAO, 2014) and many are susceptible to aeromonad infections. The diversity of aeromonads in habitat, host susceptibility and pathogenic properties is considerable. Environmentally acceptable measures to control diseases are required for the continuous development of sustainable aquaculture, to prevent the transmission of diseases between wild and farmed fish, and to prevent the introduction of disease to new areas. In addition, the zoonotic spread of motile aeromonads and distribution of antibiotic resistance are major concerns.

The diagnosis of infection relies on accurate bacterial identification, but the taxonomy and identification of atypical *A. salmonicida* and motile aeromonads are uncertain and need clarification. Therefore, rapid and reliable methods to correctly

identify and classify these bacteria are important for disease diagnosis and for epidemiological and prophylactic purposes. More whole genome sequencing is needed to establish multinational methods for the standardization of taxonomic and diagnostic tools. Furthermore, information on natural habitats of *A. salmonicida* is needed to reveal whether it has important reservoirs other than fishes.

Multidisciplinary measures are necessary to increase the prevention and control of disease caused by aeromonads. There still remain considerable gaps in understanding their pathogenicity, but it is hoped that the fast-growing knowledge of the genome sequences of pathogens and hosts, gene functions and their interactions will help to bridge these gaps.

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