4 Infectious Diseases of Coldwater Fish in Fresh Water

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Introduction

Raising fish in fresh water is an ancient practice and the earliest records of aquaculture date from 2000 BC in China, although these relate to aquaculture in fresh warm water (Brown, 1977). The rearing of animals in a cold freshwater environment is a relatively recent phenomenon and dates from the 1930s when trout were first raised in ponds in Denmark (Shepherd, 1988). Since then, coldwater aquaculture has grown exponentially and in 1996 the global cold freshwater aquaculture production including trout, salmon, eels and sturgeon was in excess of 1.5 Mt (New, 1999).

In addition to fish that are cultured exclusively in fresh water, juvenile salmonids are raised in a freshwater environment prior to smoltification and transfer to seawater. Research was first conducted on salmonid rearing and salmonid disease in enhancement hatcheries, and the technology and information accrued were later transferred to the commercial cage culture industry.

For the purposes of this chapter, fresh water refers to zero salinity, and diseases in estuarine environments are included in Chapter 3. Freshwater fish are reared in water that originates from streams or rivers, or in groundwater, and maintained in flow-through or recirculation facilities. The book concerns diseases of finfish and we shall examine those diseases that have relevance to cage and tank culture. Diseases specific to channel or earthen pond culture will not be discussed.

To avoid excessive repetition of information given elsewhere, we have defined infectious diseases of cold fresh water as those that rarely, if ever, occur in water whose temperature exceeds 15°C. The majority of infectious diseases discussed are those that are normally associated with the dominant species cultured in cold fresh water: trout and juvenile salmonids. Many pathogens have been isolated in fish cultured both in seawater and fresh water and for some diseases it was decided that most cases are seen in fresh water and thus are in this chapter. Furunculosis, caused by *Aeromonas salmonicida*, is an example, as is viral haemorrhagic septicaemia. There are other examples where the majority of cases are seen in seawater and we have merely noted the importance of the disease in this chapter. In these cases the reader is referred to the relevant seawater chapter. In cases where the disease may occur equally in fresh or seawater, the pathogen is discussed in this chapter when transmission of the agent in fresh water either horizontally or vertically is the most important aspect. Bacterial

 Diseases and Disorders of Finfish in Cage Culture (eds P.T.K. Woo, D.W. Bruno and L.H.S. Lim)

kidney disease, caused by *Renibacterium salmoninarum*, is an example.

Diseases Caused by Viral Pathogens

Viral haemorrhagic septicaemia

Introduction

Viral haemorrhagic septicaemia (VHS) is the most serious, systemic, contagious disease of farmed rainbow trout (*Oncorhynchus mykiss*) in Europe. The aetiological agent, viral haemorrhagic septicaemia virus (VHSV), trout plague or Egtved virus is an enveloped RNA rhabdovirus (Jensen, 1963; Zwillenberg *et al*., 1965). Significant and recurring losses in infected fry are reported, although surviving fish are usually immune to reinfection. Disease signs are variable, but an acute to chronic profuse internal haemorrhaging and pale viscera are typical.

Characterization

VHSV is a member of the newly accepted genus *Novirhabdovirus* (cited in Olesen *et al*., 1999) of the family *Rhabdoviridae*. In Europe five serotypes of VHSV have been described using infectivity neutralization assays (de Kinkelin, 1988). Type 1 (strain F1 from Denmark and the most common), type 2 (Heddedam strain, also from Denmark), type 3 (French strain 23/75) and type 4 represented by the strain 02-84. Both type 3 and 4 were isolated from brown trout (*Salmo trutta*) (de Kinkelin and Le Berre, 1977; Le Berre *et al*., 1977; Castric *et al*., 1992). The North American strains of VHSV are genetically different from Egtved virus. An attempt to define serogroups that avoided the overlap of strains within and between these serotypes was undertaken by Olesen *et al*. (1993).

Impact

VHS still remains a serious threat to fish culture in Europe and a disease notifiable to the Office International des Épizooties (OIE). In Denmark, for example, losses in the range of £5–7 million (Sterling) were estimated for 1992–1993. Currently, annual losses are estimated to be £1.5–2.0 million (N.J. Olesen, 1997, personal communication). In Italy, losses attributed to VHS in coldwater areas have also declined over the last 10 years (G. Bovo, 1997, personal communication). In both countries eradication programmes and improved management are contributing factors to the decline of VHSV.

In general, rainbow trout are most susceptible, followed by northern pike (*Esox lucius*) (Meier and Vestergård-Jørgensen, 1980). Natural outbreaks occur in grayling (*Thymallus thymallus*) and whitefish (*Coregonus* spp.). Freshwater species reported as susceptible to VHSV are summarized in Table 4.1. The most

Table 4.1. Susceptible freshwater fish hosts of viral haemorrhagic septicaemia virus (VHSV).

Host/species	Specific name	Reference
Atlantic salmon	*Salmo salar*	Rasmussen (1965)
Brook trout	*Salvelinus fontinalis*	Rasmussen (1965)
Brown trout	*Salmo trutta*	Ghittino (1968)
Chinook salmon	*Oncorhynchus tshawytscha*	Winton *et al*. (1989)
Coho salmon	*Oncorhynchus kisutch*	Winton *et al*. (1989)
Golden trout	*Salmo aguabonita*	Ahne *et al*. (1976)
Grayling	*Thymallus thymallus*	Wizigmann *et al*. (1980)
Hybrid rainbow trout × coho salmon	*O. mykiss* × *O. kisutch*	Chevassus and Dorson (1990)
Lake trout	*Salvelinus namaycush*	Ghittino (1973)
Pike	*Esox lucius*	Meier and Vestergård-Jørgensen (1980)
Rainbow trout	*Oncorhychus mykiss*	Jensen (1963)
Whitefish	*Coregonus* sp.	Ahne and Thomsen (1985)

pathogenic strain of VHSV is type 1, with outbreaks in freshwater fish occurring at 14°C or below (Meier and Vestergård-Jørgensen, 1979; Meier *et al.*, 1986).

Geographical distribution

VHS was first recorded in the 1930s and is now present in farmed trout throughout most of Western Europe (Wolf, 1988). Outbreaks of VHS have been reported in farmed turbot in France and Germany (Schlotfeldt *et al.*, 1991), Scotland (Ross *et al.*, 1994) and Ireland (J. McArdle, 1997, personal communication). The isolation of VHSV from returning adult chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) in the Puget Sound area and Gulf of Alaska, North America (Eaton and Hulett, 1990), was the first indication there was a marine source of VHSV, possibly involving one or more fish species. Currently VHSV has been isolated from an increasing number of marine fish species (Meyers *et al.*, 1992; Ross *et al.*, 1994; Meyers and Winton, 1995; Dixon *et al.*, 1997). The use of molecular techniques including T1 nuclease fingerprinting and sequencing analyses (Oshima *et al.*, 1993; Stone *et al.*, 1997; Mortensen *et al.*, 1999; Smail, 1995; 2000) has confirmed that these North American isolates are distinct from those found in Europe. The significance of these findings to farmed salmonids and European Union (EU) fish health legislation (Directive 91/67/EEC) is reviewed in Chapter 3.

Clinical signs

VHS occurs as an acute to chronic disease. The acute phase is associated with a rapid onset of heavy mortality. Fish are lethargic, anaemic, show dark body colour and exophthalmos, and occasionally an intermittent period of erratic spiralling. Haemorrhaging may be evident in the ocular tissues, skin and the viscera, including the skeletal muscle and intestinal submucosa. Severe glomerular changes with focal necrosis and leucocytic infiltration have been recorded. The liver sinusoids become engorged with blood, with an extensive necrosis and increased pyknotic and karyolytic nuclei. Ascites is present and food is absent from the gastrointestinal tract. Clinical signs in pike fry are generally similar to those described in trout, with the exception of extravasation or bloody swelling, deposition of blood in the muscle and pancreatic necrosis (Meier and Vestergård-Jørgensen, 1980). It is possible that these signs represent a later stage of the infection.

The chronic phase is correlated with lethargy, dark skin colour and bilateral exophthalmia. A haemorrhagic anaemia is reported with a markedly distended abdomen due to oedema in the liver, spleen and kidney. In chronically infected fish the liver sinusoids remain congested, with some hyperplasia of the haematopoietic tissue.

During the latent infection, or nervous phase, there is low mortality and some fish are hyperactive, but otherwise they appear normal.

Diagnostic techniques

Cell culture. A number of serological techniques are used to tentatively identify VHSV; however, definitive diagnosis requires culture of the virus in an established susceptible cell line. A variety of established fish cell lines are susceptible to VHSV and the most sensitive for freshwater isolates from rainbow trout are bluegill fry (BF-2) (Olesen and Vestergård-Jørgensen, 1992). Other cell lines suitable for VHSV detection include chinook salmon embryo (CHSE-214), epithelioma papulosum cyprini (EPC), fathead minnow (FHM), pike gonad (PG) and rainbow trout gonad (RTG-2) (Wolf, 1988; Olesen and Vestergård-Jørgensen, 1992). Biopsy samples or sex products are homogenized, diluted and centrifuged to remove cell debris, filtered and the material added to a growth medium in flasks containing a tissue culture cell monolayer. The cytopathic effect (CPE) results in pronounced cell shrinkage with some cell rounding and in RTG-2 cells staggered edge plaques. When a viral (CPE) is recognized, the supernatant virus is harvested and identified using a serum neutralization test and a susceptible cell line.

Enzyme-linked immunosorbent assay (ELISA). An ELISA is an accepted technique for the detection of virus in culture supernatant. Way and Dixon (1988) described a polyclonal direct antigen-capture system for VHSV. Further development by Mourton *et al.* (1990) resulted in three forms of the ELISA for virus detection: an indirect ELISA, a direct ELISA and an antigen-capture ELISA that used a variety of monoclonal antibodies to the viral glycoprotein G. Using a highly specific double sandwich protocol, they were able to detect VHSV to a sensitivity of $TCID_{50} = 10^4\ g^{-1}$ tissue (Mourton *et al.*, 1990). A comparative study of tissue culture and immunohistochemistry was reported by Evensen *et al.* (1994), who noted that tissue culture was more sensitive in rainbow trout-infected tissues. When the former was compared with PCR, tissue culture was more sensitive in most rainbow trout carriers (Bruchof *et al.*, 1995).

Virus gene probes. The nucleotide sequences of the nucleoprotein (N) gene from the Pacific Makah strain of VHSV and the virulent French 07-71 strain were compared by Bernard *et al.* (1992). Two strains showed differences of around 13% in sequence homology and enabled Batts *et al.* (1993) to design three cDNA probes. The first probe, universal to all VHSV isolates, was 29 nucleotides long and synthesized to the messenger RNA (mRNA) sequence from nucleotides 430–458 in the open reading frame (ORF) of the *N* gene. A second probe, specific to the Makah strain, was synthesized to a unique 28 base sequence that occurred after the true coding sequence in the *N* gene of the Makah strain, but not in the same region of the 07-71 strain. The third probe, specific to the 07-71 strain, was made to a 22 nucleotide sequence within the *N* gene, where six mismatches occurred with the Makah strain. Each probe was labelled with three biotin molecules at the 5′ end of the primer.

Monoclonal antibody (mAb) capture and polymerase chain reaction (PCR). A highly sensitive and specific mAb capture assay combined with PCR detection of VHSV, using sense primers to the glycoprotein (G) gene in a defined region of 379 base pairs (amino acids 64–195), was reported by Estepa *et al.* (1995). This method gave virus-specific gel electrophoresis products for VHSV with *N* and *G* gene primers.

The identification of a reverse transcriptase-dependent polymerase chain reaction (RT–PCR) specific for the detection of VHSV sequence data and differentiation of serologically similar strains was made by Bruchof *et al.* (1995) and Einer-Jensen *et al.* (1995). The latter group used two primers that amplified sequences from the *N* gene of European and Makah strains of VHSV. An additional primer, which amplified only the American strains, revealed a unique non-coding intron of 20 nucleotides near to the *N* gene. Gene probe work established that the Makah and European strain of VHSV formed two genetically distinct groups and that American and European strains of VHSV could be distinguished using this methodology.

Methods of prevention and control

Legislation introduced within the EU is designed to prevent the movement of potentially infected VHS fish to areas free of this disease. Control and prevention by avoidance and keeping stress to a minimum remain the most effective means of control. In Brittany, parts of Denmark and Italy, eradication of VHSV has been successful using systematic programmes of hatchery disinfection with chlorine, formalin, hypochlorite and iodophore, quarantine measures and restocking with specific virus-free fish and eggs (Enzmann, 1983). The use of ultraviolet (UV) radiation to inactivate virus in the inflowing water is also a practical method for VHSV control; a dose of $1–3 \times 10^3\ \mu W\ s^{-1}\ cm^{-2}$ is required to inactivate VHSV (Yoshimizu *et al.*, 1986).

Host factors in genetic control. Triploid hybrids produced using heat shock of either male coho salmon or brook trout (*Salvelinus fontinalis*) × rainbow trout females are less susceptible to VHSV than either of the

diploid or triploid rainbow trout (Dorson and Chevassus, 1985; Dorson *et al.*, 1994). This approach shows that host susceptibility of rainbow trout to VHSV has a genetic basis, and this manifests in the uptake of virus and the rate of virus multiplication in the target organs.

Killed vaccine. The testing of a killed vaccine was evaluated by de Kinkelin (1988). β-Propriolactone and formalin at a dilution of 1/5000 and 1/2000, respectively, inactivated strain 07-71. At 10°C intraperitoneal injection was essential to promote an immune response. The range of protection in 5 g fish lasted between 30 and 100 days at 10°C or below. Cross-protection between three serotypes was shown and a high titre of virus-neutralizing antibodies in the sera of vaccinated fish reported.

Live vaccines. The use of a live VHSV vaccine was suggested by Jørgensen (1976). Later, Vestergård-Jørgensen (1982) attenuated the Reva strain (related to the F1 reference strain) through 240 successive subcultures in RTG-2 cells at 14°C and reported a genetically stable strain after 20 backpassages in rainbow trout fry. Fry immersed in 10^4 plaque-forming units (p.f.u.) ml^{-1} water for 1 h below 10°C showed a protective effect up to 150 days after vaccination.

An attenuated F1-related strain obtained by subculture at 25°C in EPC cells was produced and called the F25 strain. Immunization with F25 resulted in an average increase of 30% survival compared with non-immunized fry. Later work showed that a neutralizing antibody response was made to the immunizing variant virus; however, there was no protection against the wild-type virulent virus (de Kinkelin and Bearzotti, 1981).

Recombinant protein vaccines. Advances towards the development of subunit vaccines against VHSV have been described (Lorenzen *et al.*, 1993; Heppell *et al.*, 1998), following the cloning and sequencing of the gene encoding the G of a Danish isolate of VHSV (Benmansour *et al.*, 1997). The major part of this protein, without the leader segment, was expressed in *Escherichia coli* as a protease-cleavage fusion protein. When this protein was renatured and purified, it could stimulate VHSV-specific antibodies when injected into rainbow trout. This provides a method of mass producing viral antigens, and if additional tests are successful, a commercial VHSV vaccine may become possible.

The development of a recombinant vaccine by expressing a glycoprotein in insect cells, using a baculovirus vector, has been examined (Lecocq-Xhonneux *et al.*, 1994). The baculovirus-encoded protein was shown to induce the synthesis of virus-neutralizing antibodies in trout, but stimulates only moderate protection from viral challenge when injected intraperitoneally. A lack of protection after immersion vaccination may limit this approach as a vaccine.

Progress with vaccination. The history of VHS vaccination features a 20 year period of research into the testing of candidate strains, either killed or attenuated (de Kinkelin, 1988). Research is underway to understand more of the disease interactions and the susceptibility of new species, and to determine if the protection from DNA vaccines for VHSV correlates with the presence of neutralizing antibodies.

Infectious pancreatic necrosis

Introduction

Infectious pancreatic necrosis (IPN) is a contagious, economically important viral disease of farmed salmonids, especially juvenile fish. Infectious pancreatic necrosis virus (IPNV) is widespread and occurs in most areas where these fish are cultured. Diseased fish are characterized by acute catarrhal enteritis and are generally dark, anorexic and emaciated. IPNV is a member of the *Birnaviridae*, and particularly affects rainbow trout and brook trout in fresh water. However, this virus has also been isolated from a wide range of fish species in

brackish water and seawater, in particular salmon post smolts.

Characterization

IPNV is assigned to the family *Birnaviridae*, which are naked icosahedral viruses. IPNV, like other birnaviruses, has a bi-segmented, double-stranded RNA genome (Dobos and Roberts, 1983). Several serotypes are known to be pathogenic to fish (reviewed by Hill and Way, 1995). The genome segment A contains two overlapping ORFs that encode a 106 kDa polyprotein (NH_2–pVP2–NS protease–VP3–COOH), which is cotranslationally cleaved by the protease to generate the major capsid proteins VP2 and VP3. The second ORF overlaps the amino end of the large ORF but in a different reading frame, and encodes a 17 kDa arginine-rich minor polypeptide (Dobos, 1995). Analyses of viral proteins showed that VP2, a capsid protein, is the major structural and immunogenic polypeptide of the virus (Christie *et al.*, 1990; Dobos, 1995).

Impact

IPNV is a disease of fry and fingerlings, with brook and rainbow trout being highly susceptible. Although they can be infected, brown trout, lake trout (*Salvelinus namaycush*), coho and Atlantic salmon (*Salmo salar*) are less likely to develop clinical disease. IPNV also infects non-salmonid species including eels, molluscs and crustaceans, all of which may act as carriers. Mortality and morbidity among marine-reared Atlantic salmon with IPNV is of increasing concern and this is discussed in Chapter 9. Reports of IPNV in *Salmo*, *Oncorhynchus* and *Salvelinus* spp. have been summarized (Reno, 1999).

Infection of susceptible species in fresh water can result in high mortality. It is estimated that over the period 1994–1997, this disease was responsible for fry, parr and presmolt losses of approximately 20 million NKr per annum (T. Poppe, personal communication, 1999). Virus-associated mortality is rapid between 10 and 14°C, and at lower temperatures is prolonged. Water temperature, fish age and the virus strain affect the severity of the disease, as well as the establishment of covert infections in fish. IPNV replicates in kidney, pancreas, gonad, spleen and intestinal epithelium and may be shed from carrier fish via faeces, as well as through the seminal and ovarian fluids. Virus shedding appears to be cyclic and dependent on temperature and/or other environmental stresses. Although stress may induce a recurrence of IPN in fish 6–11 months old, many older fish show no clinical signs.

The apparent increased incidence of IPN is considered to be the result of widespread fish and egg movements between countries, and because of increased sensitivity of diagnostic methods, resulting in improved surveillance practices. It is possible that other commercially important fish species may be infected (Reno, 1999).

IPNV is transmitted horizontally and vertically. However, vertical transmission has been confirmed only in brook trout (Wolf, 1988). The exact mechanism of egg entry or location of the virus within the egg is still unclear. Bebak *et al.* (1998) infected rainbow trout with IPNV by immersion challenge. The fish started to excrete the virus within 2 days of infection and shedding increased and then declined in less than 12 days post-exposure. From this study it was estimated that within 14 days, more than 75% of the population can be infected (Bebak *et al.*, 1998). This gives rise to a rapid spread of IPNV.

Ethanol, methanol, iodophore and chlorine inactivate IPAV (Inouye *et al.*, 1990), but it retains more than 90% of its infectivity after treatment with chloroform or ethyl ether, at pH 3.0 for 60 min. IPNV has been shown to be infective for several years at –70°C and for several months at 4°C. Smail *et al.* (1993) found that IPNV was not deactivated by an acidic pH unless the sample (silage) was heated for at least 2 h at 60°C. This confirms that IPNV is a robust virus with long survival in the environment.

Clinical signs

IPN in young fish is characterized by a sudden onset of increasing mortality. Affected

fish become dark and rotate their bodies while swimming. The fish show a distended abdomen, exophthalmos and some petechial haemorrhaging on the ventral surface, particularly at the base of the fins. Internally, petechiae, pale liver and spleen are noted with evidence of anaemia confirmed by a reduction in haematocrit. The presence of a gelatinous material in the stomach and anterior intestine and the production of mucoid faecal casts are common.

Histologically, the principal lesions are focal or coagulative necrosis of the pancreatic acinar tissue with acute enteritis, necrosis and sloughing of the intestinal epithelium. Eventually the whole pancreas may be destroyed. Some affected acinar cells show pyknosis and basophilic inclusions and are recognized as products of cell breakdown (Hong *et al.*, 1998). This pathology may extend into the gut mucosa and the renal haematopoietic elements. Necrotic areas may be replaced with fibrous/fatty tissue, and cell sloughing in combination with increased mucous results in a pink to white exudate in the lumen. Hyaline degeneration of skeletal muscle has also been reported. Survivors of an outbreak may exhibit fibrous replacement of the exocrine pancreas. In moribund fish, necrosis of the renal tubules, haematopoietic tissue and liver has been recorded.

Diagnostic techniques

The diagnosis is based on clinical signs, histological examination of tissues and isolation and identification of the virus in kidney and other tissues using cell culture. Established cell lines for IPNV isolation include CHSE-214 (Lannan *et al.*, 1984), BF-2 (Wolf and Quimby, 1966) and RTG-2 (Wolf and Quimby, 1962). *In vivo* it is postulated that the virus causes CHSE cells to undergo apoptosis, followed by post-apoptotic necrosis (Hong *et al.*, 1998). Virus identification can also be accomplished using a staphylococcal co-agglutination (COA) test (Taksdal and Thorud, 1999), and a variety of immunodiagnostic techniques including serum neutralization (Ishiguro *et al.*, 1984), Western blotting (Williams *et al.*, 1994), immunoperoxidase (Nicholson and Henchal, 1994), immunofluorescence (Swanson and Gillespie, 1981), immunohistochemistry (Evensen and Rimstad, 1990) and immunodot (Kirsinger *et al.*, 1999) using polyclonal and monoclonal IPNV antisera. The detection of IPNV by hybridization, using either oligonucleotide DNA probes or cloned cDNA probes (Dopazo *et al.*, 1994), has been reported, although cell culture is considered a sensitive method.

Prevention

There is no effective therapeutic for the control of IPNV. Vertical transmission of the virus can be prevented or at least reduced through the testing of broodstock. This is an important procedure to limit the spread of IPNV, and the use of virus-free stock remains an essential means of eliminating or reducing IPNV. In some cases, increasing water temperature has been recommended to reduce mortality in fresh water.

Immunity against IPNV can be transferred with serum in rainbow trout (Agneil, 1975), establishing a humoral response as an important factor in protecting against IPNV. Some success with the cloning, characterization and expression in insect cells of virus-like-particles (VLPs) of IPNV has been reported (Magyar and Dobos, 1994). Such VLPs are formed by the self-assembly of the coat proteins of a virus and are ideal candidates for vaccines (referred to as subunit vaccines) because the proteins of VLPs retain the characteristic antigenicity of the live virus. However, since they consist only of the virus's protein coat they are non-pathogenic. The virulence associated with IPNV comes from gene segment A, and portions of this segment have been cloned, and recombinant VP2 (rVP2), with structures resembling these epitopes, has been a candidate vaccine; a commercial injectable IPNV vaccine is now licensed in Norway (Frost and Ness, 1997). When salmon were immunized with rVP2, a strong humoral immune response against rVP2 and a moderate response against IPNV was recorded (Frost *et al.*, 1998). The pooled antisera from

salmon challenged with IPNV neutralized the virus, demonstrating that purified *E. coli*-expressed rVP2 of IPNV induces production of specific antibodies in salmon.

Future studies

Recombinant DNA vaccines are likely to offer long-term immunity, but have to be reliable, safe and acceptable to the regulatory authorities. The development of a subunit vaccine for IPNV, combined with studies on its potential to confer protection against vertical transmission of the virus, will make important contributions to fish health management

Sleeping disease of rainbow trout

Introduction

Sleeping disease (SD) is an infectious viral condition that has been described in farmed rainbow trout and occasionally in coho salmon (Boucher and Baudin Laurencin, 1994, 1996). The first record was in Italy (Ghittino, 1987), but an occurrence in Brittany, France, a few years later prompted more detailed observations. The disease has recently been confirmed in farmed trout in Scotland using light microscopy and PCR (Bruno, 2002, unpublished observations). SD is characterized by the unusual behaviour of affected fish, which remain on their sides at the tank bottom. A toga-like virus has been isolated from infected rainbow trout (Boucher *et al.*, 1994; Castric *et al.*, 1997) and the name sleeping disease virus (SDV) has been proposed (Castric *et al.*, 1997).

Characterization

Stained viral suspension has revealed intact enveloped particles with an external diameter between 55 and 65 nm (Castric *et al.*, 1997). Using cDNA cloning and nucleotide sequencing of part of the SDV RNA genome it has been possible to assign it to the genus *Alphavirus* (family *Togaviridae*) (Villoing *et al.*, 2000a).

Impact

Boucher and Baudin Laurencin (1994) reported that SD affected 15% of the freshwater rainbow trout in cage culture farms in Brittany, France, although mortality was low. Infected fish may stop feeding for several weeks, resulting in a loss of growth.

Farmed rainbow trout and occasionally coho salmon reared in fresh water are the only known species naturally affected by SD (Boucher and Baudin Laurencin, 1994, 1996), although experimental infection in Atlantic salmon has been successful (Boucher and Baudin Laurencin, 1994). Stress induced by *Gyrodactylus* spp. feeding on the fish was proposed as a contributing factor to SD (Ghittino, 1987). The similarity between SDV and salmon pancreas disease virus (SPDV) and the subsequent pathology (Nelson *et al.*, 1995; McLoughlin *et al.*, 1996) in marine-reared Atlantic salmon has promoted the idea that these agents might be similar (Boucher and Baudin Laurencin, 1996). Moreover, an acquired cross-protection against SDV and SPDV in laboratory studies supports this hypothesis (Boucher and Baudin Laurencin, 1996). At present, SD has been described only in France (Boucher and Baudin Laurencin, 1994) and Italy (Ghittino, 1987). A review of SPDV is given in Chapter 3.

Clinical signs

Clinical signs include fish lying or resting on the bottom of the tank or raceway. When disturbed the fish swim for short periods and then return to the tank bottom. In experimental studies, necrosis of the exocrine pancreas occurred in the first week after injection. Tissue changes included a rounding of acinar cells, and later lymphocyte and fibrocyte infiltration. Focal heart lesions can develop and these are characterized by hyalinization and loss of striation. Within the red muscle an increase in cellularity and disappearance of fibres have been recorded (Boucher and Baudin Laurencin, 1996). The histopathology observed in experimental and natural outbreaks of SD is similar (Boucher and Baudin Laurencin, 1994).

Diagnostic techniques

Provisional diagnosis is based on the characteristic behaviour of the fish and associated pathology. Recently, a virus responsible for causing SD has been isolated on a CHSE-214 cell line (Castric *et al.*, 1997). Kidney homogenate in Glasgow minimal essential medium (GMEM) inoculated on to cell lines with fetal calf serum and antibiotics showed small groups of refringent round cells after three passages. The development of a sensitive RT–PCR-based method for the diagnosis of SD has recently been reported (Villoing *et al.*, 2000b).

Prevention

No treatment for SD is available. Boucher and Baudin Laurencin (1996) have shown cross-protection between SD and SPDV, and this may allow young fish to be exposed to SD with the benefit of protection against SPDV in seawater.

Future studies

Comparative studies of the viruses causing SD and SPD in fish will be valuable and help in our understanding of these conditions. Furthermore, long-term protection recorded in salmon parr against SPDV following transfer to seawater (Houghton, 1994) may also be similar for SD in fresh water. The cross-protection between SDV and SPDV may allow young fish to be exposed to SD while protecting against SPDV.

Infectious haematopoietic necrosis

Introduction

Infectious haematopoietic necrosis (IHN) is an acute systemic rhabdovirus infection. IHN occurs primarily among cage-cultured Pacific salmonids in fresh water from coastal North America, although outbreaks in wild stocks have been documented. The first reports of serious epizootics attributed to IHN were made in the late 1940s and 1950s (Rucker *et al.*, 1953). Losses of up to 100% have been reported (Wolf, 1988).

Characterization

The causative agent of IHN was first isolated from sockeye salmon (*Oncorhynchus nerka*) and subsequently from rainbow trout (Parisot *et al.*, 1965). IHN virus (IHNV) was isolated from salmonids in Japan (Sano *et al.*, 1977), Italy, France (Baudin-Laurencin, 1987; Bovo *et al.*, 1987) and in other parts of Europe, evidently spread through fish transportation (Miller *et al.*, 1998). However, it is possible that the virus was already present in these countries and detected because of improvements in methodology. IHNV shares the bullet shape of other members of this group (McCain, 1970). IHNV has an unsegmented, negative-sense, single-stranded RNA genome of approximately 11,130 nucleotides. These encode a nucleoprotein, a phosphoprotein, a matrix protein, a glycoprotein, a non-virion protein and a polymerase (Morzunov *et al.*, 1995; Schutze *et al.*, 1995).

Clinical signs

Clinical disease is most common in salmonid fry with extensive yolk-sac haemorrhage, swollen abdomen, darkening of the skin and petechial haemorrhage on the abdomen and around the eyes. One characteristic sign is the long gelatinous faecal casts, which can be seen trailing from the vent of affected fry. Anaemia can also be seen, as well as haemorrhage in the visceral tissues. Dark red areas may develop behind the head. Ascites and stomach distension can also be observed. However, gross clinical signs are frequently absent and the characteristic necrosis of the haematopoietic tissues from which the virus derives its name is seen only by histopathological examination.

Impact

In recently hatched fry, mortalities due to IHNV can reach 80–100% within 8–15 days at 10–12°C. Mortality in fish older than

1 year does not usually exceed 25% (Winton, 1997). Generally, fish less than 100 g have the highest susceptibility to IHNV, such as rainbow trout and Pacific salmon, and particularly sockeye salmon. Fish that survive IHNV infections can be chronic carriers of the virus with 2–4% of surviving fish exhibiting scoliosis, which renders them unmarketable (Winton, 1997).

Husbandry

Millions of eggs from fish diagnosed for IHNV have been destroyed on the assumption that the virus is transmitted within eggs. Transmission within the yolk has not yet been conclusively determined. However, screening for infected stocks, and the use of virus-free water and eggs that are derived from certified virus-free broodstock, are common practice. Salmonid enhancement or production hatcheries generally couple these practices with iodophore surface-disinfection of the certified eggs and continuation with a programme of iodophore disinfection at regular intervals throughout egg incubation. At present, these are the only effective control measures available for IHNV (Winton, 1991).

Vaccines

Considerable progress has been made in research on vaccine development (Winton, 1997). The glycoprotein of the virus elicits neutralizing antibodies that are protective (Engelking and Leong, 1989a,b). The neutralizing epitopes of the glycoprotein have been studied and mapped (Xu *et al.*, 1991; Huang *et al.*, 1996). Subunit vaccines have been developed, using part of the IHNV glycoprotein cloned into *E. coli*. These have been reported to be safe, effective and inexpensive (Noonan *et al.*, 1995). Emmenegger *et al.* (1995) developed synthetic peptides that included amino acid sequences representing the neutralizing domains on the glycoprotein. These were coupled to bovine serum albumin carriers or synthesized on lysine cores to produce multiple, eight-branched antigenic peptides. Trout injected with these peptides (with Freund's complete adjuvant) did not produce sera with neutralizing activity (with the exception of one individual); control fish injected with an attenuated strain of IHNV demonstrated high neutralizing activity in their sera. This suggests that the peptides alone have low immunogenicity in trout.

DNA vaccines are relatively new for fish health. Anderson *et al.* (1996) produced plasmid vectors that encoded the IHNV *N* and *G* genes, with a cytomegalovirus immediate early promoter. The constructs were injected into rainbow trout fry, which were subsequently challenged with IHNV by water-borne exposure. Fish injected with the *G* or *G* + *N* constructs produced higher neutralizing antibody titres and were significantly protected against the challenge. DNA vaccines delivered by injection hold promise for large fish, although a more cost-effective delivery system is required for smaller fish.

Spring viraemia of carp

Introduction

Spring viraemia of carp (SVC) is an acute, systemic rhabdovirus disease of the common carp (*Cyprinus carpio*), its varieties and other cyprinids. Significant losses occur during the spring in young and adult fish. The causal agent of SVC is a member of the *Rhabdoviridae* and is known as rhabdovirus carpio or spring viraemia of carp virus (SVCV) (Lenoir and de Kinkelin, 1975). This disease is notifiable to the OIE.

Characterization

Several conditions are now recognized as synonyms of SVC including infectious dropsy of carp, red contagious disease, infectious ascites and haemorrhagic septicaemia (Fijan, 1972). Several groups have established the aetiology of this virus (Bucke and Finlay, 1979; Ahne, 1980). SVCV is a typical bullet-shaped virus measuring 90–180 nm in length with a regular

array of spicules on the surface. Work on the characteristics of the large (L) gene (Björklund *et al.*, 1995), glycoprotein gene and internal gene junctions place the SVCV firmly in the genus *Vesiculovirus* of the family *Rhabdoviridae* (Björklund *et al.*, 1996). Recently, Johnson *et al.* (1999) reported the nucleotide and predicted amino acid sequences of the *G* genes of the warmwater rhabdovirus of penaeid shrimp (RPS) and snakehead rhabdovirus (SHRV), and found over 99% similarity to the *G* nucleotide sequence from SVCV. From this study they suggested that RPS and SVCV may be the same virus.

Impact

SVC is prevalent in farmed carp in Italy, France, Spain, Romania, Slovak Republic, Hungary and Israel, and is believed to be restricted to Europe and parts of Asia. In Germany, serologically positive fish have been recorded, but without clinical disease (Wizigmann *et al.*, 1980).

The main species affected by SVCV are crucian carp (*Carassius carassius*) (Kölbl, 1975), grass carp (*Ctenopharyngodon idella*) (Roudikov, 1980), bighead carp (*Hypophthalmichthys molitrix*) (Roudikov *et al.*, 1975) and sheathfish (*Siluris glanis*) (Fijan *et al.*, 1984). Young carp are the main susceptible group (Shchelkunov and Shchelkunov, 1989), followed by grass carp and bighead × silver carp hybrids. SVC is prevalent during periods of rising water temperature with mortality occurring above 7°C, and a maximum between 10 and 15°C. Above 23°C mortality is rare and clinical disease absent (Fijan, 1976). Losses attributed to SVC are in the order of 30%, but may reach as high as 70%. SVCV enters through the gill epithelium following adsorption to the plasma membrane and receptor-mediated endocytosis (Granzow *et al.*, 1997). The infection is maintained in the population through the shedding of virus during the winter months (Baudouy *et al.*, 1980). The carp louse (*Argulus foliaceus*) and leech (*Philometra geometra*) can also act as vectors (Ahne, 1985).

Clinical signs

Clinical signs vary and may even be absent, particularly during the early stages of infection. Signs in infected fish include dark pigmentation, lethargy, loss of balance, swollen abdomen, exophthalmia, pale gills, petechial haemorrhage and trailing white or yellowish faecal casts (Bachmann and Ahne, 1974; Fijan, 1975). Internally, haemorrhaging, peritonitis, copious mucus in the intestine and splenomegaly have been reported. Histologically, varying degrees of oedematous perivasculitis leading to multifocal degeneration, necrosis, clogged kidney tubules, hyaline degeneration and vacuolation have been described from experimentally infected carp (Negele, 1977). Osad haja and Rudenko (1981) also reported myocardial necrosis with lymphocyte infiltration, haemorrhagic spleen and acute enteritis.

Diagnostic techniques

The diagnosis of SVC primarily requires the isolation of the virus in cell culture. Although other diagnostic methods have been developed, virus isolation in cell culture is the most sensitive and therefore widely used. Diluted tissue samples of encephalon or ovarian fluid from brood fish are inoculated on to epithelioma papillosum of carp (EPC) or other sensitive cell lines and held at 15°C for 7 days (OIE, 1997). The CPE involves clear cell rounding and quick spreading across the cell sheet. The diagnosis of SVC by ELISA has been documented (Rodak *et al.*, 1993), but has a lower sensitivity than that achieved with cell culture. Similarly, the indirect fluorescent antibody test (IFAT) has been used (Faisal and Ahne, 1984; OIE, 1997) but this technique does not distinguish between SVCV and the closely related contagious disease, pike fry rhabdovirus (PFRV) (Jørgensen *et al.*, 1989). A competitive immunoassay for detection of SVCV fish antibodies has been tested on experimentally and naturally infected carp and is considered a sensitive method when compared

with a neutralization test (Dixon *et al.*, 1994). Furthermore, Ahne *et al.* (1998) have described a ribonuclease protection assay (RPA) using an SVCV *G* gene probe that differentiated isolates of SVCV and PFRV.

Prevention

No vaccine is available for SVC, although trials with experimental live and attenuated strains have been encouraging in laboratory studies (Fijan *et al.*, 1977; Kölbl, 1980; Dixon *et al.*, 1994). Work by Macura *et al.* (1983) with inactivated vaccines showed increased survival rates in field trials, but less success under laboratory conditions. Unfortunately, the promising live vaccine experiments have not been followed by appropriate licensing documentation (Midtlyng, 1997). In some countries farmed carp are serologically positive for SVCV and therefore the feasibility of using live attenuated strains in these areas remains problematic (Kölbl, 1980). Wolf (1988) proposed a genetic selection programme, but this was not developed. As with many diseases, overcrowding, water quality and handling stress increase susceptibility to SVC. Currently, prevention relies on restricting the movement of infected fish, carrying out health checks, purchasing certified healthy stock, disinfecting eggs and practising good husbandry. SVC may be avoided in farms supplied with spring or well water.

Recommendations

SVC is widespread in Europe and eradication appears unlikely. Information is required to identify vaccine effectiveness under different regimes. The susceptibility of selected fish strains to SVCV and an expansion of the genetic selection programme are priorities. Future vaccination strategies involving immersion or oral vaccination of fry were suggested by Clem *et al.* (1996). Improvements to ELISA and PCR diagnosis would also be beneficial. Johnson *et al.* (1999) has strongly suggested that SVCV is identical to the rhabdovirus RPS of penaeid shrimp in Hawaii. Further studies as recommended by Johnson *et al.* (1999) are required to establish the enzootic nature of the virus in the Pacific region and the occurrence of the virus in both vertebrate and invertebrate hosts.

***Oncorhynchus masou* virus**

Introduction

Oncorhynchus masou virus (OMV) or salmonid herpesvirus is a virulent pathogen of salmonids of the genus *Oncorhynchus*, particularly affecting juvenile fish in fresh water. Infection is reported in wild and cage-cultured fish, but only from Japan. The virus typically causes an acute systemic infection in juvenile fish, especially alevins. Fish surviving infection frequently become subclinical carriers of infection. Kimura and Yoshimizu (1989) have published a review article on OMV.

Characterization

Electron microscopy shows OMV to be an enveloped Type II salmonid herpesvirus of 200–240 nm in diameter (Tanaka *et al.*, 1987). Two closely related strains of a herpesvirus have been isolated from trout in North America. These isolates are distinct from OMV and other Japanese herpesviruses, indicating they are not the result of the transfer of infected fish or eggs between continents.

Impact

Infection with OMV is listed by the OIE as a notifiable disease. OMV was first reported from ovarian fluids of a landlocked population of adult masou salmon (*O. masou*) in Hokkaido, Japan and is now widespread in the northern regions of that country. Under natural conditions, outbreaks of disease due to OMV affect only *Oncorhynchus* spp., but several other salmonid species are susceptible to OMV including fry of coho salmon, kokanee salmon (*O. nerka*), chum salmon (*Oncorhynchus keta*), and rainbow trout. Furthermore, the viral agent has limited survival outside the host. The most

significant losses are recorded in coho salmon (Kimura and Yoshimizu, 1989). The disease is more common in the freshwater phase of the salmonid life cycle and alevins of 1 month old are particularly susceptible to infection. However, larger fish (up to 1 kg in weight) may also be affected by the disease. Horizontal and vertical infection may play a role in the transmission of OMV (Kimura and Yoshimizu, 1989).

Clinical signs

Infection with OMV is a systemic and frequently lethal condition that is associated with oedema and multiple haemorrhage. The virus multiplies in endothelial cells, haematopoietic tissue and hepatocytes. Affected fish are dark and often have severe exophthalmos with petechial haemorrhage under the lower jaw and along the ventral surface. A white mottled appearance of the liver is recorded, progressing to a pearly white colour of the whole organ. A pale kidney and a multifocal, severe necrosis of the liver is also common (Tanaka *et al.*, 1984). Gill epithelial cells become swollen and slough. There is a marked splenomegaly with associated necrosis of the ellipsoids, and the digestive tract is generally devoid of food.

Infected juvenile coho salmon develop ulcers on the skin, lesions in the liver and tumours on the mouth, caudal fin, operculum and body surface. Infected rainbow trout show few signs, mainly limited to ulcerative lesions of the skin, intestinal haemorrhage and lesions in the liver (Kimura *et al.*, 1981).

Studies involving experimental infection with OMV have shown that there is some variation in histopathological findings between species of juvenile salmon. The kidney is the apparent target organ in chum salmon, with necrosis of the haematopoietic tissue, hyaline droplet degeneration and pyknosis. Partial necrosis occurs in the spleen, liver, pancreas and stomach, whereas in masou salmon, haematopoietic necrosis has been reported (Tanaka *et al.*, 1984).

OMV has oncogenic potential and induces a mandibular epithelial neoplasm and other tumours of the fins, body surface and cornea in surviving fish (Ishigaki *et al.*, 1987; Yoshimizu *et al.*, 1988). These tumours are characterized as papillomatous and consist of several layers of proliferating epithelial cells supported by thin connective tissue. Multiple mitotic figures confirm the proliferative nature of the tumour. Post-infection, tumours may be found for up to a year (Yoshimizu *et al.*, 1988). Fish surviving the septicaemia frequently become carriers of infection and may shed the virus in the faeces, urine, sexual products and, probably, in the skin mucus. In clinically infected fish, the highest titre of virus occurs in the kidney, liver, spleen and in tumour tissue (Ishigaki *et al.*, 1987). OMV may be transmitted horizontally, via exposure to a significant titre of virus in the freshwater environment. Exposure to a higher titre of virus would be required to initiate infection in adult fish.

Diagnostic techniques

The diagnosis of OMV involves virus isolation from diseased fish, using cell lines such as CHSE-214 or RTG-2 and a serum neutralization test with a specific OMV antiserum. Optimal temperature for virus isolation is 15°C (Kimura and Yoshimizu, 1989). Work by Gou *et al.* (1991) reported a DNA probe able to detect 10 copies of viral DNA per cell.

Prevention

The spread and prevalence of infection of OMV may be managed by disinfecting eyed ova and treating hatchery water with UV radiation. Fish-to-fish transmission of OMV is effected by holding 5-month-old fry with fry infected by immersion. The resulting rate of mortality was similar to that observed as a result of infection by immersion (Kimura and Yoshimizu, 1989).

Direkbusarakom *et al.* (1996) showed that some traditional Thai herb extracts reduced the CPE due to OMV in CHSE cells by 50%. The extracts demonstrated low toxicity to the CHSE cells; however, considerably more research is warranted before the

efficacy of herb treatments for salmon is known.

Erythrocytic inclusion body syndrome

Introduction

Erythrocytic inclusion body syndrome (EIBS) and other inclusion syndromes, including those caused by viral erythrocytic necrosis virus (VENV) (Evelyn and Traxler, 1978) and intraerythrocytic virus (Landolt *et al.*, 1977), are cytoplasmic viral infections of the erythrocytes of salmonids and other fish groups. In Japan, EIBS is characterized by a severe anaemia in cultured coho salmon, whereas in farmed Atlantic salmon from Scotland and Ireland there is no significant association between the EIBS virus and clinical disease (Rodger and Richards, 1998).

Characterization

Natural infections of EIBS virus have been described in pink (*Oncorhynchus gorbuscha*), chum and chinook salmon in the Pacific Northwest of the USA (Piacentini *et al.*, 1989). EIBS has been reported in farmed Atlantic salmon in Norway (Lunder *et al.*, 1990) and Scotland (Rodger and Richards, 1998), coho salmon in Japan (Okamoto *et al.*, 1992) and rainbow trout in Ireland (Rodger *et al.*, 1991). The virus particles that have been examined are icosahedral, enveloped and in salmonids measure 70–80 nm in diameter (Arakawa *et al.*, 1989; Thorud *et al.*, 1990; Rodger *et al.*, 1991). Tentatively, the viruses causing EIBS and EIBS-like conditions are placed in the *Iridoviridae* (Reno *et al.*, 1978).

Impact

Natural disease outbreaks have been reported in Atlantic salmon, coho salmon, chinook salmon and rainbow trout (Lunder *et al.*, 1990; Michak *et al.*, 1992). The virus can be transmitted in fresh water (MacMillan and Mulcahy, 1979; Leek, 1987) and its presence in young yolk-sac fry and alevins could suggest vertical transmission (Rohovec and Amandi, 1981). Fish reared from eggs in borehole water have been confirmed positive for EIBS virus, supporting the likelihood that vertical transmission of this virus occurs. EIBS has been associated with mortality exceeding 25% (Piacentini *et al.*, 1989); however, virus inclusions are also noted in apparently healthy fish (Rodger *et al.*, 1991; Rodger and Richards, 1998). Mortality directly attributed to EIBS virus is difficult to establish as other pathogens including *Flavobacterium psychrophilum* and IPNV often occur concurrently (Thorud *et al.*, 1990; Evensen and Lorenzen, 1997). Field observations suggest that EIBS affects the susceptibility of salmonids to secondary pathogens (Arakawa *et al.*, 1989; Piacentini *et al.*, 1989).

Clinical signs

Lethargy, pale livers and internal haemorrhage have been reported in chinook and coho salmon (Leek, 1987; Takahashi *et al.*, 1994). The initial stages of EIBS begin with an 11 day incubation period (at 12°C) but with no pathological changes, followed by the appearance of inclusions in increasing numbers, leading eventually to cell lysis and anaemia (Piacentini *et al.*, 1989). Although cell lysis may be virus-directed, the fish immune system may also contribute to the lysis of infected cells by sensitized lymphocytes and lymphokines being released in response to viral antigens. Fish with EIBS may appear healthy, but in North America and Japan anaemia has been consistently reported with an increased incidence of other infections (Piacentini *et al.*, 1989; Takahashi *et al.*, 1992; Maita *et al.*, 1998). Lunder *et al.* (1990) reported no liver lesions in Atlantic salmon or other specific clinical signs associated with this infection. Increased haemosiderin has been reported in experimentally and naturally infected chinook salmon in Idaho, USA (Foott *et al.*, 1992), but this may also result from other infections including vibriosis. Lipid peroxidation (Sakai *et al.*, 1994) and fatty acid changes in hepatic phospholipid

(Maita *et al.*, 1996) occur in sea-cultured coho salmon naturally infected with EIBS virus. However, Maita *et al.* (1998) suggested that diet could also influence these factors.

Two types of inclusions in red blood cells are described, type A and B. The former is characterized by large single inclusions and type B by small multiple inclusions (Michak *et al.*, 1992; Rodger and Richards, 1998). Salmon infected in fresh water mainly show type A inclusions, whereas type B inclusions are dominant in seawater (Rodger and Richards, 1998). This may reflect a level of maturation of the virus or increased infection levels resulting in more type B inclusions. Although many post-smolts carried EIBS-like virus prior to transfer to seawater, there was no correlation between type B inclusions, clinical disease and mortality (Rodger and Richards, 1998). Similarly, Jarp *et al.* (1996) found no correlation between EIBS and plasma chloride levels following transfer to seawater.

Electron microscopy of erythrocyte inclusions in naturally infected chinook salmon showed viral particles 75 nm in diameter, randomly scattered throughout the cytoplasm (Leek, 1987). In Atlantic salmon, particles measure 80 nm, are hexagonal in shape with an electron-dense outer margin and are located in packages within an adjacent membrane (Thorud *et al.*, 1990). Viral particles occurred between the inner and outer layer of the nuclear membrane.

Diagnostic techniques

The examination of methanol-fixed and Leishman–Giemsa-stained blood smears for cytoplasmic inclusion bodies in red cells is a diagnosis for EIBS (Thorud *et al.*, 1990). Staining with pinacyanol chloride may increase staining consistency (Leek, 1987; Yasutake, 1987). Acridine orange-stained blood films examined by fluorescent microscopy provide an additional method for displaying intraerythrocytic inclusions (Piacentini *et al.*, 1989). To date, the virus has not been grown in tissue culture, nor is it known if other cell types are infected.

Prevention

There are insufficient data on EIBS and EIBS-like conditions to formulate adequate preventive measures. Coho salmon infected in fresh water are able to recover and are apparently resistant to reinfection for at least 242 days without any evidence of anaemia. Acquired resistance is maintained after transfer to seawater (Okamoto *et al.*, 1992).

Recommendations

In some cases, or in certain groups of fish, severe anaemia, pale liver and internal haemorrhage are associated with erythrocytic inclusions, whereas in other groups inclusions may be present but with no apparent effect (Rodger and Richards, 1998). The reason for this is unclear and further study is necessary. Similarly, gaps in our knowledge concerning the possible correlation between a focal cardiomyopathy, infectious dose and the possibility that other cell types might be infected should be examined. Long-lasting immunity has been described in coho salmon (Okamoto *et al.*, 1992) and such studies could be extended to other susceptible groups.

Diseases Caused by Bacterial Pathogens

Furunculosis

Introduction

Aeromonas salmonicida, the causative agent of furunculosis, is one of the most studied bacterial pathogens of fish. It is widespread, having been documented in Europe, North America, Japan, Korea, Australia and South Africa. Furunculosis has been an important disease in wild and cultured stocks of fish since the 1890s (Emmerich and Weibel, 1894). In fact, losses due to furunculosis were of such a magnitude in wild salmon in Scotland that the Furunculosis Committee was created. The reports of that committee were the basis

of the Diseases of Fish Act in 1937, the earliest fish disease legislation in the world.

Although *A. salmonicida* is primarily a freshwater pathogen, outbreaks of furunculosis are often seen in seawater. The pathogen is not limited to salmonids and many species of fish are affected. Several excellent reviews of *A. salmonicida* and furunculosis are available (Bernoth, 1997; Wiklund and Dalsgaard, 1998; Hiney and Olivier, 1999).

Characterization

A. salmonicida is a non-motile bacterium and readily isolated from the kidney and other organs. Most isolates produce a characteristic dark brown diffusing pigment in culture. It is traditionally described as Gram-negative, non-motile, fermentative, producing catalase and oxidase with no growth at 37°C. Classification of the subspecies of *A. salmonicida* has been the subject of much discussion. McCarthy and Roberts (1980) proposed a division of *A. salmonicida* into three subspecies. This view was supported by subsequent studies (Belland and Trust, 1988; Munro and Hastings, 1993). The following is the classification scheme as summarized by Munro and Hastings (1993).

- Group 1 strains. *A. salmonicida salmonicida*. Arbitrarily described typical strains derived from salmonid fishes.
- Group 2 strains. *A. salmonicida achromogenes*. Atypical strains derived from salmonids that represent aberrant strains that show variation in some biochemical properties and include the former species *achromogenes* and *masoucida*.
- Group 3 strains. *A. salmonicida nova*. Atypical strains associated with disease in non-salmonid fishes.

The definition of typical or atypical refers to biochemical and morphological characteristics, such as the production of pigment and extracellular proteases (McCarthy and Roberts, 1980; Böhm *et al.*, 1986; Wiklund and Dalsgaard, 1998).

Phage typing is used extensively to distinguish different isolates of *A. salmonicida*. Popoff (1984) showed 14 phage types of *A. salmonicida* using eight phages.

The physical map of a pathogenic strain A449 was recently determined by members of the Canadian Bacterial Diseases Network, and was shown to be representative of eight other furunculosis-causing strains isolated from salmonids in Canada, the USA, Europe and Japan (Umelo and Trust, 1998). These eight strains belong to a larger group of more than 40 strains that have been shown to be homogeneous by a variety of classical and molecular methods (Vaughan, 1997). These strains are considered to belong to the subspecies *A. salmonicida salmonicida*.

More than 63 unique genes have been sequenced from *A. salmonicida* (Table 4.2), and some have been subjected to functional analysis including overexpression, directed mutagenesis or allelic replacement (Vaughan, 1997). The best-characterized strain of *A. salmonicida* is A449 (Umelo and Trust, 1998). Its 4658 ± 30 kbp genome has a G + C composition of 55%. Approximate map positions have been determined for 26 protein-coding genes (including some associated with virulence), large and small subunit rDNAs, and four insertion elements. Although some associations were noted, overall their distribution around the circular genome appears random (Umelo and Trust, 1998).

Extensive research has been conducted on the pathogenicity of *A. salmonicida*. The regular surface protein layer (the A-layer) is an important factor in pathogenicity. So too are the lipopolysaccharide (LPS) layer and the possession of cytotoxic activity, as measured in *in vitro* studies (Garduño *et al.*, 1993, 1997; Daly *et al.*, 1996).

Researchers have examined the mechanisms of innate resistance in salmonids to *A. salmonicida*. Hoover *et al.* (1998) identified pentraxins and other lectin-like proteins that bound to the LPS of *A. salmonicida*. Ewart *et al.* (1999) isolated and characterized a mannose-binding lectin from Atlantic salmon sera, and then

Table 4.2. Published gene sequences from *Aeromonas salmonicida*. Data from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nih.gov).

16 S rRNA gene
5 S rRNA
abcA protein (ATP-binding cassette membrane transporter)
Acetyl-CoA carboxylase subunit
asoA protein
asoB protein
Autoinducer synthesis protein asaI
Chaperonins groEL, groES
2′,3′-cyclic phosphodiesterase
3-Dehydroquinase (*aro*D gene product)
Dihydrofolate reductase
DNA gyrase
DNA gyrase subunit A (*gyr*A gene product)
5-Enolpyruvylshikimate-3-phosphate synthase
exeC protein
exeD protein
exeE protein
exeF protein
exeG protein
exeN protein
Ferric siderophore receptor
Flagellins flaA, flaB, flaG
General secretion pathway proteins (or precursors thereof) C, D, N
Glycerophospholipid–cholesterol acyltransferase
Haemolysins (or precursors thereof) 1, 2, 3, 4 and β
Hypothetical 21.5 kDa protein in *asa*R–*cdp*D intergenic region
Hypothetical protein in *exe*N 3′ region (not transcriptional regulator)
Hypothetical proteins, other
Hypothetical transcriptional regulator in *exe*N 3′ region
Major cold-shock protein (*csp*A gene product)
Major outer membrane proteins ompAI, ompAII
N-acyl homoserine lactone synthase (*asa*I gene product)
oriC replication inhibitor (*ici*A gene product)
3-Phosphoshikimate 1-carboxyvinyltransferase (*aro*A gene product)
Pilin proteins pilC, pilD
Pilus assembly proteins tapB, tapD
Polytopic cytoplasmic membrane protein
Porin, maltose-inducible (*lam*B gene product)
Prepilin peptidase tapD
Prepilins sfpA, tapA
Proteolysis tag (coded portion)
recA protein
Ribosomal protein S20
satA protein
Secretion proteins, including *aps*E gene product
Serine proteases and protease precursors, including *asp*A gene product
Transcriptional activator (*asa*R gene product)
vapA (S-layer protein precursor; tetragonal surface virulence array protein, or A-protein)
yggA protein

Ottinger *et al.* (1999) demonstrated *in vitro* activity against *A. salmonicida* by this lectin. They postulated that this and possibly other lectins are important as part of the innate defence system against *A. salmonicida*.

Impact

All life stages of salmonids are susceptible to furunculosis, although young fry are less frequently infected (Munro and Hastings, 1993). Serious losses have been seen in farmed and wild salmonid stocks.

Infections by *A. salmonicida* have been documented in non-salmonids. The source of these infections is unknown. Infection by *A. salmonicida* subsp. *nova* is indigenous in cyprinids and other non-salmonids (Cornick *et al.*, 1984; Morrison *et al.*, 1984; Antychowicz and Rogulska, 1986; Evenberg *et al.*, 1986).

MacKinnon has undertaken an extensive study to determine the degree of species specificity in virulence of atypical *A. salmonicida* isolates from non-salmonids. She found that the isolates were the most virulent within the species from which they were derived, and that cross-challenges with other species showed varying degrees of virulence (A.-M. MacKinnon, Department of Fisheries and Oceans, Moncton, New Brunswick, personal communication).

Clinical signs

External signs of furunculosis depend upon the time course of the disease (Munro and Hastings, 1993). Fish may die from an acute infection without any signs of disease; chronically infected fish may present signs such as darkening, lethargy and petechial haemorrhage at the base of the fins. Fish may also exhibit furuncles from which the disease derives its name (Fig. 4.1). These furuncles may ulcerate to release necrotic tissue debris and bacteria. Internally, ascites may be seen. Frequently the intestine is empty of food, but may contain mucus and bloody cellular debris. Blood vessels around the pyloric caeca and intestine may be inflamed and the kidney swollen and liquefied. The haematocrit is often depressed and frequently the leucocyte cell layer is significantly reduced or absent. A widespread acute or subacute haemorrhaging occurs in the viscera. The kidney is soft, the spleen swollen and the liver pale. Toxins released by the bacteria liquefy the tissue causing an intense inflammatory reaction, with associated swelling causing ulceration with an exudate of blood stained tissue rich in bacteria. Bacterial invasion of the compact myocardium results in necrosis and an increase in monocytes within the subepicardial space. Foci of bacteria occur in many organs and become the centre of a generalized tissue necrosis.

Fig. 4.1. Dorsal lesion on farmed rainbow trout attributed to infection by *Aeromonas salmonicida*.

Diagnosis

Diagnosis is based on clinical signs, and isolation of *A. salmonicida* from fish tissues. The bacterium is grown on tryptic soy agar (TSA), or brain heart infusion agar (BHI) at temperatures below 20°C. The presence of brown, diffusable pigment is a useful tool for identification of *A. salmonicida salmonicida*; however, the pigment may be absent from some strains. On Coomassie blue agar, colonies of *A. salmonicida* are a characteristic blue colour. Upon initial isolation, colonies of *A. salmonicida* may be easily pushed across the plate by means of a bacteriological loop. Atypical colonies appear shinier and are viscous. The appearance of the bacteria in haemotoxylin and eosin (H&E)-stained sections is characteristic (Bruno and Poppe, 1996).

Within a population some fish may be infected with *A. salmonicida* but without showing clinical signs of furunculosis. In order to identify these carriers, fish can be subjected to stress tests. Elevated temperatures and other stresses are applied to induce an infectious state within a population (Bullock and Stuckey, 1975). PCR and nested-PCR techniques have also been developed to identify carriers (Hiney *et al.*, 1992; Oakey *et al.*, 1998).

Treatment

A. salmonicida is susceptible to a number of antibiotics, including oxytetracycline, oxolinic acid, trimethoprimsulphadiazine and amoxyxillin. However, antibiotic-resistant isolates have been identified and multiple resistance is common (Richards *et al.*, 1992). Both plasmid-mediated and mutational drug resistance have been detected (Aoki *et al.*, 1983; Barnes *et al.*, 1992). Oxytetracycline (OT) is one of the most extensively used antibiotics in aquaculture (Austin and Austin, 1993) and OT resistance in *A. salmonicida* has increased significantly in recent years (Smith *et al.*, 1994). Adams *et al.* (1998) have identified two plasmids of the bacterium that confer resistance to OT. In Scotland, the total number of distinct *A. salmonicida* strains isolated from cage-cultured fish by the Marine Laboratory, Aberdeen, declined from 164 in 1989 to five in 2001. The number of strains that were OT-resistant in 1990 was 52/164 isolates and 1/6 were OT-resistant in 2000.

Prevention

Duff (1942) was the first to attempt to develop a vaccine. Since then work to identify antigens of the pathogen that would be potential vaccine candidates, including those in activated whole cells, soluble extracts, immune serum and attenuated live cells with and without a modified A-layer, has continued. At present the majority of commercial bacterins are emulsified cells in an oil-based adjuvant. The side effects of this form of vaccination are discussed in Chapter 9.

There has been growing interest in the immunostimulatory properties of polysaccharides associated with the cell wall of yeasts. Some β-1,3 glucans have been tested as adjuvants in injectable furunculosis vaccines (Rorstad *et al.*, 1993). It was found that the glucan enhanced antibody formation and specific protection, and Midtlyng *et al.* (1996) found that glucan-adjuvanted furunculosis vaccines confer protection that is similar or even slightly higher than an aluminium-salt adjuvant. Culture of *A. salmonicida* in iron-depleted media results in the expression of highly protective antigens and is a technique currently used in commercial vaccines.

Work has been done on passive immunization of salmonid fry via pre-spawning vaccination of the broodstock. Kawahara *et al.* (1993) reported that when white-spotted char (*Salvelinus leucomaenis*) were vaccinated with *A. salmonicida* extracellular proteins before spawning, the eggs from these fish had increased levels of total antibodies. Furthermore, there was an increased survival upon challenge with *A. salmonicida* among fry from the broodstock. Other work has demonstrated the feasibility of depositing specific antibodies within salmonid eggs via broodstock injection (Brown *et al.*, 1997a). This may

represent an alternative approach for immunization.

Future studies

A. salmonicida is one of the most extensively studied bacterial fish pathogens. Despite significant advances in knowledge regarding pathology, virulence and epizootiology, there are still outbreaks of the disease, and *A. salmonicida* continues to be an important pathogen in freshwater aquaculture. It is unlikely that *A. salmonicida* will be eliminated, but recent advances in understanding the molecular and biochemical basis of the host–pathogen interaction may help to develop techniques to ensure consistent and reliable control of this bacterium.

Motile aeromonad septicaemia (*Aeromonas hydrophila*)

Introduction

Motile aeromonad infections are ubiquitous and loosely encompass several species and subspecies including *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria*, although the classification of some of the group remains contentious. The bacteria are opportunistic pathogens and cause a loosely described haemorrhagic septicaemia of cultured and wild freshwater fish throughout the world and, to a lesser extent, in marine fish species. Reference here is made to the most widely described, *A. hydrophila*, a common inhabitant of aquatic systems and the causative agent of a motile aeromonad septicaemia (Roberts, 1993).

Characterization

A. hydrophila is Gram-negative, measures 0.4–1.0 µm in length and is motile by a monotrichous flagellum (Roberts, 1993). It is aerobic, catalase, oxidase and aesculin positive, produces indole, reduces nitrate and hydrolyses starch. Newman (1982) and Popoff (1984) reported biochemical profiles. Colonies are round with entire margins, and pale white to cream on TSA culture at 15°C.

Impact

Aeromonas spp. induce the following conditions in fish: a motile *Aeromonas* septicaemia, bacterial haemorrhagic septicaemia, tail and fin rot, and redsore disease. These bacteria are in soil and most natural freshwater environments. Most freshwater fish are susceptible, including rainbow trout, brown trout, tilapia (*Oreochromis* spp.), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatilis*) and carp (Fijan, 1972; Thune *et al.*, 1982). Outbreaks are associated with rising water temperatures, crowding, handling or transfer, low oxygen levels and poor nutritional status of the fish. These stress-related conditions can lead to a rapid rise in mortality in susceptible fish (Fijan, 1972).

A. hydrophila multiplies in the intestinal epithelium and large numbers are shed in the faeces. The bacterium occurs widely in Europe, the Americas and Asia. Acute losses are reported at 20–22°C and mortality of 80% can occur in 2–3 weeks. At low temperature and in older fish, the mortality is reduced and the infection is often chronic in nature.

Toxins and extracellular enzymes, together with some structural features of the bacteria, are considered important virulence factors for *A. hydrophila* and related species (Nieto *et al.*, 1991; Mateos *et al.*, 1993). Their production is influenced by specific environmental conditions (Mateos and Paniagua, 1996). Virulent strains display cell-surface characteristics, including a paracrystalline protein array (S-layer) assembled from the monomeric S-protein (Thomas and Trust, 1994). Intracellular location of the bacterium, in tissue culture, may help to maintain a systemic infection (Low *et al.*, 1998).

Clinical signs

Motile aeromonad infections occur throughout the year, but epizootics are most

common in the spring and early summer as the water temperature rises. These outbreaks may be a generalized septicaemia with superficial to deep grey-red ulcers, local haemorrhage, particularly from the gills and vent, abscesses, exophthalmia and abdominal distension. Fish may appear sluggish and dark in colour with tail rot. Internally, clear or blood-stained ascitic fluid, anaemia, haemorrhage around the caeca and intestine, and swollen kidneys are reported (Roberts, 1993).

Diagnostic techniques

A provisional diagnosis is based on non-specific signs. Definite diagnosis requires biochemical identification of the bacterium (Popoff, 1984). A range of commercially available media will support growth of *A. hydrophila*, including TSA and BHI after 24–48 h incubation at 20–22°C. An ELISA for detection of highly virulent strains of *A. hydrophila* and *A. sobria* serotype 0:11 has been developed by Merino *et al.* (1993).

Prevention

Infection by *A. hydrophila* often results from poor husbandry, and improvements in management practices decrease the likelihood of disease outbreaks. Antibiotic therapy has been effective, but widespread and incorrect use has resulted in antibiotic-resistant strains that have restricted the usefulness of antibiotic treatment (Mitchell and Plumb, 1980). Overall improvements in general fish health practices linked to reducing stress factors have contributed to a decline in outbreaks and consequently a reduction in use of antibiotics.

Vaccination is an alternative strategy to antibiotic use, but the antigenic diversity of *Aeromonas* challenges vaccine development. Several experimental vaccines for *A. hydrophila* have been tested, with varying degrees of success (Ascencio and Wadstrom, 1994; Ramadan *et al.*, 1994; Yin *et al.*, 1996). A marked immunopotentiating effect on both humoral and cell-mediated immune responses was noted by Ramadan *et al.* (1994), who fed ascogen (5 g kg^{-1} feed) to tilapia after vaccination.

Summary

Outbreaks involving *Aeromonas* spp. are frequently the result of poor husbandry, as the bacteria are often secondary or opportunistic pathogens (Roberts, 1993). Given the widespread occurrence of this group and the heterogeneity of strains, the development of commercial vaccines remains problematic. Extracellular products and proteins from the S-layer require further examination with respect to determining their role in pathogenesis and potential as vaccine candidates.

Enteric redmouth disease

Introduction

Enteric redmouth (ERM) disease or yersinosis is an economically important disease in freshwater aquaculture worldwide. The causative bacterium, *Yersinia ruckeri*, generally affects farmed rainbow trout fry reared in fresh water. Outbreaks are characterized by escalating losses, with congestion and haemorrhage in the kidney, liver, pancreas, musculature and intestinal tract. The severity of the disease is dependent upon the biotype of the pathogen and the salmonid host. Fish as carriers are important in the epizootiology of ERM disease, and outbreaks are associated with stressed populations. Commercial vaccines are successful.

Characterization

Y. ruckeri is a short, motile, Gram-negative, rod-shaped bacterium placed in the Enterobacteriaceae (Ewing *et al.*, 1978). Five major serovars have been recognized of which Type I (Hagerman strain) is commonly isolated and the most virulent. Stevenson *et al.* (1993) expanded this to six whole-cell serovars (I–VI) using LPS profiles. Later, Romalde *et al.* (1993) proposed four serogroups, using antigenic determinants,

that incorporated the serovars suggested by Stevenson *et al.* (1993). A recent study to characterize Portuguese strains found that ribotyping using three restriction endonucleases was more discriminatory than outer membrane protein (OMP) or plasmid profiling (Sousa *et al.*, 2001).

Impact

ERM disease was first recorded in Idaho, USA in the 1950s among hatchery-reared rainbow trout (Ross *et al.*, 1966). Shortly afterwards the bacterium was reported from Alaska and then Canada (Wobeser, 1973). During the mid-1980s ERM disease was introduced into Europe and is now present in the UK, Norway, Denmark, France, Germany, Italy and South Africa (Bragg and Henton, 1986). Llewellyn (1980) described a bacterium similar to *Y. ruckeri* among salmonids in Australia.

The most susceptible group is young rainbow trout. Losses may be from 30 to 70% of the population (Wobeser, 1973). In the USA during 1998, of the 34.3 million total fish mortalities, 84% were attributed to ERM disease (Hinshaw, 1999). Outbreaks of ERM disease are common between 15 and 18°C, and linked with stress-related conditions such as low oxygen, handling and high stocking densities.

The movement of carrier fish is implicated as a principal cause for the spread of yersinosis and likely reservoirs of infection are therefore farmed salmonids (McDaniel, 1971). However, *Y. ruckeri* has also been isolated from wild salmonids (Petrie *et al.*, 1996) and other fish, including sturgeon (*Acipenser baeri*), walleye (*Stizostedion vitreum vitreum*), carp, goldfish (*Carassius auratus*) and minnow (*Pimiphales promelas*) (McArdle and Dooley-Martin, 1985; Michel *et al.*, 1986; Enriquez and Zamora, 1987; Vuillaume *et al.*, 1987).

Clinical signs

ERM disease is typical of other Gram-negative septicaemias and varies in severity from subclinical to subacute to acute infection with deaths in small fry (Kawula *et al.*, 1996). Predominant gross signs include darkening of the skin pigmentation and lethargy. The gills may show haemorrhage with petechiae on the liver surface, pancreas and musculature. Inflammation of the vent region, splenomegaly and a yellowish mucoid fluid in the intestine are common. Progressively blood-tinged ascites, pale gills, exophthalmos, haemorrhage of the ocular cavity and eventual rupture of the eye may also occur. At necropsy characteristic observations include profound venous and capillary congestion, particularly of the brain and blood vessels, intestinal haemorrhage, diffuse petechial haemorrhage of the musculature, splenomegaly and yellowish discharge from the vent. Necrosis of the haematopoietic tissue is the principal histological sign. Bacteria spreading to the gills, musculature and liver result in capillary dilation and haemorrhage, tissue oedema and focal necrosis. A spreading necrosis and associated pyknosis occur within the haematopoietic tissue and the splenic ellipsoids following infection by *Y. ruckeri*.

Diagnostic techniques

A provisional diagnosis made on clinical signs can be confirmed by culture of the bacterium from infected tissues. Regular cyclical shedding of *Y. ruckeri* from the intestinal tract may delay isolation (Bruno and Munro, 1989). On TSA, colonies of *Y. ruckeri* are circular with entire edges, non-pigmented and with a butyrous type of growth. The bacterium is fermentative, oxidase and cytochrome oxidase negative, catalase and citrate positive. No indole is produced in tryptone broth. Biochemical properties have been described by Hastings and Bruno (1985), Davies and Frerichs (1989) and Austin and Austin (1993). The development of selective media (Waltman and Shotts, 1984) to aid culture has not been proposed, but some biochemical tests are variable between isolates and may yield false positives (Hastings and Bruno, 1985). Immunodiagnostic approaches include the development of ELISA for specific antibodies and latex-agglutination tests (Romalde

et al., 1995). More recently, sensitive, specific DNA primers and PCR protocols for the detection of *Y. ruckeri* in the kidney of infected fish have been reported (Argenton *et al.*, 1996; Gibello *et al.*, 1999; Temprano *et al.*, 2001).

Prevention

Commercial immersion vaccines based on a suspension of killed bacterial cells are now used. Healthy fish, generally weighing more than 4 g, are immersed or sprayed within an appropriately diluted vaccine. A booster vaccination may be included at the fingerling stage (Larsen and Pedersen, 1997). The efficacy of ERM vaccines is dependent upon species of fish, weight and water temperature, although vaccination does not eliminate mortality. Disease prevention involves adequate sanitary measures combined with regular removal of dead or moribund fish, disinfection of nets and equipment and reduction of stress to a minimum. Ozonation has been shown to be effective against *Y. ruckeri* in laboratory trials (Liltved *et al.*, 1995). Oxytetracycline, ciprofloxacin (oxolinic acid) and amoxicillin are antibiotics effective against *Y. ruckeri*. These are incorporated into the diet. Prolonged or incorrect use may have selected for strains resistant to these antibiotics (Busch, 1983). The trend may, however, be decreasing, as in Scotland (isolated by the Marine Laboratory, Aberdeen) the number of oxolinic acid-resistant *Y. ruckeri* isolates from farmed fish has declined from 67 to 14% since 1990. Similarly, no oxytetracycline-resistant strains have been found since 1992 and this parallels the decline in the amount of antibiotics prescribed.

Recommendations

Different approaches to serotyping *Y. ruckeri* have led to difficulties in comparing results between laboratories. Standardization is therefore required (as is the case with all pathogens). Development of programmes in vaccine research that identify the mechanisms of cross-protection and the antigens involved are also warranted.

Coldwater disease/rainbow trout fry anaemia

Introduction

Rainbow trout fry anaemia syndrome (RTFS) or bacterial coldwater disease (BCWD) is caused by *F. psychrophilum* (Bernardet *et al.*, 1996) and has been implicated as one of the most serious infectious diseases within the early freshwater stages of salmonids (Holt *et al.*, 1993). The first description of bacterial coldwater disease was made in West Virginia, USA, from farmed rainbow trout (Davis, 1946). Losses of more than 50% have been reported in alevins (Holt *et al.*, 1993). The disease can be seen as external lesions involving the epidermis and muscle tissue, or as a systemic infection. *F. psychrophilum* has been isolated from hatchery water, non-salmonid freshwater fish and vertebrates. Recently, the bacterium has been isolated from within newly spawned eggs of infected steelhead trout broodstock suggesting vertical transmission occurs from adult fish via eggs to alevins (Brown *et al.*, 1997b).

Characterization

The pathogen *F. psychrophilum* was initially named *Flexibacter psychrophilus* and *Cytophaga psychrophila* (Bernardet *et al.*, 1996). It was first isolated and described by Borg (1960). The bacterium is a slender Gram-negative rod, with gliding motility. Cells from young broth cultures are 0.3–0.75 μm × 2–7 μm, with some long filamentous rods of 10–40 μm (Fig. 4.2). *F. psychrophilum* does not form microcysts or fruiting bodies. The bacterium grows on Shieh's medium (Shieh, 1980), modified *Cytophaga* agar (Wakabayashi and Egusa, 1974), tryptone yeast extract (TYE) agar (Holt *et al.*, 1993) and modified Anackers medium (Lorenzen, 1993).

The salt tolerance of *F. psychrophilum* varies with the strain; however, 1.0–2.0% NaCl appears to be the maximum concentration for bacterial viability (Pacha, 1968; Bernardet and Kerouault, 1989; Holt *et al.*, 1993). *F. psychrophilum* produces a protease that is an important virulence

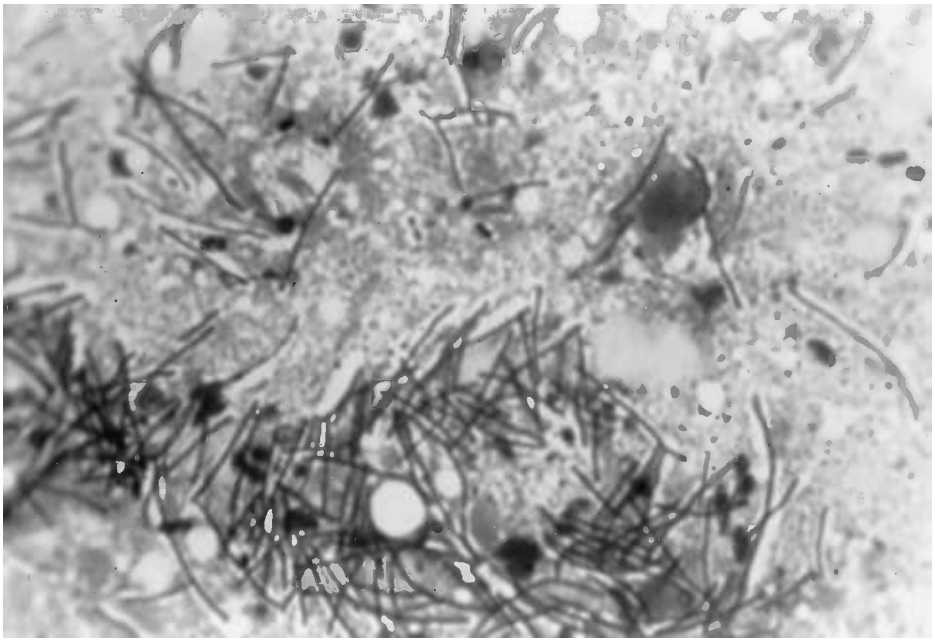

Fig. 4.2. Long filamentous rods of *Flavobacterium psychrophilum*. Fresh preparation.

factor. The optimal temperature for protease production corresponds to the environmental temperature recorded for many outbreaks of coldwater disease.

Lorenzen *et al.* (1997) applied DNA hybridization, plasmid profiling and examination of biochemical, physiological and morphological characteristics. They found similarities between Danish isolates and the type strain (NCIMB 1947) and with previously described French and American strains (Pacha and Porter, 1968; Holt, 1987; Bernardet and Kerouault, 1989). Lorenzen and Olesen (1997) further characterized *F. psychrophilum* isolates using serological methods. They found one major serotype (serotype Th), which could be further differentiated into one major (Th-1) and one minor (Th-2) subtype, and two minor serotypes (serotypes Fd and Fp^T). Serotype Fp^T included isolates from subclinical asymptomatic fish or from fish species other than rainbow trout. Chakroun *et al.* (1997) used random amplified polymorphic DNA (RAPD) analysis to differentiate strains of *F. psychrophilum* from different fish species and different geographical areas. They concluded that this method was efficient for typing *F. psychrophilum* strains and differentiation of *F. psychrophilum* from phylogenetically related species coexisting in the same environment.

Impact

The major fish affected by *F. psychrophilum* are rainbow and steelhead trout in cage culture, although outbreaks in cage-cultured coho salmon have been recorded (Borg, 1960). *F. psychrophilum* has also been isolated from the European eel (*Anguilla anguilla*), carp, tench (*Tinca tinca)* and crucian carp (Lehmann *et al.*, 1991). Fry and fingerlings are generally affected particularly if the skin is damaged (Madetoja *et al.*, 2000). Mortality can range from 20 to 90% (Bruno, 1992a). An increasing number of reports of coldwater disease in salmonids greater than 50 g have been reported; however, mortality is frequently lower (Brown *et al.*, 1997b).

Clinical signs

Disease signs are variable and include necrotic lesions in the dorsal and caudal areas, dark pigmentation, anaemia and exophthalmos (Bruno, 1992a; Holt *et al.*, 1993). In fingerlings, later stages show skin ulceration, most commonly on the peduncle and anterior portion of the body (Fig. 4.3). Internally, the fish show signs of general septicaemia, anaemia, severe splenomegaly, and haemorrhage in the body cavity and internal organs. During chronic infection the fish may exhibit spiral swimming behaviour (Kent *et al.*, 1989) or physical deformities such as lordosis or scoliosis (Conrad and Decew, 1967).

Clumps of weakly stained Gram-negative bacteria occur loosely on and around the gill arch and secondary lamellae, which show some hypertrophy (Bruno, 1992a). The lateral skin lesions display necrosis, pyknosis and lymphocyte infiltration of the dermis and underlying muscle blocks. These signs are apparently associated with relatively low numbers of *F. psychrophilum*. Filamentous rods may be located within the spleen, liver and kidney with evidence of focal necrosis. The liver also shows increased vacuolar degeneration, pyknotic nuclei and scattered necrotic hepatocytes. In the kidney an enhanced eosinophilia of the tubules, also with some pyknotic nuclei, is noted. Colonies of *F. psychrophilum* can occur within the lumen of the olfactory capsule and in association with the epithelial lining (Bruno, 1992a).

Diagnosis

F. psychrophilum is readily cultured and a presumptive identification is based on yellow colonies, growth at 17°C but not at 30°C, and confirmation by agglutination with polyclonal antisera against *F. psychrophilum*, or by a fluorescent antibody test (FAT) using anti-*F. psychrophilum* sera. Recently, Wakabayashi and Izumi (1997) have developed a PCR to detect the pathogen within tissues. Evensen and Lorenzen (1997) have also shown that immunohistochemistry can be a sensitive and specific technique for detecting *F. psychrophilum* in fish tissues, and have suggested that it may be used as a supplementary diagnostic tool. A sensitive nested-PCR assay for the detection of *F. psychrophilum* in water samples taken from a rainbow trout farm has recently been reported (Wiklund *et al.*, 2000).

Prevention and treatment

At present there is no vaccine for RTFS and various antibiotics are used to control the bacterium.

Conclusions

One major difficulty in the management of BCWD or RTFS is that the pathogen can survive in a wide range of environmental conditions. For example, *F. psychrophilum* can be isolated from hatchery water, from the surface of eggs and from within salmonid eggs (Brown *et al.*, 1997b), possibly having entered the egg during

Fig. 4.3. Gross lesion on farmed rainbow trout attributed to *Flavobacterium psychrophilum*.

water-hardening (Kumagai *et al.*, 2000). The possibility of vertical transmission of *F. psychrophilum* adds another dimension to the control of this disease. In addition, the bacterium is resistant to low concentrations of iodophore (Brown *et al.*, 1997b). The range of *F. psychrophilum*-like isolates has made classification confusing (Chakroun *et al.*, 1997; Lorenzen and Olesen, 1997; Lorenzen *et al.*, 1997) and further work is necessary before effective vaccines and rapid and sensitive diagnostic techniques can be formulated.

Bacterial kidney disease

Introduction

Bacterial kidney disease (BKD) is a systemic, chronic, bacterial infection of salmonids in both the fresh- and saltwater stages of their life cycle and a major bacterial problem of cage-cultured salmonids. Although known since the 1930s, the first major description of BKD was as Dee Disease following outbreaks in wild Atlantic salmon in the River Dee in Scotland (Smith, 1964). Since then, BKD has been reported throughout the world wherever salmonids are cultured or occur naturally, except Australia, New Zealand and the former USSR (Evelyn, 1988; Evenden *et al.*, 1993). The importance of vertical transmission of the causative agent, *R. salmoninarum*, will be discussed here and was mentioned briefly in Chapter 3.

Characterization

R. salmoninarum, the causative agent of BKD, was first cultured by Ordal and Earp (1956) and characterized by Sanders and Fryer (1980). The bacterium is a small (1.0 μm in length), Gram-positive, non-acid-fast, periodic acid Schiffs (PAS)-positive, non-sporulating, non-motile rod that is fastidious in its nutrient requirements (Bruno and Munro, 1986a). *R. salmoninarum* is slow-growing and a primary isolate often requires 8–12 weeks to establish growth at 15°C. Some refinements have been made in the techniques used to culture this bacterium, most notably the development of the nurse-culture technique by Evelyn *et al.* (1990). McIntosh *et al.* (1997) described a method of propagating *R. salmoninarum* in cell culture, specifically in EPC cells, thereby providing an alternative system for the study of *R. salmoninarum*.

Clinical signs

Clinical signs of BKD vary greatly and external signs include lethargy and darkening of the fish, exophthalmos, petechial haemorrhage (principally along the lateral line) and haemorrhage at the base of the fins (particularly notable on spawning salmon upon their return to fresh water). Internally, bloody ascites and enlargement of the kidney and spleen are often noted. White-grey granulomatous lesions can be seen in the internal organs, frequently in the kidney (Fig. 4.4). A pale membrane often encapsulates the spleen, kidney, heart and other organs (Bruno, 1986a). However, *R. salmoninarum* has been isolated from outbreaks where the fish have exhibited few or none of these signs.

Light microscopy

Histological lesions are reported elsewhere but notes on changes in the kidney are included here. During infection, tissue necrosis extends to large areas between the kidney tubules. Spherical granulomatous lesions containing *R. salmoninarum*, leucocytes and cellular debris can be distinguished. An opaque membrane may develop externally to the kidney capsule, appearing as thin layers of fibrin and collagen, which trap macrophages containing the bacterium, and are similar to the splenic membrane. Histologically, the glomerulus appears oedematous and *R. salmoninarum* can be detected intracellularly in endothelial cells lining the glomerular blood vessels and the lumen of the collecting ducts, although it is generally not within the proximal tubules. The nuclei of endothelial cells

containing *R. salmoninarum* are diffusely stained and slightly cloudy (Bruno, 1986a).

Transmission

The route of invasion, attachment mechanisms and intracellular survival of the pathogen are not fully understood. Young and Chapman (1978) were the first to describe the ability of *R. salmoninarum* to survive within macrophages. Viable bacteria are within the cytoplasm of host macrophages (Fig. 4.5). Bruno and Munro (1986b) located *R. salmoninarum* in developing oocytes of experimentally infected juvenile rainbow trout, and Gutenberger *et al.* (1997) provided additional evidence for an intracellular survival mechanism. This is thought to be important in survival of the bacterium, and a number of studies have investigated the interactions between *R. salmoninarum* and salmonid macrophages. Campos-Pérez *et al.* (1997) determined that live *R. salmoninarum* elicited respiratory burst activity in rainbow trout macrophages, and that this response was enhanced when heat-killed from naive or recovered fish, but not UV-killed bacteria were used. The exact mechanism that enables *R. salmoninarum* to survive in activated macrophages is not yet known. Evenden *et al.* (1993) suggested that this intracellular survival indicates that cellular transport is involved in spreading the pathogen throughout the host; however, Flaño *et al.* (1996) speculated that *R. salmoninarum* merely incubates within phagocytes and is disseminated via the blood.

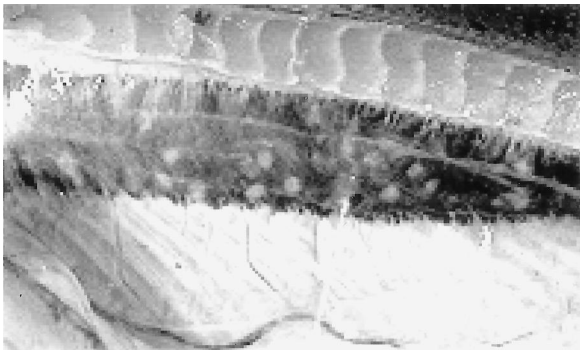

Fig. 4.4. White granulomatous lesions in kidney of farmed rainbow trout attributed to *Renibacterium salmoninarum*.

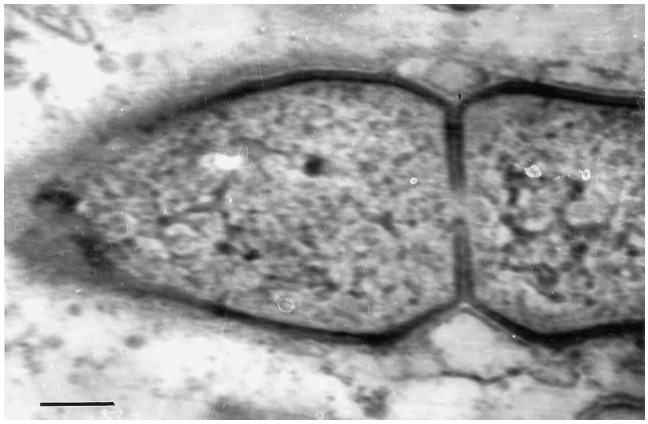

Fig. 4.5. Viable *Renibacterium salmoninarum* undergoing cell division within host macrophage. Bar, 0.1 µm.

Virulence mechanisms

Although the pathogenic mechanisms are poorly understood, progress has been made in recent years. Characterization of virulence factors or other compounds (e.g. immunosuppressive factors) produced by *R. salmoninarum* is of interest to researchers. The main virulence factor is the 57 kDa protein, known as antigen F, or more commonly known as p57 (Getchell *et al.*, 1985; Turaga *et al.*, 1987; Bruno, 1990). This protein is the major secretory antigen produced by *R. salmoninarum* (Kaattari *et al.*, 1987) and has been associated with virulence of the bacterium (Bruno, 1988). Furthermore, it has been shown *in vitro* to be haemagglutinating (Daly and Stevenson, 1987), leucoagglutinating (Wiens and Kaattari, 1991), able to adhere to salmonid erythrocytes (Kaattari *et al.*, 1986) and to agglutinate salmon spermatozoa (Daly and Stevenson, 1989). Additional properties for p57 include contribution to the hydrophobicity of the bacterial cell surface (Bruno, 1988; Daly and Stevenson, 1990). An attenuated non-hydrophobic strain of low virulence displaying little or no surface-extractable p57 has been described (Bruno, 1988). In this case virulence may be related to the reduced levels of putative protein p57 (O'Farrell *et al.*, 2000). Cell multiplication of all strains, but particularly non-hydrophobic strains, of high-affinity chelators is reduced, and Grayson *et al.* (1995) suggested that iron reductase is an important component of its iron acquisition mechanism. Rose and Levine (1992) demonstrated that p57 is involved in the attachment of *R. salmoninarum* via opsonization by complement component C3b and subsequent binding to the CR1 receptor. Brown *et al.* (1996) have shown that vertical transmission of p57 can result in partial immunotolerance to the protein, and that fry exposed as eggs to p57 have an increased susceptibility to subsequent horizontal transmission of the pathogen. Brook trout splenic cells incubated with 0.1 or 1.0 mg extracellular protein ml^{-1} showed significantly decreased respiratory burst activity, but not phagocytic activity, as compared with untreated controls (Densmore *et al.*, 1998). Barton *et al.* (1997) further showed that p57 and some of its derivatives undergo autolytic cleavage, releasing proteolytically active polypeptides, and that these peptides may have a common mechanism characteristic of a thiol proteinase. Two copies of the gene encoding p57 (*msa1* and *msa2*) have been found. Sequence data for these were identical in the attenuated and virulent strains. While p57 may be important for *R. salmoninarum* virulence, these data suggest that differences in localization and total p57 expression are not due to differences in *msa* sequence or differences in steady-state transcript levels (O'Farrell and Strom, 1999).

Barton *et al.* (1997) also detected a soluble polysaccharide-like material in extracellular products and tissues from infected fish. This polysaccharide may have structural similarity to the O-polysaccharide of *A. salmonicida*. Sørum *et al.* (1998) showed that a galactose-rich polysaccharide and a peptidoglycan are quantitatively the most important carbohydrate constituents of the *R. salmoninarum* cell wall. Antibodies against these components are readily detectable in the sera of Atlantic salmon immunized with cell wall preparations. Maulén *et al.* (1996) have suggested that invasin molecules are involved in adhesion and attachment to host cells.

Impact

BKD is responsible annually for direct losses due to direct mortalities and losses from poor growth rates in chronically infected fish (Bruno, 1986b; Evelyn, 1988). In addition to losses within cage-cultured salmon, *R. salmoninarum* has been responsible for significant losses within valuable stocks of wild fish (Elliott *et al.*, 1989; Jónsdóttir *et al.*, 1998; Kent *et al.*, 1998). Researchers are now attempting to investigate the effect of chronic BKD infections on salmonid populations (Mesa *et al.*, 1998, 1999).

R. salmoninarum has been isolated from salmonids worldwide. All species of salmonids can be affected, although Pacific salmon species are the most susceptible. The

pathogen has not been found in other fishes, or aquatic invertebrates (Evelyn, 1988; Sakai and Kobayashi, 1992). Although Evelyn (1988) has shown that *R. salmoninarum* can survive for a limited time in fresh water and saltwater, the bacterium can certainly survive outside the host for sufficient time to ensure transmission to other salmonids, possibly within sediments and/or faecal matter (Austin and Rayment, 1985; Balfry *et al.*, 1996). The occurrence of *R. salmoninarum* within tissue adhesions attributed to vaccine adjuvant in the body cavity has been highlighted (Bruno and Brown, 1999) and it has been suggested that subclinical infections may not be detected in these fish.

Diagnostic techniques and prevention

Culture is a sensitive method for detecting *R. salmoninarum*; however, the slow growth of the bacterium often makes this method impracticable. Immunoassays, e.g. FATs, are routinely performed on tissues taken from salmonids, using polyclonal antisera against *R. salmoninarum* (Evelyn *et al.*, 1981). ELISAs are generally sensitive and are used for screening wild and farmed populations (Olea *et al.*, 1993). Commercially available kits have been shown to vary in their efficacy (Bandin *et al.*, 1996). In North America most broodstock are screened for *R. salmoninarum* with ELISA or FATs (Elliott *et al.*, 1989), on kidney or ovarian fluid. These are carried out using polyclonal or monoclonal antisera against p57. However, there are limitations to the efficacy of these tests. Several researchers have reported cross-reactions with other bacterial species when using the antisera against *R. salmoninarum* or against p57 (Bullock *et al.*, 1980; Austin and Rayment, 1985; Brown *et al.*, 1995). To avoid false-positive reactions, monoclonal antibodies directed against p57 have been used (Wiens and Kaattari, 1989; Hsu *et al.*, 1991). Others have used Western blot analysis (Olivier *et al.*, 1992) to demonstrate the serologically reactive antigen migrating in an SDS–polyacrylamide gel to a position identical to that of p57. However, Olivier *et al.* (1992) concluded that the technique was not sufficiently sensitive to detect small amounts of p57 and thus would be useful only in diagnosing active *R. salmoninarum* infections. Other limitations of the ELISA screening method include the difficulties associated with establishing a reliable and consistent negative–positive threshold optical density value (Meyers *et al.*, 1993).

Molecular techniques hold promise for sensitive assays. PCR has been used to detect nucleic acid amplified from a variety of pathogens of aquatic vertebrates and invertebrates (Gustafson *et al.*, 1992; Kellner-Cousin *et al.*, 1993). It has been applied to detect *R. salmoninarum* within salmonid tissues (Brown *et al.*, 1994; Léon *et al.*, 1994; Magnússon *et al.*, 1994), even within individual salmonid eggs (Brown *et al.*, 1994), and the technique is sensitive and specific (Brown *et al.*, 1995). 16S RT–PCR has also been shown to be very effective for screening tissues, including blood and ovarian fluid (Rhodes *et al.*, 1998). Pascho *et al.* (1998) showed nested-PCR to be a significantly more sensitive method than membrane FAT and ELISA for detecting *R. salmoninarum* in ovarian fluid of broodstock salmon. Recently, Cook and Lynch (1999) have used a nested-RT–PCR assay to detect mRNA from between one and ten *R. salmoninarum* cells seeded into kidney homogenate. They suggested this approach was a better indicator of viable bacteria than PCR.

At present, the only preventive measure is avoidance of infection, as an effective vaccine has yet to be developed for widespread use. Recently, there have been a number of reports suggesting progress in this area. The work by Sørum *et al.* (1998) on surface polysaccharides and peptidoglycans suggests a promising area for vaccine research. Griffiths *et al.* (1998) reported promising results when they vaccinated Atlantic salmon with an avirulent strain of *R. salmoninarum* developed by Bruno (1988). Piganelli *et al.* (1999a) decreased the amount of cell surface p57 by incubating at 37°C for > 4 h. They subsequently showed that coho salmon immunized (intraperitoneal or intramuscular injection) with these p57 reduced cells showed a significantly increased mean

time to death when challenged with live *R. salmoninarum*. Fish orally immunized with the p57 reduced cells demonstrated a significantly lower concentration of the protein 150 days after bath-challenge with *R. salmoninarum* (Piganelli *et al.*, 1999b). These findings suggest that the p57 *R. salmoninarum* may be effective as an oral BKD vaccine.

Future studies

There are gaps in our knowledge of the host–pathogen interactions, the mechanisms of *R. salmoninarum* pathogenicity and intracellular survival. We suggest that this area of research could prove fruitful. Similarly, the most sensitive diagnostic assay is not effective unless the epizootiology of the pathogen is known. For example, what is the minimum number of eggs required within a population that must be positive for *R. salmoninarum* before horizontal transmission will occur within that population? Under what conditions of density, temperature change, handling and other stressors is an outbreak most likely to occur? What is the significance of vertical transmission of antigens of *R. salmoninarum* to disease resistance?

Salmonid rickettsial septicaemia (piscirickettsiosis)

Introduction

Piscirickettsiosis caused by *Piscirickettsia salmonis* is primarily a disease of fish reared in the marine environment (see Chapter 3); however, reports from freshwater hatcheries are discussed briefly in this chapter. Furthermore, this disease is considered as an emerging problem for cage culture (Chapter 9).

Transmission

Natural outbreaks of piscirickettsiosis occur a few weeks after smolts are transferred to the sea, suggesting that the oral route is not the normal method of infection, particularly as Smith *et al.* (1999) have demonstrated that intact skin and gills are penetrated by *P. salmonis*, the causative organism. Sea lice have also been implicated in transmission, but at the present time this area is unclear.

Vertical transmission is suspected in coho salmon, and consequently salmon farmers in Chile now routinely screen their broodstock to limit spread of infection. In the laboratory (L.L. Brown, unpublished data), culturable *P. salmonis* were recovered from Atlantic salmon eggs and embryos that had been microinjected with *P. salmonis* cells just before fertilization. The eggs were fertilized after injection, incubated in flowing water and examined at the eyed and hatch stage of development for *P. salmonis*. The presence of the pathogen within each egg or embryo was determined by culture on CHSE cells, by IFAT and by Giemsa stain. These results suggest that *P. salmonis* survives within the salmonid egg at least until after hatch, and further, that the bacterium may be able to enter eggs via infected ovarian fluid and/or milt.

Natural outbreaks in fresh water

Piscirickettsiosis was initially described from fish in the marine environment, but natural outbreaks among rainbow trout and coho salmon in fresh water have also been reported in Chile (Bravo, 1994). The lesions in moribund trout were similar to those observed in marine outbreaks and *P. salmonis* was confirmed using a fluorescent antibody test.

Summary

P. salmonis is a Gram-negative obligate intracellular bacterium that causes a serious, systemic infection of salmonids in seawater in Chile and to a lesser extent in other countries. *In vitro* isolation requires culture on fish cells without antibiotics. Horizontal and vertical transmission of *P. salmonis* has been implicated. Similar pathogenic rickettsial organisms have been reported affecting salmonids cultured in

saltwater sites in Canada, Ireland, Norway and South Africa.

Diseases Caused by Pseudofungi

Saprolegnia

Introduction

The *Oomycetes* are widespread in fresh water and are the most important group of water moulds or pseudofungi affecting wild and cultured fish. The most significant genera, particularly with respect to their impact on sexually mature broodstock and eggs, is *Saprolegnia* (Hatai and Hoshiai, 1993). Mycelial growth may occur anywhere, but normally appears as conspicuous cottony tufts of non-septate filaments around the head, caudal and anal fin (Noga, 1993). For a recent review on *Saprolegnia* and other *Oomycetes* see Bruno and Wood (1999).

Characterization

A current accurate taxonomic status of *Saprolegnia* and other *Oomycetes* is uncertain, because it relies upon the morphology of reproductive structures (Dick *et al.*, 1984; Beakes *et al.*, 1994). Consequently, identification of asexual pathogenic *Saprolegnia* isolates using classical taxonomic criteria is impractical. Willoughby (1978) subdivided *Saprolegnia diclina* into three subspecific groups based upon oogonial morphology. *S. diclina* Type 1 infects salmonids and is synonymous with *Saprolegnia parasitica* and *Saprolegnia* sp. Type 1 (Kanouse, 1932; Pickering and Willoughby, 1977). Type 2 occurs as a parasite of coarse fish and Type 3 is entirely saprophytic, with the zoospores showing direct germination (Hatai and Hoshiai, 1992). The *Oomycetes* isolated from fish are generally assigned to a single major cluster forming a coherent, separate taxon, *S. parasitica* Coker (synonym *S. diclina* Humphrey Type 1) (Willoughby, 1978). Several characteristics including cell wall composition and rDNA sequences differentiate the water moulds from the true fungi. Molecular sequences show their phylogenetic roots with the *Chromista*, the chromophyte algae and other *Protista*, rather than the true fungi (Dick, 1990; Kwon-Chung and Bennett, 1992).

Impact

Infection of eggs, fry and larger fish by water moulds is a widespread problem in cultured fish. Overcrowding, handling, temperature changes, increased organic loading, parasitism and sexual maturation increase the possibility of *Saprolegnia* infection (Pickering, 1994). Losses up to 50% of farmed catfish occur during severe winters in the USA, with an annual economic cost of US$40 million (Bly *et al.*, 1994). Among commercial catches of Atlantic menhaden (*Brevoortia tyrannus*), up to 80% of the stock may have an ulcerative mycosis (Lilley and Roberts, 1997). The economic loss is estimated at US$27 million per annum. In Japan annual losses may exceed 50% of farmed coho salmon (Hatai and Hoshiai, 1993).

Species affected

Saprolegnia sp. has been isolated from Atlantic salmon, rainbow trout, brown trout, Arctic char (*Salvelinus alpinus*) and coho salmon (Pickering and Christie, 1980; Wood and Willoughby, 1986; Hatai and Hoshiai, 1993). *S. parasitica* and *S. diclina* have been implicated in mortality of cultured rainbow trout, coho salmon and ayu (*Plecoglossus altivelis*) in Japan (Yuasa and Hatai, 1995) and infections of *S. diclina* have been reported in spawning rainbow trout in Taiwan (Chien, 1980).

Predisposing factors

The *Oomycetes* are ubiquitous, therefore fish are continually exposed to potentially pathogenic zoospores. However, infection normally only results from a change in environmental factors, predisposition or immunosuppressive components (Bly *et al.*, 1994). In wild Atlantic salmon concurrent infestations with *Saprolegnia* sp. and

Gyrodactylus salaris (Johnsen, 1978) have been reported following sustained damage to the skin by the parasite. The association of sexual maturity with the elevated occurrence of infection is attributable to skin damage resulting from spawning activities (Richards and Pickering, 1978). Susceptibility to saprolegniasis may also be associated with an increase in cortisol and certain reproductive hormones (Pickering, 1977). Quiniou *et al*. (1998) proposed that a reduction in water temperature reduced mucus cell numbers allowing the attachment and germination of *Saprolegnia* cysts.

Clinical signs

Cotton wool-like circular tufts have been recorded on the integument and gills of host fish or eggs (Willoughby, 1989). Infection normally occurs in the epidermis and dermis where more than one species may be present (Pickering and Willoughby, 1982). Pathogenic members of the *Saprolegniaceae* may also be seen to surround and penetrate gill tissue (Fig. 4.6) (Hatai and Egusa, 1977; Bruno and Stamps, 1987).

Diagnostic techniques

Identification of oomycete fungi has relied on morphology and sporulation characteristics (Seymour, 1970; Willoughby, 1978). Sexual reproductive stages are required to enable accurate identification (Wood and Willoughby, 1986). However, some isolates take long periods to produce oogonia and this makes identification difficult (Pickering and Willoughby, 1982). Features such as size, shape and nature of the oogonial surface and wall have been used to identify *S. parasitica* (Hatai *et al*., 1990).

Low-nutrient culture media with or without antibiotics are used for the isolation and culture of fungi. Agar plates are inoculated and incubated between 5 and 37°C and observed for newly emerging hyphae tips (Noga and Dykstra, 1986).

A PCR to examine rDNA from wide-ranging *Saprolegnia* isolates was developed with the use of the endonuclease *Bst*UI, which produced identical fingerprints from all strains of *S. parasitica* (Molina *et al*., 1995). The authors suggested this could be used as a diagnostic test in the absence of antheridia and oogonia.

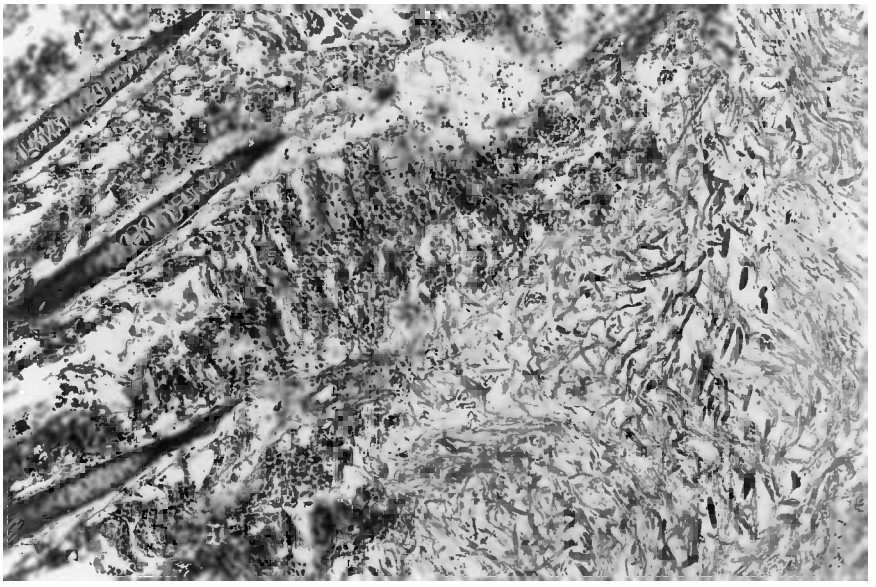

Fig. 4.6. Widespread necrosis and sloughing of gill lamellae following infection by *Saprolegnia* sp.

RAPD using PCR with DNA from *S. diclina–parasitica* isolates was developed by Diéguez-Uribeondo *et al.* (1996). Spanish isolates have a genetic similarity of 85–100%, compared with a 20–45% similarity with other strains of the complex. Yuasa *et al.* (1977) highlighted a simple method to distinguish between *S. parasitica* and *S. diclina* based upon spore-germination type. Observations of a zoospore suspension in diluted Griffin's (GY) broth revealed a distinction between *S. diclina* (i.e. direct germination) and *S. parasitica* (i.e. indirect germination) (Yuasa *et al.*, 1977).

Prevention

The control of fungi on eggs requires the removal (by hand) of dead or infected eggs at regular intervals, and/or chemical bath treatments. Malachite green has been the traditional chemical used in fish aquaculture facilities to control or prevent external fungal outbreaks (Foster and Woodbury, 1936).

Treatment is successful in fish culture (Bailey, 1984) at concentrations of 3–5 mg l^{-1} for a 60 min exposure (Marking *et al.*, 1994). However, because of toxicological and possible mutagenic properties, the use of malachite green has been curtailed or prohibited in many countries (Schnick and Meyer, 1978).

Buffered bicarbonate iodophors are generally used to disinfect eyed ova (Marking *et al.*, 1994). Eggs are flushed using 100 mg available iodine l^{-1} in the disinfectant bath for 5–15 min and then rinsed thoroughly. For small numbers of fish, a dip treatment in the iodophors of around 30 s is adequate.

Formalin has been effective in controlling fungal infections on rainbow trout and on eggs at 250 mg l^{-1} for a 60 min exposure (Bailey and Jeffrey, 1989; Marking *et al.*, 1994). Schreier *et al.* (1996) concluded that *S. parasitica* was controlled on rainbow trout eggs that received prophylactic formalin treatments on alternate days.

Hydrogen peroxide has been successfully tested as fungicide against *Saprolegnia* on developing rainbow trout and chinook salmon (Marking *et al.*, 1994; Schreier *et al.*, 1996). Dawson *et al.* (1994) noted that exposure of eggs to a prophylactic treatment of 250–500 ml hydrogen peroxide l^{-1} (based on 100% active ingredient) for 15 min, on alternate days, inhibited fungal infection on healthy eggs.

The fungistatic effect of sodium chloride has been examined and improvements in hatching rates recorded (Phelps and Walser, 1993). Using a continuous sea-salt bath treatment, Phelps and Walser (1993) reported an improvement in the hatching success of channel catfish eggs.

Pottinger and Day (1999) used a biocide, bronopol (2-bromo-2-nitropropane-1,3-diol), formulated as Pyceze, to prevent or reduce infection with *S. parasitica* in rainbow trout and to protect eggs from infection, and suggest this is a safer alternative to malachite green. The effects of the biocide on embryonic stages of fish and the growth and survival of developmental stages need to be determined.

An alternative approach using elevated water flow for egg incubation was presented by Rach *et al.* (1995). They reported that eggs maintained at a flow rate of between 300 and 600 ml min^{-1} did not roll in the water and had a reduced rate of infection and hatching success. At 1200 ml min^{-1} the eggs were lifted into the water column and rolled moderately, resulting in an improved hatch with an absence of fungal growth.

Future studies

Oomycetes have their phylogenetic origins with the chromophyte algae, rather than the true fungi, and PCR techniques continue to enhance our taxonomic knowledge of this group. Further development of these and other techniques, such as RAPD–PCR, may help to resolve taxonomic difficulties, particularly as new species are being described (Willoughby, 1998; Bangyeekhun *et al.*, 2001). Research is also required into the lack of immune response to infection (e.g. leucocytic infiltration into pseudofungal-infected tissue as seen in channel catfish during the winter months and the inactivity of the complement system).

Diseases Caused by Parasites

Whirling disease

Introduction

Whirling disease (WD) is a widespread, chronic parasitic infection of salmonids caused by *Myxobolus cerebralis* (Protozoa: Myxosporea). This parasite infects the head cartilage and is responsible for major economic losses to some wild and cultured trout in Europe and North America. WD was first reported in 1893 in central Europe and named for the erratic, tail-chasing, whirling motion in young fish (Hofer, 1903). Lesions in skeletal tissues and spores, particularly in the head cartilage, are used for diagnosis. In advanced stages of the disease, spores are in the spine and infected fish appear black and may be twisted posterior to the dorsal fin. Alevins often suffer 100% mortality, although there is a reduced mortality and infection in older trout.

Characterization

WD is caused by the sporogonic stage of *M. cerebralis* and is the only myxosporean in the cartilage of salmonids. A two-host life cycle involves a fish and the aquatic oligochaete worm, *Tubifex tubifex* (Markiw and Wolf, 1983; Wolf *et al.*, 1986). A re-examination of the ultrastructural features and ribosomal subunit gene sequences of myxozoans was made by Siddall *et al.* (1995) and they concluded the myxozoans were a class of highly derived parasitic cnidarians.

Impact

A heavy infection of *M. cerebralis* in young fish can result in high mortality or unmarketable, deformed individuals. Mortality among newly hatched fish may approach 90% following exposure to the infective agent as sac fry. However, 1-day-old rainbow trout appear refractory (Markiw, 1991). In many areas of the USA, WD has been implicated in the severe decline or elimination of some year classes (Vincent, 1996).

Susceptibility and host range

Young rainbow trout are most susceptible, although lake trout are apparently refractory (O'Grodnick, 1979). Other salmonids can be infected, but clinical signs of the disease may not develop. Susceptibility varies with age, species, strains and even among fish within a population (Markiw, 1992). The following list ranks species in descending order of apparent susceptibility: rainbow trout, sockeye salmon, golden trout (*Oncorhynchus aguabonita*), cutthroat trout (*Oncorhynchus clarki*), brook trout, chinook salmon, Atlantic salmon, brown trout, coho salmon, lake trout and splake (hybrids between brook trout and lake trout).

Reservoir of infectivity

The source of the infective agent for fish is usually the water supply or earthen ponds inhabited by the tubificid worm, *T. tubifex*. Predators and scavengers such as birds, which consume infected fish, can release viable spores into the environment and therefore distribute the parasite (El-Matbouli and Hoffmann, 1991a). Trout and salmon infected with WD may harbour spores throughout their life. El-Matbouli and Hoffman (1991b) found *M. cerebralis* spores can survive freezing for at least 3 months.

Geographic range

WD originated in rainbow trout in Germany, but trade in the movement of fish has helped spread the disease to many countries (Halliday, 1976), including New Zealand (Hewitt and Little, 1972) and the USA (Modin, 1998; Hoffman, 1990).

Clinical signs

In infected fish, abnormal whirling behaviour and dark or black caudal peduncle and tail fin can be used as a presumptive diagnosis. The whirling behaviour is attributed to impaired coordination caused by neural damage. Deformities, including shortening of the snout, axial skeleton or head, and

cranial depressions, may occur and persist through the life cycle; however, injury or deficiency in dietary ascorbic acid can result in similar signs (Wolf *et al.*, 1981). Clinical signs are also influenced by the intensity of infection and water temperature. During gross examination internal organs appear normal. The pathogen is difficult to eradicate, and the use of earthen ponds for rearing juveniles is an ideal habitat for tubifex. Fish should be cultured in spore-free water, using smooth concrete raceways, plastic-lined raceways or in ponds that are regularly disinfected.

Histological sections of cartilage, particularly the skull, gill and vertebrae, stained with methylene blue, Giemsa or May–Grünwald Giemsa, show areas of lysis, inflammation and digestion of the cranial cartilage. The presence of *M. cerebralis* spores in the cartilage is considered pathognomonic for WD. However, infection in older fish is usually asymptomatic.

Life cycle

The WD protozoan has a two-host life cycle involving a fish and the aquatic oligochaete *T. tubifex* (Wolf *et al.*, 1986). Two separate stages of sporogony occur, one in each host. Spores of *M. cerebralis* are released into the environment when infected fish die or are consumed by predators. The myxosporean-type spores are ingested by these oligochaetes where they develop in the gut epithelium to the actinosporean triactinomyxon, after which infected worms release many mature forms into the water. The triactinomyxon stage enters susceptible fish through the epithelium and buccal cavity, particularly at the base of the gills and the oesophagus. The parasite reaches the cartilage via peripheral nerves and the central nervous system (El-Matbouli *et al.*, 1995). Scanning electron microscopy studies on the triactinomyxon of *M. cerebralis* have shown that attachment and complete penetration of their sporoplasm germs occur as early as 1 min post-exposure (El-Matbouli *et al.*, 1999). This was correlated with increased convulsive movements and increased mucus from the trout host (El-Matbouli *et al.*, 1999). Development time for the myxosporean in fish and the actinosporean in tubificids is directly related to temperature. Trout fry fed infected worms or exposed to water-borne triactinomyxon show black tails after 35–45 days at a water temperature of 12.5°C. Whirling behaviour frequently appears at the same time. Fully mature spores can be detected after 2.6–3.5 months at 12.5°C.

Identification

During hatchery inspections young, susceptible fish from earthen ponds instead of concrete raceways should be examined for *M. cerebralis*. The head of an anaesthetized fish is cut sagittally and a smear examined at ×20 or ×40 magnification.

Fresh or frozen gill arches should be removed, ground in several volumes of water, allowed to settle and then drops of the supernatant examined microscopically. Negative samples or lightly infected carriers (about 100 spores per head) should be subjected to a spore-concentrating procedure (O'Grodnick, 1975) or the pepsin–trypsin–dextrose (PTD) digestion method (Markiw and Wolf, 1974). Purified DNA has been used in a nested-PCR for detecting *M. cerebralis* from oligochaetes (Rognlie and Knapp, 1998) and all stages of infection in fish (Andree *et al.*, 1998).

Mature spores are lenticular in side view and nearly circular when viewed from the front. The spores are 8–10 μm in diameter and have two prominent ovate polar capsules with coiled filaments, which may be extruded in certain situations (Lom and Hoffman, 1971). The identification of *M. cerebralis* in sections from lesions of skeletal tissue is recommended for diagnosis. However, this may not be reliable with lightly infected fish that have only a few spores present. FAT examination of resin-embedded sections of infected rainbow trout has been used to detect *M. cerebralis* spores and pre-spore stages (Hamilton and Canning, 1988). The specific fluorescence of older specimens stored in formalin for a week or more is reduced and in older specimens is insignificant or non-existent.

Control

Rainbow trout produce antibodies against *M. cerebralis*, although protection against infection has not been shown (Halliday, 1974). However, host-tissue reaction against the pathogen can decrease or even eliminate myxosporean infections in lightly infected rainbow trout (Markiw 1992), showing that immunization might be practical. Hoffman (1990) has presented a review of management practices for infected fish in hatcheries.

There is a decrease in spore development in young trout fed furazolidone or fumagillin (Taylor *et al.*, 1973; O'Grodnick and Gustafson, 1975). However, El-Matbouli and Hoffmann (1991a) reported that fumagillin, fed to experimentally infected rainbow trout, caused defects in the morphology of *M. cerebralis* spores and prevented a clinical outbreak of WD.

Future studies

WD is determined by the intensity of infection and not simply by the presence of spores. Therefore, control measures do not need to eradicate the parasite completely. Measures such as culturing resistant species, filtering the water supply, chemotherapy and periodical disinfection of the facility help in WD control. Although control of *M. cerebralis* is difficult, preventive measures can decrease the intensity of the disease and perhaps eliminate the spread to non-infected areas. Research is also required to link known species of actinosporidia to species of myxosporidia. Further research into the possible mechanical and chemical stimulation of the triactinmyxon spores of *M. cerebralis* in locating and attaching to the host is also suggested.

Proliferative kidney disease

Introduction

Proliferative kidney disease (PKD) is an economically important myxozoan of cultured salmonids in fresh water that primarily affects the kidney and spleen. It is caused by the extrasporogonic stage of an enigmatic myxozoan, referred to as PKX (Kent and Hedrick, 1985). PKX is presumed to be in the family Sphaerosporidae, genus *Sphaerospora* (Hedrick *et al.*, 1993). This disease causes severe losses in hatchery-reared salmonids in Europe and western North America (Clifton-Hadley *et al.*, 1984; Smith *et al.*, 1984, Hedrick *et al.*, 1993). Mortalities are highest during the summer months and fry are the most severely affected. Recently, our understanding of the parasite has improved with confirmation that some bryozoans harbour the mature myxospore stages (Anderson *et al.*, 1999; Longshaw *et al.*, 1999; Canning *et al.*, 1999; Feist *et al.*, 2001). Reviews on PKD and a description of the disease and its causative agent have been published (Clifton-Hadley *et al.*, 1984; Hedrick *et al.*, 1986, 1993; Morris *et al.*, 2000).

Characterization

There are two developmental stages in the salmonid host kidney: the extrasporogonic and the sporogonic stages. The extrasporogonic stage proliferates in the kidney interstitium and is often seen in tissue sections or in stained kidney imprints. PKX typically appears as large cells (≥ 20 µm) with a distinct plasmalemma (Ferguson and Needham, 1978). Sporogenesis occurs in the renal tubules. Also characteristic of PKX is the frequent appearance of cells within a cell, or endogeny (Seagrave *et al.*, 1980). This can be seen in both extrasporogonic and sporogonic stages; internal secondary cells are within primary cells. The secondary cells frequently contain internal tertiary cells and form the sporogonic stages in the kidney tubule lumen (Kent and Hedrick, 1986).

Anderson *et al.* (1999) have shown that there is a significant similarity between the reference 18S rDNA sequence of PKX in the databank and sequences obtained from myxozoan parasites in North American bryozoans and European and North American PKX. They suggested that Bryozoa (*Pectinatella magnifica*, *Cristatella mucedo*

and *Plumatella rugosa*) are natural hosts for PKX, and that transmission and ultrastructural studies were warranted. Recently, Longshaw *et al.* (1999) reported the occurrence of PKX in a further two species of Bryozoa, *Plumatella emarginata* and *Fredericella sultana*, in the UK. Canning *et al.* (1999) identified a new species of the myxozoan genus *Tetracapsula* as the PKX organism, and proposed the name *Tetracapsula bryosalmonae*. They also successfully experimentally transmitted *T. bryosalmonae* from infected bryozoans (*F. sultana*) to naive rainbow trout

Impact

PKX is thought to be restricted to the family Salmonidae, although there is some evidence that similar infections, possibly related to other bryozoan myxozoans (*Tetracapsula* spp.), may be involved in pike and carp infections with PKX-like organisms (Bucke *et al.*, 1991; Voronin and Chernysheva, 1993). PKD affects Arctic char and rainbow trout and to a lesser extent brown trout, Atlantic salmon and Pacific salmon species. During summer months, morbidity can reach 60–100%.

Clinical signs

Clinical signs associated with PKD outbreaks include exophthalmos, pale gills, abdominal swelling and darkening. Internal gross signs include renal swelling and discoloration, ascites production, often with associated blood, splenomegaly and yellowish liver (Hedrick *et al.*, 1993).

Diagnostic techniques

Light microscopy examination is used for a presumptive diagnosis. The PKX extrasporogonic stages can be visualized directly in tissue squashes or stained imprints. Also, the characteristic 'swirls' can be seen within the kidney interstitium. These are caused by the progressive replacement of stem cells by a mixed cell infiltrate of macrophages and lymphocytes (MacConnell *et al.*, 1989).

Histopathological signs range from a mild haematopoietic hyperplasia early in the infection to a severe granulomatous response (Ferguson and Needham, 1978). This corresponds the proliferation of the extrasporogonic stages (Clifton-Hadley *et al.*, 1987). The first PKX cells can be seen 2–3 weeks post-exposure, at water temperatures greater than 15°C. These cells can most often be seen in the blood sinuses (Kent and Hedrick, 1986).

Discovery of a lectin that binds to PKX and the development of an mAb against the parasite has greatly aided diagnosis of PKD (Castagnaro *et al.*, 1991; Adams *et al.*, 1992). Saulnier and de Kinkelin (1996) produced 11 mAbs specific for PKX to identify several proteins of PKX, one of which is thought to have important antigenic properties. The study resulted in the establishment of a set of immunological probes that can be useful for diagnosis, expression and antigenicitiy studies. Saulnier *et al.* (1996) cloned, sequenced, and expressed a cDNA encoding a PKX antigenic protein of *c.* 13 kDa. This protein can be used in diagnostic applications as well as for research on the antigenicity, pathology and epizootiology of the parasite.

Saulnier and de Kinkelin (1997) have developed primers for PCR-based detection of PKX. These primers were deduced from the sequence of the small subunit rRNA gene (SSUrDNA). They suggested that the primers could be used for PCR detection of PKD in subclinical infections, for screening populations in fisheries management, and for further studies on the intermediate or definitive hosts of PKX.

Prevention and treatment

There is no vaccine to prevent PKX, therefore screening is the only available preventive measure. Molecular studies described point to some candidates for vaccine research; however, to date this has not been accomplished.

Higgins and Kent (1996) found that oral treatment with fumagillin (3.0 mg kg^{-1} fish) for 14 days after the first detection of PKX within kidney tissue resulted in a

significant reduction in the prevalence of infection.

Ceratomyxa shasta

Introduction

Ceratomyxa shasta is a freshwater myxosporean and the causative agent of ceratomyxosis. The parasite causes high mortality in wild and captive-reared juvenile and adult salmonids in the western USA, particularly in the Columbia River basin. A massive infiltration, occlusion and destruction of several tissues by the infective stage are a direct cause of mortality.

Characterization

The aetiological agent, *Ceratomyxa shasta* (Protozoa: Myxosporea), was first described by Noble (1950) from hatchery-reared rainbow trout. This work established *C. shasta* as a new species and the first of this genus to parasitize freshwater fish.

Impact

Ceratomyxosis is recognized as an economically important condition and significant losses can occur in hatchery-reared and wild juvenile salmonids, with prespawning mortality in adult salmon. In the latter, a disease incidence as high as 94% has been reported (Chapman, 1986). In young fish, up to 100% mortality may occur. Epizootics have been reported in chinook, chum and coho salmon, and rainbow and cutthroat trout, but with variability between strains of fish (Ibarra *et al.*, 1994). Infected anadromous salmonids retain the parasite and continue to die during their marine migration. Prevalence data varies considerably with species, season and specific sampling site in a river (Margolis *et al.*, 1992).

The life cycle of *C. shasta* has not been fully described, although it is known that susceptible fish become infected while residing in or migrating through waters that contain the infective stage of the parasite (Johnston, 1980). At low temperatures (4–6°C), the progress of the disease is slow and accounts for the seasonal nature of ceratomyxosis that is generally reported between May and November (Ching and Munday, 1984).

The parasite occurs within a well defined geographic area including areas of the states of Oregon, Washington and Idaho; California north of a line due east from the southern tip of San Francisco Bay; British Columbia, south of a line east from the northern tip of Vancouver Island; and the Yukon River in Canada and Alaska (Hoffmaster *et al.*, 1988; Follett *et al.*, 1994). Distribution of *C. shasta* in these regions has apparently expanded, although this could be related to increased spread of the disease, although conversely may have occurred through improved monitoring and detection methods.

Clinical signs

The parasite manifests itself in the gut, liver, spleen and muscle, causing haemorrhage. In rainbow trout, anorexia, lethargy, dark appearance and exophthalmia are recorded, with abdominal distension due to production of a spore-containing fluid (Schafer, 1968). The intestinal tract of juvenile fish becomes swollen and haemorrhagic and the content becomes mucoid, with caseous material present in the intestine and pyloric caeca. The entire digestive tract including the liver, gall bladder and spleen, and the kidney, heart, gills and skeletal muscle may also become haemorrhagic and necrotic (Wales and Wolf, 1955). In infected juvenile chinook salmon, the fish become emaciated and then later develop large fluid-filled blebs and kidney pustules (Conrad and Decew, 1966). Infected adult salmon can develop nodular lesions in the intestine that perforate and contribute to fish mortality. These nodules may be accompanied by gross lesions in the liver, spleen, kidney and musculature.

Trophozoites in the mucosa cause a strong inflammatory response in the lamina propria. As the infection progresses, the parasite multiplies in all layers of the intestine and causes severe inflammation and desquamation of the mucosal epithelium.

Trophozoites penetrate the intestinal tract, spread into the surrounding adipose tissues and enter the bloodstream, where they are carried to other tissues.

Histologically there is enlargement of the connective tissue within the intestinal caeca and massive infiltration by developing trophozoites and other developmental stages. Granulomatous lesions may develop in the viscera, causing peritonitis. The occlusion and destruction of the intestinal lumen is considered to be the cause of the rapid rise in mortality among infected fish.

Life cycle

The actinosporean stage of the cycle of *C. shasta* is completed through a freshwater polychaete annelid worm, *Manayunkia speciosa* (Bartholomew *et al.*, 1997).

Characterization

Spores are evident only at the terminal stages of infection and are identified by their size, shape and location. Several techniques have been suggested for spore preparation (Amos, 1985), and include wet mounts from the lower intestinal wall, ascitic fluid or gall bladder. Alternatively, air-dried smears stained using Ziehl Neelsen (ZN) without heat fixation, or smears fixed with Schaudin's fixative and then stained with Heidenhain's iron haematoxylin can be used. In stained smears the polar capsules stain red against a bluish sporoplasm and background. The trophozoites are rounded but variable in shape, and mature to form a sporoblast that usually contains 12 nucleated cells producing two groups of six cells (sporonts) and the formation of two spores in each mother cell (Yamamoto and Sanders, 1979).

Diagnosis

A presumptive clinical diagnosis, based on inflammation of the intestine, is confirmed by microscopical examination of spores in intestinal scrapings (Zinn *et al.*, 1977). The spores are evident at the terminal stages of infection and are identified by their size, shape and location. The ends of the spores are rounded, reflected posteriorly with a distinct suture line (Noble, 1950) and measure 14–23 m by 6–8 m.

The variability in size and shape of the trophozoites and their similarity to this stage in other myxosporea makes diagnosis using light microscopy difficult. Consequently, serological techniques have been developed using mAbs. The antibodies produced react specifically with the pre-spore stages and do not cross-react with trophozoite or spore stages of other myxosporeans. Hence, the use of mAbs and fluorescein or enzyme-conjugated secondary antibodies has enabled the reliable detection of early infections (Bartholomew *et al.*, 1989). A primed PCR designed to generate a specific marker for *C. shasta* DNA has been developed by Bartholomew *et al.* (1995) and is useful for detecting low levels of the parasite. The sequencing of the single-stranded rDNA (Bartholomew *et al.*, 1997) allowed the development of a sensitive PCR assay for *C. shasta* based on this sequence (Palenzuela *et al.*, 1999).

Prevention

There is no current chemotherapy for ceratomyxosis, although a selection factor on fish migrating through enzootic areas may confer some resistance. Sanders *et al.* (1972) suggested that contaminated water supplies may be filtered and treated using UV sterilization or chlorine, and that controlling the movement of eggs or live fish from *C. shasta*-endemic areas to those free of this disease can be used to contain the pathogen. The latter measure is now being used with significant effect, and recently it has been possible to introduce fish to parasite-free areas using PCR as an assay technique (Palenzuela *et al.*, 1999).

Future studies

The effect of salt water on the progress of this disease is largely unknown although mortality is believed to continue during the marine phase of the host's life cycle. Furthermore, it is unclear if anadromous

fish are infected before they enter salt water or on returning to fresh water. Research in these areas would increase our understanding of ceratomyxosis. The increased sensitivity afforded using PCR is valuable for monitoring early and subclinical infections; however, it is unknown if the increased incidence of the disease signifies an increase or an improvement in detection methods.

Henneguya salminicola

Introduction

Henneguya salminicola (synonym *Henneguya zschokkei*) is a common internal myxosporean parasite of salmonids and coregonids. The cysts in the somatic musculature ruin the aesthetic appearance of the flesh and make it unsuitable for commercial purposes, with a consequent economic impact on salmon fisheries (Kent *et al.*, 1994).

Characterization

Several species of *Henneguya* (Protozoa, Myxozoa: Myxobolidae) are described in cultured freshwater fish. These include *H. salminicola* in salmonids, *Henneguya waltairensis* in green snakehead (*Channa punctatus*) (Kalavati and Narasimhamurti, 1985), *Henneguya exilis* and *Henneguya* sp. in channel catfish (Current, 1979; Smith and Inslee, 1980), and *Henneguya laterocapsulata* n.sp. and *Henneguya suprabranchiae* n.sp. in the catfish (*Clarias lazera*) (Landsberg, 1987). However, only *H. salminicola* is significant in cage culture (Boyce *et al.*, 1985).

Impact

H. salminicola infections are contracted in fresh water and are not usually lethal. The cysts are unsightly in the flesh and the parasite contributes to poor growth and organ displacement. The production of a heat-labile protease from the spores during the host's sea migration causes hydrolysis of the muscle resulting in liquefaction (Bilinski *et al.*, 1984; Boyce *et al.*, 1985). The marketability of some fresh, frozen or smoked products is affected, although the cysts are not readily evident in canned products. Five species of salmon along the Pacific coast of North America and Asia are susceptible to *H. salminicola* (Boyce *et al.*, 1985).

Clinical signs

Grossly visible, white, subspherical cysts up to 15 mm in diameter, with a creamy content, occur within the musculature and other tissues (Boyce *et al.*, 1985). As the cysts mature and grow, they eventually rupture through the integument, releasing many infective spores into the water. These open ulcers provide an excellent port of entry for secondary pathogens. Boyce *et al.* (1985) reported that the prevalence of infection was highest in coho and sockeye salmon and was correlated with the length of time the juvenile fish spent in fresh water.

Diagnostic techniques

Microscopic examination is required for confirmation of this infection, and diagnosis of *H. salminicola* requires the demonstration of the characteristic spores, which contain two polar capsules and two caudal projections. In addition, ultrasonic equipment has been used to detect *Henneguya* spores in whole fish. Clouthier *et al.* (1997) used molecular and antigenic characterization of spores from skeletal muscle of chum salmon to provide a potential peptide and ORF2 fusion protein that may be suitable for candidate vaccine trials.

Prevention

There are no treatments available against *Henneguya*, and the current approach is through the selection of fish from uninfected stocks.

Future studies

Continued development work on fusion proteins (Clouthier *et al.*, 1997) with the

aim of developing a vaccine suitable for testing will be beneficial.

Ichthyobodo necator

Introduction

Ichthyobodo necator is an ectoparasite of freshwater salmonids. Mortalities of infected salmonid fry or ornamental fish can occur with moderate to severe infections; however, chronic outbreaks can result in loss of growth, and secondary gill and skin infections.

Impact

Many fish species are susceptible to *I. necator*, but infestation is particularly important among salmonid fry. Morbidity can be moderate to severe and can lead to serious secondary infections by opportunistic pathogens.

Characterization

I. necator is a small, bean-shaped flagellate, approximately 5–18 μm (Fish, 1940) in length. This protozoan belongs to the order Kinetoplastida, Family Bodonidae, and its taxonomic status was determined using electron microscopy (Joyon and Lom, 1969). Although *I. necator* is suggested as acquiring salinity tolerance and surviving and reproducing in seawater, it should be noted that the morphologically similar bodonid, *Ichthyobodo* sp., from marine Japanese flounder (*Paralichthys olivaceus*) and others from the marine environment probably represent separate species (Urawa and Kusakari, 1990).

Clinical signs

Externally, there is a white-blue haze on the skin of infected fish. Gill tissues show lamellar fusion and hyperplasia. In Atlantic salmon and rainbow trout the parasite causes hyperplasia of the malpighian cells and exhaustion of the goblet cells below infested surfaces (Robertson *et al.*, 1981). The epidermal plaque is sloughed off, leaving just a single layer of basement membrane cells. Robertson *et al.* (1981) also showed that *I. necator* caused a significant increase in cell division below parasite-infested surfaces, a pattern that is markedly different from normal cell proliferation. Pickering and Fletcher (1987) showed that *I. necator* effected the production of sacciform cells in salmonids. These cells, located in the epidermis of brown trout and Arctic char, contained an acidophilic, proteinaceous secretion. The cell number increases during chronic infections in immature trout and decreases during sexual maturation of trout and char.

Prevention

In a study of parasites on wild and cultured fishes in two lakes and a fish farm in central Finland, Valtonen and Koskivaara (1994) determined that *I. necator* was the most prevalent parasite. It was found on a wide range of fish species, including salmonids, whitefish, roach and perch. In a study examining the epizootiology of protozoans in farmed salmonids at northern latitudes, Rintamaki-Kinnunen and Valtonen (1997) showed that, unlike other protozoan parasites, *I. necator* infections had a higher prevalence within fingerling sea trout than yearlings. *I. necator* can also be transferred to seawater sites (Urawa and Kusakari, 1990) and can cause morbidity and losses within sea cages, although in some reports a separate species is also likely to be involved (Urawa and Kusakari, 1990; Bruno, 1992b; Lamas and Bruno, 1992). Under some circumstances it may be practical to move rainbow trout yearlings to a lower water temperature to prevent parasite development (Dyková, 1995).

Future studies

I. necator is a ubiquitous parasite of teleost fish, with a wide host and geographic range. There has been surprisingly little work done on the pathology and epizootiology of this flagellate. Current molecular techniques could be applied to gain valuable

information about antigenic proteins and carbohydrates produced by the parasite, aiming towards a goal of vaccine production.

Microsporidium takedai

Introduction

Microsporidium takedai is a highly pathogenic Microspora parasite and specific to the Salmonidae (Awakura, 1974). All reports of microsporidiosis by this parasite occur in freshwater salmonids from Japan, with other species occurring in seawater fish.

Characterization

M. takedai is diagnosed by finding ovoid spores with subapically attached polar tubes that measure 2×3.4 μm in length. The target tissues are the heart and skeletal muscle, where whitish, spindle-shaped cyst-like lesions form, which are generally visible through the skin.

Impact

Eight species of salmonids are known to be susceptible and they include sockeye, pink, chum and masou salmon, rainbow and brown trout and Japanese char. Prevalence may be 100% in rainbow trout, but is usually lower in other species. A seasonal prevalence of this parasite is recognized, with the initial outbreak during the summer at water temperatures around 15°C (Urawa, 1989). The life cycle is direct and *M. takedai* is transmitted directly by ingestion of the spores present in food, or in the water.

Clinical signs

In chronic cases, the heart shows an extreme hypertrophy and deformation of the tissue with inflammatory oedema. Acute cases frequently result in high mortalities and are characterized by massive numbers of cysts within the musculature. The tissue response in skeletal muscle is similar to that observed in the heart muscle. Within each cyst numerous ovoid, proliferating microsporidia occur and measure 2.5–4.0 μm when mature (Dyková, 1995). No xenoma formation occurs. Fish surviving this infection appear to acquire protective immunity for up to 1 year.

Diagnostic techniques

Provisional identification is based on gross examination of the musculature, and confirmed by microscopical examination of the cysts.

Prevention

There is no commercially available treatment for *M. takedai*, and avoidance is the most effective means of control.

Diagnosis

Diagnosis is by gross external signs, and by identification of the flagellate using light microscopy. Skin or gill scrapings can be prepared and examined at a magnification of greater than ×25. The parasite is approximately the same size as salmonid skin cells and swims with a staggering movement.

Prevention and treatment

There is no vaccine, and prevention is largely reliant on good animal husbandry measures. Tojo and Santamarina (1998) found metronidazole, secnidazole and triclabendazole were 100% effective against the parasite in experimentally infected trout; however, the effective doses were too high to be economically viable, although there was no observed toxicity associated with the drug.

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