**Piscirickettsia salmonis** infection in Atlantic salmon *Salmo salar* in Norway—epidemiological, pathological and microbiological findings

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**ABSTRACT:** A rickettsia-like organism was isolated from diseased Atlantic salmon *Salmo salar* in Norway. Because of morphological and serological similarities to the type strain the suggested name of the organism is *Piscirickettsia salmonis*. The bacterium is considered the most probable cause of a systemic disease diagnosed in 51 farms along the west coast of Norway. Most of the cases occurred in the autumn of 1988. The disease was only recorded in smolts after exposure to sea water and cumulative mortality has been low. In 63 % of fish with gross lesions examined during outbreak of disease in 14 of the affected farms, the typical macroscopic finding was a normal coloured liver with white, circular, sometimes haemorrhagic foci. Of fish with gross lesions, 35 % showed pale gills, a yellow, mottled liver, and haemorrhages scattered throughout the skeletal muscles, pericysternal fat, the stomach wall and the swimbladder. Histomorphological changes were most often observed as necrosis and granulomatous inflammation in the liver. Intracellular, intravacuolar bacteria-like inclusions with an affinity for phagocytic host cells were observed. Transmission electron microscopy revealed individual or paired organisms enclosed in membrane-bound vacuoles.

**KEY WORDS:** *Piscirickettsia salmonis* · Atlantic salmon · Norway · Epidemiology · Pathology · Microbiology

**INTRODUCTION**

Diseases caused by rickettsia are well known among terrestrial species. Rickettsia-like organisms (RLO) have also been observed worldwide in aquatic organisms like molluscs and crustaceans (Fryer & Lannan 1994). Rickettsial organisms are Gram-negative bacteria which usually multiply within host cells, and have an association with arthropod vectors (Weiss & Moul-der 1984).

A rickettsia shown to cause disease in fish was first isolated in Chile in 1989 (Cvitanich et al. 1990, 1991, Fryer et al. 1990). The suggested name of this organism is *Piscirickettsia salmonis* and it causes significant losses of farmed coho salmon *Oncorhynchus kisutch* (Fryer et al. 1992). Epizootics have subsequently occurred in all salmonid species farmed in Chile, i.e.

Atlantic salmon *Salmo salar*, chinook salmon *O. tsha-wyt'scha*, masou salmon *O. masou* and rainbow trout *O. mykiss* (Enriquez 1995). The disease has been called 'salmonid rickettsial septicaemia' (SRS) on the basis of the typical changes characterized by swollen internal organs and haemorrhage (Cvitanich et al. 1991). Intracellular bacteria have been identified in the blood and internal organs of infected fish.

RLO have been observed in connection with low-mortality disease in cultured Atlantic salmon in Ireland (Rodger & Drinan 1993). Similar organisms have also been observed sporadically associated with mortality in salmonids since 1970 in British Columbia, Canada (Evelyn 1992). Recently, RLO-infection has been reported in all species of tilapia (*Oreochromis* and *Tilapia* spp.) reared in Taiwan (Chern & Chao 1994). Organisms resembling rickettsia are also observed in the freshwater-ornamental fish blue-eyed plecostomus *Panaque suttoni* (Eigenmann & Eigenmann) from...
The smears were made by applying a drop of medium instructions) was incubated for 30 to 45 min at room temperature. After an additional wash, the specimens were incubated for 15 min with AEC (3-aminobenzazide; 0.27 g 1⁻¹, Sigma A-5754). AEC was dissolved in N,N-dimethylformamide (67 ml 1⁻¹) in 0.1 M acetate buffer, pH 5.2, and 0.03% H₂O₂ was added. The sections were incubated for 15 min. The sections were then washed in running tap water for 10 min and counterstained with Mayer’s haematoxylin for 2 min. Finally the sections were washed in tap water and coverslipped with an aqueous medium (Aquamount, Merck). All incubations, unless otherwise stated, were performed at room temperature in a humidity chamber. The control consisted of the application of a non-immune serum (normal rabbit serum) at the same dilution as the antiserum.

Transmission electron microscopy. Two blocks of paraffin-embedded liver with characteristic histopathological findings were postfixed in 2% OsO₄ in 0.1 M cacodylate buffer, pH 7.2, for 2 h, dehydrated, and embedded in LX 112 Resin (Ladd Research Industries Inc., USA). Ultrathin sections were stained with uranyl acetate (Frasc & Parks 1965) and lead citrate (Venable & Coggeshall 1965), and examined with a Jeol 100 S electron microscope.

Haematology. Blood samples from 17 healthy and 22 moribund fish were sampled from the Vena caudalis. Samples were placed on ice, and microhaematocrits were measured within 2 h. Blood smears were stained by Leishman Giemsa and examined by light microscopy.

Microbiology. Isolation in antibiotic-free cell cultures: Samples were taken from 7 fish with typical signs of the disease in October 1992. Tissue samples were removed aseptically, kept on ice and processed the following day. They were then treated according to the recommendations given by Lannan & Fryer (1991), with some modifications. In brief, kidney, spleen and liver samples were homogenised at 1:20 in antibiotic-free Glasgow modification of Minimum Essential Medium (GMEM) using a stomacher. The homogenate was further diluted 1:10 (final dilution 1:200). No centrifugation was performed. Subsequently, 100 ml of the suspension was transferred to a 25 cm² tissue culture flask containing a monolayer of CHSE-214 cells (American Tissue Culture Collection CRL 1681) and 5 ml antibiotic-free GMEM with 10% fetal bovine serum. The flasks were incubated at 15°C for up to 28 d. Flasks showing typical CPE were passaged by transferring 100 ml cell culture medium onto 1 flask with antibiotic-free medium and 1 flask containing 50 mg gentamycin per ml medium.

Identification by IFAT: For identification, a slightly-modified indirect fluorescent antibody test (IFAT) as described by Lannan et al. (1991) was used on CHSE-214 monolayers and smears of cell culture medium. The smears were made by applying a drop of medium...
on adhesive-coated slides, while the monolayers were prepared by passaging infected cell culture medium onto cell culture flasks equipped with a removable plastic microscope slide growth area.

Slides with smears or cell monolayers were air dried and fixed in 96% ethanol followed by incubation with rabbit anti-Piscirickettsia salmonis antiserum diluted 1:1000 in Hanks' balanced salt solution, and incubated in the dark at room temperature for 30 min. After washing with PBS and air drying, the slides were counterstained with methyl green, rinsed, dried and incubated with a fluorescein-conjugated goat anti-rabbit IgG F(ab')2 fragment specific (Cooper Biomedical, No. 1212-0111) in the dark at room temperature for 30 min. After rinsing and drying, the slides were coverslipped in PBS glycerol (1:1) and examined by epifluorescence microscopy (Leitz) equipped with an N1 filter (excitation at 450 to 490 nm and emission at 520 nm). Performance testing included the use of non-immune serum, and incubation of non-infected cells with immune serum. Smears were prepared as described above and also Gram stained according to standard procedures.

Additional microbiological studies: Inocula were streaked from kidney and liver onto 4% cattle-blood-agar plates with and without 2% NaCl, incubated aerobically and anaerobically at 15°C and 22°C, respectively, and inspected for growth of bacteria daily for 21 d. Inocula were also tested on kidney disease medium (KDM2; Evelyn 1977) and selective kidney disease medium (SKDM; Austin et al. 1983). These plates were incubated at 15°C for 12 wk. Sabouraud's medium incubated at 15°C for 20 d was used to test for fungi. Liver and kidney samples for virus isolation were filtered (0.22 μm) and tested on RTG-2 cell cultures.

RESULTS

Epidemiology

The RLO infection was diagnosed in 51 Atlantic salmon farms along the west coast of Norway (Fig. 1) during 1988 to 1992. Most of the outbreaks (71%) occurred in 1988. Almost all of the cases (91%) occurred in the autumn, between August and December.

The disease was only diagnosed in Atlantic salmon smolt, except for one case in adult fish. Diseased fish are only seen after exposure to sea water, i.e. after sea transfer or in tanks supplemented with non-disinfected sea water.

A disease outbreak usually lasted for 1 to 3 mo, with low cumulative mortality. In cases with high mortality, other diseases were also diagnosed (clinical infectious pancreatic necrosis). An obvious pattern of spread within a farm or between farms was not observed.

The epidemiological investigation showed that diseased fish were of different stocks and were fed on different commercial diets. Vaccination status and application methods varied and there was no consistency in the use of net-impregnating chemicals.

The disease was frequently recorded after algal blooms. Smolt pens tended to be overstocked with fish in poor condition. Fish were observed feeding on zooplankton.
Clinical findings and gross pathology

Affected fish swam sluggishly near the surface and along the side of the net. Gross changes were observed in 67 out of 163 moribund fish examined. Typical gross changes, found in 63% of these fish, were characterized by well-circumscribed, white, circular foci of varying size up to 5 or 6 mm in diameter on the liver (Fig. 2a). The foci contained either a central or a peripheral haemorrhage, and the centre was usually raised above the liver surface. The liver parenchyma was of normal colour. Splenomegaly was observed.

Of the fish with gross lesions, 35% had pale gills and haemorrhages scattered throughout the skeletal musculature, perivisceral fat, the stomach wall and the swimbladder. The liver was discoloured, yellow-

Fig. 2. *Salmo salar* infected with rickettsia-like organisms. (a) Liver of normal colour with numerous white foci, and splenomegaly, the most common post mortem findings. (b) More heavily infected fish showing yellow, mottled liver with white and haemorrhagic spots, a white spot in the epicardium, pale gills, splenomegaly, and haemorrhages scattered throughout the skeletal musculature.
green, and with white, sometimes haemorrhagic spots (Fig. 2b). Moderate amounts of haemorrhagic ascitic fluid were found, and the spleen and kidney were moderately swollen.

Small white foci were occasionally seen in the heart, kidney, spleen, and skeletal musculature in both of the groups described above. Skin lesions with small areas of raised scales or scaleless superficial white spots were sometimes observed. In a few fish, skin lesions were the only gross findings.

**Histopathology**

The organ most consistently affected was the liver. Histopathological examination revealed multifocal necrotic areas in the liver parenchyma with an accompanying inflammatory response (Figs. 3 & 4). Cases with a dominance of polymorphonuclear granulocytes and scattered macrophages were recognized as acute and subacute stages. Perivascular inflammation and necrosis were also observed. Thrombi were occasionally seen.

In chronic stages the inflammatory changes were characterized by macrophage infiltration developing into a focal granulomatous type of inflammation. Multinuclear giant cell formations were seen in granulomata. There was usually a marked infiltration of mononuclear inflammatory cells along sinusoids and large vessels. In the chronic stages, the proliferation of connective reparative tissue was extensive. In the kidney, focal degeneration and necrosis of haematopoietic tissue was observed and in severe cases degeneration and necrosis of the tubular epithelium (Fig. 5a). In the heart, multifocal inflammatory and reparative lesions occurred beneath the epicardium, and in the spongy and compact myocardium. Minor changes were observed as small foci of mononuclear inflammatory cells in the endocardium. Additional changes were observed as focal degeneration and necrosis of the white pulp of the spleen, and a granulomatous inflammation in the meninges (Fig. 6). Skin lesions were characterized by a granulomatous inflammation in the dermis. No histological tissue lesions were observed in the intestine, gills and eyes of the fish examined. In some cases fibrosis of exocrine pancreatic tissue consistent with infectious pancreatic necrosis (IPN) was found.

Intracellular, intravacuolar bacteria-like inclusions were seen by light microscopy in apparently normal cells in the perimeter of inflamed areas and in degenerated and necrotic tissue. The rickettsia-like organisms were coccoid or pleomorphic and occurred singly or in groups in cytoplasmic vacuoles. They stained Gram-negative, but not by PAS or Ziehl-Neelsen, blue by May-Grünwald-Giemsa and blue-violet by Macchiaveli’s.

Affected cells seemed to be phagocytosing host cells such as hepatocytes (Figs. 3 & 4), interstitial cells in the kidney and spleen, and endocardial cells. Inclusions were found in mononuclear cells in gill capillaries (Fig. 7) and in the tubular epithelium (Fig. 5). Inclu-
sions were also observed in mononuclear inflammatory cells in granulomatous tissue, and sometimes in vacuolated anuclear cell-like structures. The organism was never observed by light microscopy in polymorphonuclear granulocytes.

**Immunohistochemistry**

Bacterium-like organisms were identified intracellularly in macrophages and hepatocytes in inflamed and necrotic areas in the liver (Fig. 3), in macrophages,
Fig. 5. Kidney from *Salmo salar* infected with rickettsia-like organisms. (a) Advanced case with degeneration and necrosis of tubular epithelium. Intracellular, intravacuolar bacteria-like inclusions are present. Intravacuolar bacteria-like inclusions are seen (arrow). (x630). (b) Mononuclear cell with intracytoplasmic, intravacuolar bacteria-like inclusions within nephrotubular epithelium (arrow) (x1000)
Fig. 6. Brain from *Salmo salar* infected with rickettsia-like organisms showing granulomatous meningitis (long arrow) and parenchymal perivascular, mononuclear hypercellularity (short arrow). (x100)

Fig. 7. Gills from *Salmo salar* infected with rickettsia-like organisms. Intracellular, intravacuolar bacteria-like inclusions are present in blood channels (arrow). No specific tissue lesions are observed. (x630)
endothelium and myocytes in the heart, and in macrophages in the meninges. In the liver, there was a distinct association between tissue necrosis and the presence of numerous bacteria-like organisms that were intensely positive by immunostaining.

Transmission electron microscopy

Transmission electron microscopy of the liver revealed individual or paired organisms enclosed in membrane-bound vacuoles in the cytoplasm of hepatocytes and mononuclear cells (Fig. 8a). The organisms were spherical or slightly ovoid, with a diameter of 0.8 to 1.2 μm, and were bound by 2 membranes—one closely apposed inner layer, and 1 undulate outer membrane (Fig. 8b). Electron-dense material was concentrated in the periphery of the organisms. The inner area was electron-lucent, containing some fibrillar material and a centrally located electron-dense granule. An equatorial band of electron-dense material divided the organisms into 2 identical hemispheres, indicating an early stage of division. Organisms apparently undergoing binary fission along this plane were frequently observed (Fig. 8a).

Haematology

The haematocrits of diseased fish ranged from 6 to 30%. Apparently healthy fish had haematocrits of 27 to 49%. Rickettsia-like organisms were not observed in blood smears.

Microbiology

After 2 to 4 d an extensive cytopathic effect (CPE) was demonstrated in samples from 2 of 7 fish. The CPE-causing agent was identified as infectious pancreatic necrosis virus (IPNV) as revealed by an indirect immunofluorescent antibody test for IPNV. This early IPNV-related CPE made isolation of rickettsia impossible in those flasks. In 3 fish, however, CPE was not seen until Day 8 or 9, and subsequently took the form of a variable number of distinct plaques. In flasks with many plaques, these became confluent and destroyed the whole cell monolayer by Day 21 post-inoculation (p.i.), while flasks with fewer plaques had large but distinct plaques as late as Day 28 p.i.

Three flasks inoculated with organ material from 2 different fish showing late CPE were passaged onto new flasks. CPE was observed 6 to 7 d after passage in the form of numerous foci of rounded cells. Gradually the foci developed in size, and after 10 to 14 d were seen as plaques with a centre devoid of cells (Fig. 9). Control flasks with gentamycin-containing medium inoculated with the same material were CPE negative. Identification by IFAT was done on smears and monolayers showing CPE. In the smears, strongly fluorescent rod-shaped organisms about 1 μm in length were seen. In the monolayers, similar organisms were seen in large numbers in the foci of rounded cells (Fig. 10). Gram-stained smears showed Gram-negative cocci and pleomorphic rods about 1 μm long. They were particularly numerous inside or adherent to cells or cell debris.

No significant bacteria were isolated on the culture media. Fungi were not isolated. Viral examination revealed growth of IPNV.

DISCUSSION

The present study describes a systemic disease of Atlantic salmon in Norway, and the isolation and partial identification of a rickettsia-like organism from diseased fish. Morphological changes were typically confined to the liver parenchyma in association with the immunohistochemical detection of the agent.

The intracellular, intravacuolar bacteria-like inclusions observed in tissue lesions and the organism isolated from diseased fish share morphological and serological characteristics. An RLO is considered to be the most probable cause of the disease.

The intracellular organism shows morphological similarities, as observed by TEM, to the RLO named Piscirickettsia salmonis (type strain LF-89T, ATCC VR 1369) (Fryer et al. 1992), which causes disease and mortality in Chilean salmonids, and the RLO causing disease in Atlantic salmon in Ireland (Rodger & Drinan 1993). Further, the serological examination using antibodies produced against the type strain strongly indicates a close antigenic relationship to this bacterium, suggesting the Norwegian isolate to be P. salmonis. Recently 16S rDNA was sequenced and is available in GenBank with accession number NOR-92 U39942. On the basis of sequence data primers for P. salmonis detection by PCR have been published (Mauel et al. 1996). Various primers were tested and RFLP analysis of PCR-products showed no difference between the Norwegian and the type isolate. This supports the identification of the Norwegian isolate as P. salmonis.

The pathology of the RLO infection in this study is comparable to that of other RLO infections in salmonids (Branson & Nieto-Diaz-Munoz 1991, Cvitanich et al. 1991, Evelyn 1992, Rodger & Drinan 1993). Tissue necrosis, vascular damage and an affinity for reticuloendothelial and other phagocytic fixed cells and macrophages are characteristic findings; however, there are some differences. Multifocal gill hyperplasia,
Fig. 8. Transmission electron micrographs of liver from *Salmo salar* infected with rickettsia-like organisms. (a) Individual or paired organisms enclosed in membrane-bound vacuoles in the cytoplasm of a hepatocyte (arrow). (x12,000). (b) Spherical organisms bound by 2 membranes, 1 closely apposed inner layer, and 1 undulated outer membrane. (x60,000)
dilatation of the stomach and inflammation and necrosis in the gut, common findings in Chilean coho salmon (Branson & Nieto-Diaz-Munoz 1991, Cubillos et al. 1995, Neubrand et al. 1995), were not observed in our study, or from Atlantic salmon in Ireland (Rodger & Drinan 1993). Similarly, extensive thrombosis, seen in salmonids in British Columbia (Evelyn 1992) and Chile (Branson & Nieto-Diaz-Munoz 1991, Cvitanich et al. 1991), was not found. Like the Irish cases, thrombosis was occasionally observed in Atlantic salmon in Norway. The variety in the expression of the RLO infections may be due to modulations by the host species and its immune status, dose dependence and the presence and absence of concurrent disease. The differences regarding the pathological changes may indicate similar, but not identical aetiological agents. The precise relationship between the rickettsia-like organisms has yet to be clarified. Results from Chile using a PCR technique indicate the existence of at least 4 genetic variants (S. Valdebenito & B. Jauriguiberry, Veterquimica, pers. comm. 1995). In the study 6 Chilean isolates represented 3 variants, all different from the type strain LF-89. PCR assays reported by Mauel et al. (1996), distinguished one of the isolates of Piscirickettsia salmonis from the 4 others tested.

Pathogenic rickettsiae of mammals are reported to display toxin-like action (Weiss & Moulder 1984). Tissue necrosis and anaemia, prominent findings of the infection, could imply a toxic effect by the organism. A toxicological investigation of liver from RLO-infected fish in 1988 indicated a hepatotoxin (T. Aune, Norwegian Veterinary College, pers. comm. 1994). The in vitro effects of the toxin were similar to those previously induced by toxins from algae (Underdal et al. 1989). Histopathological indications of algal toxicosis (Parker et al. 1982, Roberts 1989) were not found. A water-born toxicant is considered the most likely cause of netpen liver disease (NLD) (Kent 1990) diagnosed in sea-water-reared salmonids in North America and British Columbia. Megalocytosis, the hallmark of NLD, was not observed in our study. Whether the hepatotoxic substance recognized here is connected to the pathogenesis of the RLO infection has yet to be investigated.

In this study IPNV was isolated from fish with RLO infection. This is not surprising, as IPNV is widely spread in the sea water farms in Norway (Melby et al. 1991). However, the importance of IPNV in the susceptibility of fish to other infectious agents is still uncertain. In vitro, an immunosuppressive effect of IPNV in fish leucocytes has been shown (Knott & Munro 1986, Tate et al. 1990, Johansen & Sommer 1995, Novoa et al. 1996). In vivo, no influence of IPNV was found in Atlantic salmon bath-challenged with Yersinia ruckeri, neither in fish vaccinated against Y. ruckeri nor in non-

Fig. 9. Salmo salar. CHSE-214 cell monolayer 14 d after passage showing plaques with the centre devoid of cells (o). Phase contrast
vaccinated controls (Bruno & Munro 1989). In a few cases of RLO infection with high mortality, a simultaneous outbreak of IPN was found. Clinical IPN is a major cause of mortality in Norwegian Atlantic salmon postsmolt (Jarp et al. 1994). The outbreak of IPN has probably caused the higher mortalities in these few cases as clinical IPN was not found in the majority of cases of RLO infection with low mortality.

The route of infection is not known. For the Piscirickettsia salmonis-infection in Chile transmission by coprophagy was suggested (Inglis et al. 1993). The finding of the rickettsia-like organisms in nephrotubular epithelium in this study indicates the organism may pass through the urinary tract. P. salmonis is observed in monocytes emigrating into the renal tubules of coho salmon in Chile (Neubrand et al. 1995).

In Norway and elsewhere RLO have mainly affected salmon smolts in sea water and have shown a seasonal occurrence in the late summer and autumn. Typically, but not consistently, the disease in Norway is seen after an algal bloom. Most cases in Norway occurred in 1988. The geographical distribution of the affected farms coincided with the geographical spread of a large algal bloom (Dahl & Tangen 1990). Algal blooms were also reported to precede disease outbreaks in Chile (Branson & Nieto-Diaz-Munoz 1991).

Due to an exceptionally large production of smolts in 1988, the sea farms tended to be overstocked (Anonymous 1988). Smolts were probably underfed. Fish were observed feeding on zooplankton. There may in that year have been an interaction between a favourable marine environment for the organism and its possible vector and underfed fish in the netpens leading to the high prevalence of the disease. As arthropods are common vectors for rickettsial diseases of man and other terrestrial animals (Weiss & Moulder 1984), the fish may have been exposed to the rickettsia-like organisms through marine zooplankton, e.g. copepods which followed the extensive growth of algae in that year.

Infestation with sea-louse, especially the copepod Lepeoptheirus salmonis, is very common on Atlantic salmon in Norway. The parasite is known to transmit viral infectious salmon anaemia (ISA) (Nylund et al. 1994). In this study L. salmonis was occasionally observed on fish with RLO infection. However, the infrequent occurrence of the disease compared to the very common and large problem of sea-louse infestation indicates that their possible role in transmission of the RLO-infection in Norway is minor.

Under experimental conditions horizontal transmission of the Piscirickettsia salmonis-infection in Chile is observed to take place without a vector (Cvitanich et al. 1990). Lannan & Fryer (1994) have shown extended survival of rickettsia in sea water, which may support the possibility of direct transmission in the marine environment. The normal mode of transmission of the rickettsia-diseases has, however, yet to be established.

Disease outbreaks over a short time period at different locations suggest a wide distribution of the infectious organism in the marine environment of Norway; however, there have been no diagnoses in the farming areas in the northern and most southern parts of the coastline.

Piscirickettsiosis represents a major problem for the Chilean salmonid farming industry (Enriquez 1995). The significance of the RLO infection for the Norwegian fish farming industry has so far been low, although several farms were affected in 1988. The limited
significance may be due to a steadily better smolt quality, improved rearing conditions and normally unfavourable environmental conditions for the organism or its possible vector. Furthermore the salmonid species farmed in Norway are mainly Atlantic salmon and rainbow trout, species which seem to be more resistant to infection with rickettsia-like organisms (Bravo & Campos 1989, Cvitanich et al. 1991, Brocklebank et al. 1992, Evelyn 1992, Rodger & Drinan 1993).

The organism has been found in gonads of infected fish (Inglis et al. 1993), which may indicate vertical transmission. Piscirickettsiosis is also reported to occur in freshwater in progeny of salmonids that survived a Piscirickettsia salmonis outbreak in saltwater (Geggero et al. 1995), which may also indicate vertical transmission. There is so far no indication of vertical transmission of the disease in Norway. This may have been prevented by the routine disinfection of eggs by iodophors. However, egg disinfection is perhaps not sufficient, as there is a possibility of an intraovarial localisation of the bacterium. In Norway the use of fish from affected farms for breeding is not allowed. Treatment of RLO infected fish in Norway has not been attempted.

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