

## Rickettsial Infections of Fish

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Received 02 December 2005  
Accepted 16 April 2006

### Abstract

*Piscirickettsia salmonis*, the aetiological agent of piscirickettsiosis, has recently been responsible for significant disease outbreaks in a variety of economically important freshwater and seawater fish species cultured worldwide. However, the development of effective control strategies for the disease has been limited due to a lack of knowledge about the physiology, intracellular growth, transmission and pathogenesis of the organism.

The review presented here looks at important issues relating to *P. salmonis* such as recent progress in classifying the bacterium taxonomically, examining its transmission of infection, and considering methods to detect or identify the bacterium in infected fish or in the environment. We also look at current approaches for treatment and control of Piscirickettsiosis.

**Key Words:** *Piscirickettsia salmonis*, Rickettsia, Fish Disease, Piscirickettsiosis.

### Introduction

The Rickettsias constitute a relatively small, but important group of obligate, intracellular micro-organisms that cause disease in both man and other vertebrates, and in invertebrate hosts (Buxton and Fraser, 1977; Weiss and Moulder, 1984).

The Rickettsias are classified into two orders, *Rickettsiales* and *Chlamydiales* (Weiss and Moulder, 1984). The *Rickettsiales* are clearly different from the *Chlamydiales*, which are a narrowly defined group of energy-parasitizing, obligately intracellular bacteria (Weissburg *et al.*, 1989). Gram negative, coccoid and non-motile microorganisms, *Chlamydiales* are characterised by their ability to change from a small, rigid-walled infectious agent (elementary body) into a larger, thin walled, non-infectious form (initial body) that divides by fission. The developmental cycle is complete when the daughter cells re-organise and condense into a new generation of elementary bodies, which survive extracellularly to infect other host cells (Page, 1974; Moulder, 1985). Most of the organisms which belong to the order *Rickettsiales* are pleomorphic, and may vary from coccoid, ellipsoid or coccobacillary to rod-shaped, and are occasionally found as filamentous forms with their size ranging from 0.3 to 2.0 µm in length and 0.3 to 0.5 µm in width (Buxton and Fraser, 1977). These non-motile, Gram negative micro-organisms have typical bacterial cell walls, no flagellum and only multiply within host cells (Weiss and Moulder, 1984). Although they vary widely in their characteristics, they all undergo binary fission (Turnbull, 1993).

The order *Rickettsiales* consists of a group of prokaryotes that share an intimate association with

eukaryotic cells. In most cases the relationship with their host is obligate intracellular parasitism (Weissburg *et al.*, 1989) or mutualism. The parasitic forms are associated with reticuloendothelial and vascular endothelial cells or erythrocytes of vertebrates and arthropods, which may act as either vectors or primary hosts. The mutualistic forms of rickettsias seen in insects are considered as essential for development and reproduction of the host animal (Weiss and Moulder, 1984).

Initially, only one rickettsial species, *Neorickettsia helminthoeca*, responsible for salmon poisoning disease in canines, was thought to be associated with fish. It has an extraordinary mode of transmission from salmonids through the eggs, larvae and adults of a trematode vector, *Nanophyetus salmincola*, to dogs but it is not, however, a fish pathogen (Fryer and Muel, 1997; Davies, 1986). Until the end of the eighties, the role of rickettsia in fish disease was unrecognised. Prior to 1989, Wolf (1981) reported that fish as well as terrestrial vertebrates are subjected to infections caused by bacteria, fungi, parasites and viruses. Wolf (1981) also reported that although chlamydia and rickettsia had been reported to cause several diseases in mammals and birds, only one chlamydia was known to infect fish and therefore the existence of rickettsia infection in fish was open to question since rickettsial diseases of terrestrial vertebrates require parasitic arachnoids and insects as a vector or transport agent of which there were few if any in aquatic environments. However, current literature reflects a large and diverse group of Gram negative, intracellular prokaryotic organisms which make up the group of Rickettsias that are known to infect

aquatic poikilotherms. Most of these infections are in marine or anadromous hosts, but they have also been reported in freshwater environments. In the last twenty years, at least 25 species of marine bivalves and 12 aquatic crustaceans have been reported to be infected with intracellular rickettsia-like and chlamydia-like organisms in France, Canada, Japan, Spain, Scotland, Sweden, Singapore, Malaysia and USA (Fryer and Lannan, 1994). Only recently was epitheliocystis shown to be a disease caused by chlamydia-like organisms belonging to the order *Chlamydiales* (Turnbull, 1993). However, none of these chlamydia-like organisms have been isolated from freshwater or seawater fish, and the description of their cellular morphology and pathology is limited (Fryer and Lannan, 1994). In recent years, the importance placed on these intracellular bacteria in fish disease has changed dramatically and rickettsial agents are now known to cause significant disease outbreaks in fish (Fryer and Lannan, 1994).

### Historical Background

Current literature has shown that the rickettsia-like organisms (RLO) observed or isolated from a variety of fish species in different geographical locations and aquatic environments around the world, make up an important group of fish pathogens, with the potential for significant effect on the health of both freshwater and seawater species (Fryer and Lannan, 1996). However, prior to 1989 there were few reports of RLO in fish.

The first report of RLO associated with fish, occurred during an examination of a dead *Tetradon fahaka* from the Nile River in Egypt in 1939 (Mohamed, 1939). The organism, a round coccus structure, approximately 0.25 µm in diameter, was seen in the blood and in some of the internal organs of the fish when stained with Giemsa. They were named as a new species "*Rickettsia pisces*". However, no additional cases of the disease were reported either in that particular species of fish or at the same location.

No further reports of rickettsia in fish occurred until 1975 when Ozel and Schwanz-Pfitzer (1975) first cultured an intracellular RLO from rainbow trout, *Oncorhynchus mykiss*, collected from a freshwater source in Germany, while testing for Egtved (Viral Hemorrhagic Septicemia) virus. They did not, however, characterise it morphologically or establish the nature of the agent associated with these mortalities in rainbow trout (Fryer and Lannan, 1994). In 1986, RLO was observed by electron microscopy in a marine fish, dragonet *Callionymus lyra* collected from Cardigan Bay, Wales, while examining tissue sections for a common blood parasite of dragonets (Davis, 1986).

The role of rickettsiae as emerging pathogens of fish became apparent in 1989 (Fryer and Lannan, 1996). During 1989, an estimated 1.5 million Coho salmon, *Oncorhynchus kisutch*, roughly 200 g to

market size (approximately 2 kg) located in areas around Puerto Montt and the Island of Chiloe in Chile, died of a disease with an unknown etiology. These mortalities resulted in losses of more than 10 million US dollars to the Chilean fish farming industry (Cvitanich et al., 1990). Average mortalities which occurred in some locations were approximately 60% with losses of up to 90%. No common factors were evident among the infected fish, which occurred in a variety of stocks, hatcheries, and water supplies during the freshwater phase of production. The fish had also been fed different types of food (Branson and Nieto Diaz-Munoz, 1991). The disease was only observed in Coho salmon and not in Chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo salar* or rainbow trout *O. mykiss*, also present in the affected area (Fryer et al., 1990). The disease was associated with high mortalities in Coho salmon as early as 1981, and was known as 'Coho Salmon Syndrome' or 'Huito Disease'. The epizootics typically occurred and peaked in autumn with repeat outbreaks in the following spring (Bravo and Campos, 1989; Cvitanich et al., 1990).

Bravo and Campos (1989), who first described the disease, observed an unidentified parasite in the blood and internal organs of infected fish by both light and electron microscopy, although no infectious agent was isolated from diseased fish. After the outbreaks, intensive investigations were initiated to isolate and identify the causative agent, describe the pathology caused by the agent, develop diagnostic methods, examine the fish immune response to the pathogen and conduct field trials with antibiotics (Cvitanich et al., 1990). The causative agent of the disease was first isolated from infected Coho salmon reared in seawater in Chile using fish cell lines. The organism fulfilled Koch's postulates by reproducing the disease experimentally in Coho salmon in both fresh and seawater aquaria and confirming the presence of the agent by reisolating the organisms from infected fish (Fryer et al., 1990; Cvitanich et al., 1991). Initially, the organism was thought to be a pathogen of only Coho salmon, but it was then shown to cause disease and mortality in Atlantic salmon, Chinook salmon and rainbow trout, leading to the name for the disease of 'salmonid rickettsial septicaemia' (SRS) (Cvitanich et al., 1991). Since 1989, a number of reports have documented rickettsial disease in different salmonid species: Coho salmon; rainbow trout; Chinook salmon and Atlantic salmon in both seawater and freshwater in Chile (Fryer et al., 1990; Cvitanich et al., 1991; Branson and Nieto Diaz-Munoz, 1991; Garcés et al., 1991; Fryer and Lannan, 1992; Bravo, 1994; Cvitanich et al., 1995; Gaggero et al., 1995). The previously undescribed obligate intracellular pathogen isolated from infected fish, was named *Piscirickettsia salmonis* gen. nov., sp. nov, a rickettsial organism belonging to the order *Rickettsiales*, family *Rickettsiaceae*. This became known as the agent of

the epizootic outbreaks occurring in the marine netpen-reared coho salmon in southern Chile, with the disease, referred to as piscirickettsiosis (Fryer *et al.*, 1992).

After isolation of the RLO from Chilean salmonids, rickettsial infections were identified in netpen-reared Atlantic salmon in the coastal waters of British Columbia in 1991. The clinical signs and post-mortem findings of the disease were similar to the disease reported in farmed Chilean coho salmon caused by RLO (Brocklebank *et al.*, 1992). An identical disease had been reported in pink salmon, *Oncorhynchus gorbuscha*, cultured in seawater tanks in British Columbia in the 1970's and in farmed Coho and Chinook salmon in 1989 (Evelyn, 1992). Following the disease outbreak in Chile and Canada, similar disease outbreaks were observed in Atlantic salmon in Norway (Olsen *et al.*, 1997), Ireland (Rodger and Drinan, 1993) and Scotland (Grant *et al.*, 1996).

Up until 1994, RLO had only been isolated from or observed in salmonids. However, after this time the disease was reported in a number of different fish species located in different geographic regions. The disease was reported to affect Mozambique tilapia *Oreochromis mossambicus*, Nile tilapia *Oreochromis niloticus*, blue tilapia *Oreochromis aureus*, redbelly tilapia *Tilapia zillii* and Wami tilapia *Tilapia hornorum* in freshwater culture ponds and seawater in southern Taiwan (Chern and Chao, 1994; Chen *et al.*, 1994). Khoo *et al.* (1995) also observed RLO in blue-eyed plecostomus *Panaque suttoni*, a tropical freshwater fish that was imported from South America to the USA for the pet fish industry. During the winter of 1993-1994, mortalities associated with pathological changes in farmed juvenile sea bass, *Dicentrarchus labrax*, occurred on the Mediterranean coast, Southern France. Comps *et al.* (1996) reported that a RLO was the causative agent of this disease.

Cvitanich *et al.* (1995) observed and isolated a new RLO from Atlantic salmon during December 1994 and January 1995 in Chile. The disease outbreak was observed in net pens in farms located in Lake Llanquihue (water temperature approximately 16°C) and in sea pens (water temperature approximately 14°C). Cvitanich *et al.* (1995) named this agent UA-2 or U2 meaning "unidentified agent 2" because it was different from the RLO which was previously observed and referred to as UA "unidentified agent" by Branson and Nieto Diaz-Munoz (1991). A disease associated with a RLO occurred on four Atlantic salmon sea farms in Ireland during 1995 and 1996. A bacterium consistent with *P. salmonis* was isolated from infected fish on fish cell lines, and the disease was experimentally reproduced with the isolated agent (Palmer *et al.*, 1996).

In Eastern North America, a disease began in a Nova Scotia Atlantic salmon pen site in September 1996, and continued in the fish population which had been transferred to sea pens in spring 1996.

Diagnostic findings strongly suggested that the agent responsible for the disease was a RLO. This was the second report of a RLO infection in North America, although from a different part of the country (Cusack *et al.*, 1997). The second report of RLO infections in European sea bass in the Mediterranean came from Greece (Athanasopoulou *et al.*, 1999). Twenty days after transfer to cages, fish showed erratic swimming and abnormal behavior with high mortality of up to 80% in colder months. Intravacuolar bacteria-like inclusions observed in tissue lesions showed similarities to *P. salmonis*.

In 2000, mortalities occurred in southern California, USA among hatchery reared juvenile white seabass *Atractoscion nobilis* with infections associated with a *P. salmonis*-like organism. Although, the isolated bacterium induced 80% mortality in 10 d in experimentally infected Coho salmon, it reacted weakly with polyclonal anti-*P. salmonis* serum (Chen *et al.*, 2000). Mauel and Miller (2002) reported the presence of *P. salmonis*-like organisms in tissue sampled from tilapia affected by epizootics similar to piscirickettsiosis. The samples had been sent from Jamaica, Indonesia, Southern California and Florida during 2000 and 2001. Chen *et al.* (2000) reported a Piscirickettsia-like organism in grouper *Epinephelus melanostigma*, in Taiwan. The clinical signs and histological lesions were similar to the piscirickettsiosis observed in salmonids and tilapia. The intracellular organisms involved were similar in size and morphology to *P. salmonis*, and gave a positive reaction with polyclonal antibody (PAb) against *P. salmonis*. However, they did not produce a cytopathic effect (CPE) in fish cell lines.

Mauel *et al.* (2003) reported a disease which caused high losses in tilapia (*O. mossambicus* and *Sarotherodon melanotheron*) in wild and farmed populations on Oahu, Hawaii, USA in 1994. The disease was similar but not identical to piscirickettsiosis observed in salmonids. The agent of the disease was not thought to be *P. salmonis*, but was considered as a *Piscirickettsia*-like organism referred to as Hawaiian tilapia *Piscirickettsia*-like organism (HTPLO). The HTPLO did not respond to *P. salmonis*-specific antibodies in IFAT and did not produce an amplicon with a *P. salmonis*-specific polymerase chain reaction (PCR). Also, the HTPLO did not produce a cytopathic effect in any of the cell line used in the study. However, the HTPLO exhibited many of the characteristics common with *P. salmonis*, for example they were obligate, pleomorphic coccoid bacteria, occurring free in the cytoplasm or within phagolysosomes. Both pathogens had a double cell wall with no defined nucleus and variable electron-dense and electron-lucent areas, however, the HTPLO was smaller in size than *P. salmonis*. The disease syndromes caused by the two bacteria were similar, although the HTPLO did not produce craterform lesions in the liver of infected fish, as seen with *P. salmonis*, and grew at

temperatures above 20°C.

Corbeil *et al.* (2005) observed a RLO in farmed Atlantic salmon located in South-East Tasmania, Australia. Weak staining of the Tasmanian RLO in immunohistochemistry suggested the presence of some shared antigenic determinants between the Tasmanian RLO and the *P. salmonis* type strain. Immunogold staining also supported the immunohistochemical results. Also, the Tasmanian RLO appeared to share many morphological characteristics with *P. salmonis*. However, in contrast to *P. salmonis*, cell cultures inoculated with tissue homogenates from Tasmanian RLO-infected fish did support growth of the pathogen. From sequence alignment studies, Corbeil *et al.* (2005) concluded that the Tasmanian RLO contained a 19 bp deletion at the 3'-end of the internal transcribed spacer region of the rDNA operon, thus indicating a genetic divergence from *P. salmonis* isolates which are exotic to Australia.

In the last few years, rickettsial diseases have been observed in various locations in a variety of fish species world wide. The disease has been especially important to the Chilean salmonid fish farming industry. History of rickettsial disease in fish is reviewed in Table 1.

### The Bacterium

**Taxonomy :** In the 1984 edition of Bergey's manual of Systematic Bacteriology (Weiss and Moulder, 1984) the order *Rickettsiales* was divided into three families: *Rickettsiaceae*, *Bartonellaceae* and *Anaplasmataceae* depending on their morphology, serology, mode of transmission and their association with the cells they infect. The family *Rickettsiaceae* includes tribes: *Rickettsieae* (genus *Rickettsia*, *Rochalimaea* and *Coxiella*), *Ehrlichieae* (genus *Ehrlichia* and *Cowdria*) and *Wolbachieae* (genus *Wolbachia* and *Rickettsiella*). The variation in intrinsic properties of the members of each tribe is based on the hosts that they infect. *Rickettsieae* are pathogenic for man, while *Ehrlichieae* are pathogenic for domestic animals but are also possible human pathogens. On the other hand, the tribe *Wolbachieae* are pathogens or symbiontes of arthropods (Buxton and Fraser, 1977; Weiss and Moulder, 1984; Drancourt and Raoult, 1994). Assignment of *P. salmonis* to one of the tribes mentioned above was delayed until appropriate classification of the family was carried out. It was placed in the order *Rickettsiales*, the family *Rickettsiaceae*, and is both a new genus and a new species based on its unique 16S rRNA sequence, temperature requirements, host range and serological characteristics (Fryer *et al.*, 1992).

The term rickettsia initially covered all intracellular bacteria, regardless of any other characteristics they exhibited. Early rickettsial taxonomy was based on a comparison of phenotypic characteristics such as the bacteria's interactions with

the environment and the antigenicity of isolates. However, genomic studies including 16S rRNA sequencing, DNA-DNA relatedness studies, restriction profiles and polymorphism analysis have recently been used to unravel rickettsial taxonomy (Weisburg *et al.*, 1989; Drancourt and Raoult, 1994). The 16S rRNA gene sequence showed *P. salmonis* to be a member of the gamma subdivision of the Proteobacteria like *Coxiella burnetti* and *Wolbachia persica*, while bacteria of the genera *Neorickettsia*, *Cowdria*, *Ehrlichia*, *Anaplasma* and *Rickettsia* are members of the alpha subdivision of the Proteobacteria (Mauel, *et al.*, 1999). Therefore, the genus *Piscirickettsia* has been placed in a new class within Gammaproteobacteria and a new family *Piscirickettsiaceae* (Fryer and Hedrick, 2003). Many RLO have been isolated from a variety of different fish species since 1989. Most of them are serologically identified as *P. salmonis* (Evelyn, 1992; Rodger and Drinan, 1993; Gaggero *et al.*, 1995; Olsen *et al.*, 1997, Fryer and Lannan, 1996). Although the isolates react with polyclonal antibodies (PABs) against *P. salmonis* type strain LF-89 and appear to be morphologically similar, taxonomic placement of the isolates should be based on genetic differences between the RLO isolates. Mauel *et al.* (1999) assessed the genetic variability of the 16S ribosomal DNA, the internal transcribed spacer (ITS) and the 23S ribosomal DNA of a number of different isolates from three different hosts and different locations (LF-89, coho salmon/Chile; EM-90, Atlantic salmon/Chile; ATL-4-91, Atlantic salmon/Canada; NOR-92, Atlantic salmon/ Norway; SLGO-94, rainbow trout/Chile and C1-95, coho salmon/Chile) using PCR. Phylogenetic comparison of the *P. salmonis* 16S rRNA to other bacterial genes confirmed that the organism is a member of the gamma subdivision of the Proteobacteria, and closely related to the genus *Coxiella* and *Francisella*. The authors also concluded that all isolates of *P. salmonis* were from a monophyletic group within the same subdivision of Proteobacteria, and isolate EM-90 diverges genetically from other isolates based on its 16S, ITS and 23S rDNA sequence analysis (Mauel *et al.*, 1999).

Casanova *et al.* (2003) also reported an electrophoretic analysis of ITS region of eleven *P. salmonis* isolates obtained from different salmon species and places in southern Chile. Results showed that two groups could be determined among Chilean isolates which is in agreement with Mauel *et al.* (1999). Five isolates, including LF 89, were closely related while the Chilean isolate EM-90 had diverged genetically, therefore, genetic variability within *P. salmonis* is lower than expected (Casanova *et al.*, 2003).

Reid *et al.* (2004) analysed 16S-to-23S ITS and 16S ribosomal DNA of isolates of *P. salmonis* from Scotland and Ireland. Their results showed the Irish isolates of *P. salmonis* formed two new groups of the

**Table 1.** History of natural outbreaks of rickettsial diseases in fish

Date of Outbreaks	Host species	Location	Means of identification	Mortality	Named	Reference
1939	<i>Tetradon. fahaka</i>	Egypt (Freshwater)	LM: Small coccoid forms in monocytes leucocytes and plasma in heart-blood and liver smears of a dead fish with Giemsa staining	Not reported	Rickettsia pisces	Mohamed, 1939
1975	Rainbow trout <i>Oncorhynchus mykiss</i>	Germany (Freshwater)	Isolation in fish cell line (RTG-2) and LM	Not reported	Rickettsia-like organism (RLO)	Ozel and Schwanz-Pfitzer, 1975
1970-1978 1983-1984 1984	Pink salmon <i>O. gorbuscha</i> Chinook salmon <i>O. tshawytscha</i> Coho salmon <i>O. kisutch</i>	Pacific Northwest Coast of Canada (Seawater)	LM: Gram negative, Giemsa positive, basophilic or amphophilic spheres in Haematoxylin and Eosin (H&E), best staining with Methylene blue, acid fast, Periodic acid-Schiff negative, Macchiavello negative in tissue sections from liver and kidney	Not reported	RLO/ Salmonid rickettsial septicaemia (SRS)	Evelyn, 1992
1986	Dragonet <i>C. lyra</i> L.	Cardigan Bay Wales (Seawater)	EM of spleen	Not reported	RLO	Davies, 1986
1989	Coho salmon <i>O. kisutch</i>	Chile (Seawater)	LM (Gram, Giemsa) and EM in blood and internal organs of infected fish	90%	Coho salmon syndrome (CSS)	Bravo and Campos, 1989
1989	Coho salmon <i>O. kisutch</i>	Chile (Seawater)	Isolation using fish cell lines; LM (Gram negative, stained with Pinkerton's method and modified Gimenez. and Giemsa) and EM of isolated organisms; in vitro characterisation; no reaction with a monoclonal antibody (MAb) against the group-specific LPS chlamydial antigen by Indirect fluorescent antibody technique (IFAT)	90%	RLO/Chilean Coho salmon disease (CCSD)	Fryer <i>et al.</i> , 1990
1989-1990	Chinook salmon <i>O. tshawytscha</i> Coho salmon <i>O. kisutch</i> Atlantic salmon <i>Salmo salar</i> Rainbow trout <i>O. mykiss</i>	Chile (Seawater)	Isolation using fish cell lines, LM [Gram negative, periodic acid-Schiff (PAS), Ziehl-Neelsen acid fast and Gimenez negative but stained with H&E, Giemsa and methylene blue on smears prepared from peripheral blood, fish tissue and infected cell culture] and EM of fish tissue and infected cell culture; fulfilled Koch's postulate; IFAT with MAb against Chlamydia and reproduced in fresh water	70%	RLO/CCSD/SRS	Cvitanich <i>et al.</i> , 1990 Cvitanich <i>et al.</i> , 1991
1989	Coho salmon <i>O. kisutch</i>	Chile (Seawater)	LM (Gram negative, PAS negative, not stain with Gimenez and Macchiavello) of kidney and blood smears and fish tissue; attempted to isolate with fish cell lines but with no CPE	60-90%	Unidentified agent-UA/RLO	Branson and Nieto Diaz-Munoz, 1991

Table 1. (Continue)

Date of Outbreaks	Host species	Location	Means of identification	Mortality	Named	Reference
1989	Chinook salmon <i>O. tshawytscha</i> Coho salmon <i>O. kisutch</i> Atlantic salmon <i>S. salar</i> Rainbow trout <i>O. mykiss</i>	Chile (Seawater)	Isolation; fulfilled Koch's postulates; IFAT and Acridine orange stain	Not reported	RLO designated as strain LF-89 of SRS	Fryer and Lannan, 1992
1989	Coho salmon <i>O. kisutch</i>	Chile (Seawater)	EM and 16 small subunit ribosomal ribonucleic acid (16S rRNA) - analysis of the designated strain LF-89		<i>Piscirickettsia salmonis</i> gen. nov., sp. nov./ Piscirickettsiosis	Fryer et al., 1992
1991	Atlantic salmon <i>S. salar</i>	British Columbia (Seawater)	LM: the organisms was Gram negative, acid fast, PAS negative, Giemsa positive, Macchiavello negative and blue with toluidine blue in infected tissue sections	0.06%/d	RLO	Brocklebank et al., 1992
1988-1992	Atlantic salmon <i>S. salar</i>	Norway (Seawater)	Isolation in fish cell line; LM (Gram, H&E, PAS, Ziehl-Neelsen, May-Grunwald-Giemsa, Machiavello's); Immunohistochemistry (IHC); EM; IFAT	Not reported	<i>P. salmonis</i> Piscirickettsiosis	Olsen et al., 1997
1991	Atlantic salmon <i>S. salar</i>	West of Ireland	LM (Gram, H&E, Giemsa and Acridine orange) and EM of tissue samples	Not reported	RLO	Rodger and Drinan, 1993
1993	Coho salmon <i>O. kisutch</i> Atlantic salmon <i>S. salar</i> Rainbow trout <i>O. mykiss</i>	Chile (Freshwater)	Isolation using fish cell line; LM (Gram and Giemsa staining of infected cell culture); IFAT	Not reported	<i>P. salmonis</i>	Gaggero et al., 1995
1993	Rainbow trout <i>O. mykiss</i>	Llanquihue Lake in Chile (Freshwater)	LM (Giemsa) of tissue smears and Fluorescent antibody (FA)	10%	<i>P. salmonis</i> Piscirickettsiosis	Bravo, 1994
1992-1993	Mozambique tilapia <i>O. mossambicus</i> , Nile tilapia <i>O. niloticus</i> , Blue tilapia <i>O. aureus</i> , Redbelly tilapia <i>T. zillii</i> Wami tilapia <i>T. hornorum</i>	Southern Taiwan (Seawater/ Freshwater)	Isolation using fish cell lines, LM (Gram, Liu's staining, H&E, PAS, Ziehl-Neelsen acid-fast) of blood film and spleen smear and tissue sections; EM; fulfilled Koch's postulate	20-40%; up to 95% in severe cases)	RLO	Chern and Chao, 1994 Chen et al., 1994

Table 1. (Continue)

Date of Outbreaks	Host species	Location	Means of identification	Mortality	Named	Reference
1993-1994	Sea bass <i>D. labrax</i>	France	LM (H&E) and EM of affected fish tissue	20%	RLO	Comps <i>et al.</i> , 1996
1995	Atlantic salmon <i>S. salar</i>	Scotland (Seawater)	Isolation, LM and EM; fulfilled Koch's postulates (With affected cell culture supernatant: no reaction to a latex agglutination test for <i>P. salmonis</i> ; Enzyme-linked immunosorbent assays (ELISA)-Relisa-Rickettsia, Microtex was positive; IFAT positive for <i>P. salmonis</i> ); 16S rRNA analysis	Not reported	RLO/ <i>P. salmonis</i>	Grant <i>et al.</i> , 1996 Grant, 1999
1995	Blue-eyed plecostomus <i>P. suttoni</i>	Colombia (Freshwater)	LM (Gram, H&E, Macchiavello's, Pinkerton's and Fite's acid fast stains) and EM	Not reported	RLO	Khoo <i>et al.</i> , 1995
1994-1995	Atlantic salmon <i>S. salar</i>	Chile (Seawater/ Freshwater)	Isolation; LM (Gram, Gimenez, PAS, Ziehl-Neelsen acid-fast) and EM; IFAT negative with sera against Unidentified agent (UA)/ <i>P. salmonis</i> or <i>Renibacterium salmoninarum</i> , Chlamydia and also negative in identification test for chlamydia (culture in McCoy cells, ELISA, and Gimenez stain)	4-12% /week	Unidentified agent 2 (UA2)	Cvitanich <i>et al.</i> , 1995
1995-1996	Atlantic salmon <i>S. salar</i>	Ireland (Seawater)	Isolation; LM (Gram-Twort, Giemsa, H&E) and EM of tissue sections; IFAT with serum against <i>P. salmonis</i> was positive; fulfilled Koch's postulate	Not reported	Piscirickettsiosis-like disease	Palmer <i>et al.</i> , 1996
1996	Atlantic salmon <i>S. salar</i>	Eastern North America (Seawater)	Isolation; LM (Gram, Giemsa, Toluidine blue) and EM; IFAT with serum against <i>P. salmonis</i> was positive; fulfilled Koch's postulate	6%	RLO	Cusack <i>et al.</i> , 1997
1999	Sea bass <i>D. labrax</i>	Greece (Seawater)	LM (Giemsa) of affected fish tissue	Up to 80%	RLO	Athanassopoulou <i>et al.</i> , 1999
2000	White seabass <i>A. nobilis</i>	Sothorn California USA (Seawater)	Isolation; LM (white Giemsa); IFAT with serum against <i>P. salmonis</i> was reacted weakly.	Not reported	White seabass <i>P. salmonis</i> -like organism (WSPSLO)	Chen <i>et al.</i> , 2000
2000	Grouper <i>E. melanostigma</i>	Taiwan	LM (H&E) and EM of affected fish tissue; IHC positive with polyclonal against <i>P. salmonis</i> ; attempted to isolate with fish cell lines but with no CPE.	1% in 10 d	Piscirickettsia-like organism	Chen <i>et al.</i> , 2000
1994	Hawaiian tilapia <i>O. mossambicus</i> <i>S. melsnotheron</i>	Oahu, Hawaii (Freshwater)	LM (H&E, Gram, Giemsa, Lillie Twort, Kinyoun's acid fast, Brown & Brenn, Prussion Blue, Fontana Masson); IFA; PCR; attempted to isolate with fish cell lines but with no CPE.	60%	The Hawaiian tilapia Piscirickettsia-like organism (HTPLO)	Mauel <i>et al.</i> , 2003
2005	Atlantic salmon <i>S. salar</i>	Tasmania, Australia (Seawater)	IHC, Immunoelectron microscopy; PCR; Nucleic acid sequencing; phylogenetic analysis of rDNA sequences; attempted to isolate with fish cell lines but with no CPE.	Not reported	The Tasmanian RLO	Corbeil <i>et al.</i> , 2005

organism, while Scottish isolates clustered together with the Norwegian and Canadian isolates from Atlantic salmon.

McCarthy *et al.* (2005) compared the DNA sequences of the 16S rDNA and 16S-23S ITS region of the sea bass piscirickettsia-like organism (SBPLO) isolated from European sea bass with published sequences from *P. salmonis* strains. Results showed this SBPLO to be another strain of *P. salmonis*, closely related to the salmonid pathogens.

Arkush *et al.* (2005) also reported an intracellular bacterium isolated from white sea bass in Southern California, USA identified as *P. salmonis* by sequences from 16S and 23S rDNA and ITS.

It is agreed that the taxonomic classification of *P. salmonis* isolates needs further analysis.

**Morphology :** The size of *P. salmonis* is between 0.5-1.5  $\mu\text{m}$  in diameter. It is a coccoid or ring-formed bacterium or can appear as a pair of curved rods. It is pleomorphic, non-encapsulated, non-motile and Gram negative. The bacterium usually develops within the cytoplasmic vacuoles of the host cells and can occur either singly in diffuse groups, in pairs or as dense morula-like masses. They multiply by binary fission (Figure 1). Individual or paired organisms enclosed in membrane bound vacuoles, are surrounded by a double membrane layer consisting of a highly rippled outer membrane and an inner membrane closely associated with the cytoplasm of the bacterium. It has a typical Gram negative cell wall and protoplasmic structure of a prokaryote (Fryer *et al.*, 1990; Fryer *et al.*, 1992). The cell contents are composed of numerous ribosome-like particles concentrated near the plasma membrane, a single or multiple fibrillar nucleoid(s) localized in the central region and small electron lucent vacuoles (Fryer *et al.*, 1990). The vacuoles are not bound by a membrane and are variable in size and number (Cvitanich *et al.*, 1991).

The RLO stain dark blue with Giemsa, methylene blue, retain basic fuchsin when stained with Pinkerton's adaptation of Gimenez, and are basophilic with H&E, but they do not stain with PAS, Ziehl-Neelsen acid fast or Macchiavello's Gimenez. The organisms do not react with MAb against the group-specific chlamydial LPS antigen (Fryer *et al.*, 1990; Turnbull, 1993).

**Culture Characteristics in Vitro :** *P. salmonis* produces a CPE in the following salmonid cell lines: chinook salmon embryo (CHSE-214) (Figure 2); chum salmon, *Oncorhynchus keta* heart (CHH-1); coho salmon embryo (CSE-119); rainbow trout gonad (RTG-2); and the following non-salmonid fish cell lines: common carp, *Cyprinus carpio*, epithelioma papillosum cyprini (EPC) and fathead minnow, *Pimephales promelas* Rafinesque (FHM), but not in brown bullhead, *Ictalurus nebulosus* (Lesueur) (BB) and bluegill, *Lepomis macrochirus* Rafinesque, fry

(BF-2) cell cultures. The RLO responsible for causing disease in tilapia in Taiwan was cultured in a non-salmonid fish cell line, Nile tilapia ovary origin cells (TO-2) at 25°C (Chern and Chao, 1994). Attempts to culture the organism on artificial media in different culture environments have however failed (Cvitanich *et al.*, 1991). A CPE in fish cell lines appears after 5-6 d and the monolayer completely lyses by 14 d at 15-18°C. Although some attempts to obtain a CPE by *P. salmonis* in the BB cell line have failed (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991), a CPE by the bacterium with complete destruction of the monolayer by 78 d post-infection has been reported in BB cells (Almendras *et al.*, 1997). Almendras *et al.* (1997) observed the organism free and in intracytoplasmic and extracellular locations under transmission electron microscopy (TEM).

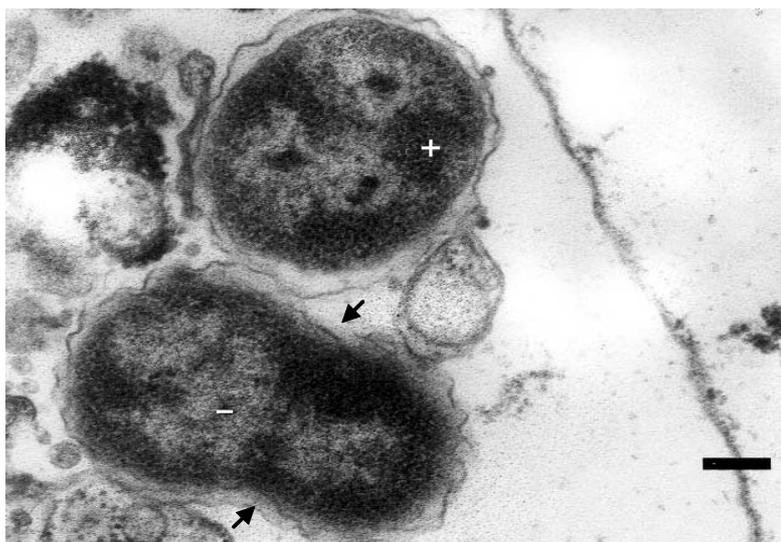
Birkbeck *et al.* (2004a) reported that *P. salmonis* could be grown in a *Spodoptera frugiperda* (Sf21) insect cell line. The yield of *P. salmonis* isolate SCO-95A, from Atlantic salmon in Scotland, was up to 100 times that obtained in CHSE-214 cells over 14 to 21 days at 18°C, and its virulence for Atlantic salmon was retained. The replication titre of *P. salmonis* is  $10^6$  to  $10^7$  50% tissue culture infective dose (TCID<sub>50</sub>) ml<sup>-1</sup> in fish cell lines.

Several antibiotics, streptomycin, gentamycin or tetracycline, but not penicillin, inhibit replication of the organism *in vitro*. The infectivity titre is destroyed by 99% after one freeze-thaw cycle. The addition of 10% dimethyl sulfoxide (DMSO) in the freezing medium helps act as a cryopreservative for the organism (Fryer *et al.*, 1990).

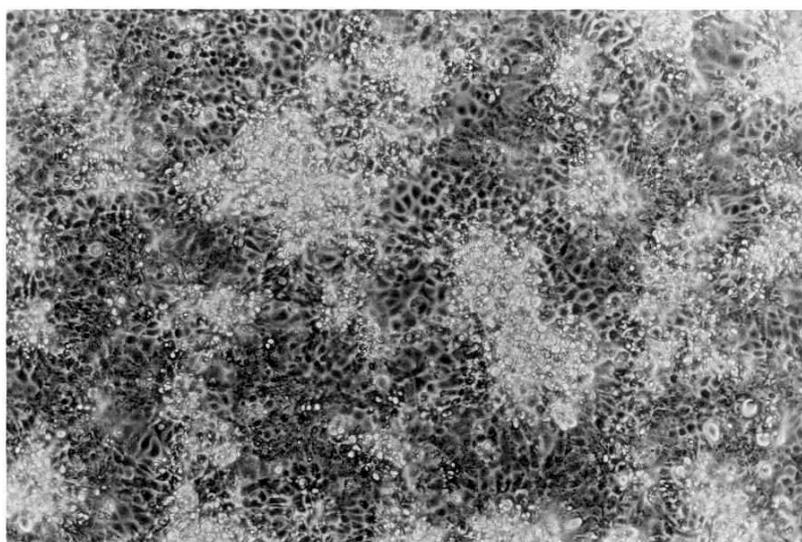
### Clinical Signs

**Gross Pathology :** The clinical signs of rickettsial disease differ between outbreaks and individual fish, although some fish die with very few signs of abnormality or exhibiting external signs of the disease (Turnbull, 1993; Cvitanich *et al.*, 1991).

Moribund fish swim near the surface or at the side of the net, with vertical or circling movement, and lethargic and anorexic behaviour (Schafer *et al.*, 1990; Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Brocklebank *et al.*, 1992; Rodger and Drinan, 1993; Bravo, 1994; Chen *et al.*, 1994; Palmer *et al.*, 1996; Cusack *et al.*, 1997; Olsen *et al.*, 1997). Enlargement of the abdominal cavity with ascites and peritonitis is also observed (Schafer *et al.*, 1990; Branson and Nieto Diaz-Munoz, 1991; Chen *et al.*, 1994). General internal pallor, necrotic stomatitis, a transparent seromucous liquid in the stomach and a yellowish mucous content in the intestine have also been reported (Schafer *et al.*, 1990; Branson and Nieto Diaz-Munoz, 1991). Petechial haemorrhages are frequently evident in the stomach, intestine, pyloric caeca, swim bladder, visceral fat and skeletal muscle and internal lesions included petechia



**Figure 1.** Progress of CPE by *P. salmonis* in CHSE cells examined by TEM (a) *P. salmonis* in CHSE cell (2 h post-incubation), showing the bacterium undergoing binary fission, a division into two similarly equal parts (arrows); note the electron dense areas resembling ribosome-like structures seen throughout the cell indicated by (+) and DNA-like material present as a filamentous network indicated by (-). Bar: 0.2  $\mu$ m



**Figure 2.** Development of CPE by *P. salmonis* in CHSE cells, 7 d post-inoculation (magnification x 100).

on serosa, fibrous adhesions in the peritoneal cavity, and white nodules on the stomach, intestine and mesenteric fat (Cvitanich *et al.*, 1990; Brocklebank *et al.*, 1992; Chen *et al.*, 1994; Palmer *et al.*, 1996; Olsen *et al.*, 1997).

The spleen, liver and kidney of infected fish are often swollen with pale discoloration. They often have a haemorrhaged liver with yellow multifocal subcapsular nodules scattered diffusely throughout and sometimes internal lesions. The kidney appears pale with inflammation and petechial hemorrhages. Occasionally, small white foci are seen in the heart,

kidney, spleen and skeletal musculature (Schafer *et al.*, 1990; Cvitanich *et al.*, 1990; Branson and Nieto Diaz-Munoz, 1991; Brocklebank *et al.*, 1992; Rodger and Drinan, 1993; Chen *et al.*, 1994; Chern and Chao, 1994; Olsen *et al.*, 1997). Extensive haemorrhages on the surface of the brain and softening of the brain tissue have also been reported (Chern and Chao, 1994; Comps *et al.*, 1996), as has the presence of a whitish pseudomembrane around the heart (Cvitanich *et al.*, 1991). Many of the infected fish are dark in colour and possess skin lesions with small areas of raised scales, areas with no scales or superficial white

spots, or ulcers and petechiae along the dorso-lateral surfaces or ventral surfaces (Schafer *et al.*, 1990; Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Bravo, 1994; Olsen *et al.*, 1997). Other clinical signs are shallow dermal ulcers on the lateral caudal peduncle and haemorrhages in the dorsal and ventral fins, punctuate ulceration on the head, pale coloured gills, white nodules on the gills in some fish and bilateral exophthalmus (Schafer *et al.*, 1990; Cvitanich *et al.*, 1991; Brocklebank *et al.*, 1992; Comps *et al.*, 1996; Turnbull, 1993; Bravo, 1994; Chen *et al.*, 1994; Chern and Chao, 1994; Cusack *et al.*, 1997; Olsen *et al.*, 1997).

**Histopathology :** Although histopathological findings associated with rickettsial disease have been described in both naturally and experimentally infected fish, the sequence of histological changes and systemic dissemination of RLO has not yet been described (Almendras and Fuentealba, 1997).

Rickettsia are commonly observed within macrophages, within cytoplasmic vacuoles, free in the cytoplasm or outside host cells. In some fish, darkly-stained basophilic cells, approximately 20 x 10 µm in size, with large nuclei and a little cytoplasm in small or large numbers but often in close groups, appear within the haematopoietic tissue, but as yet their function is not clear (Branson and Nieto Diaz-Munoz, 1991; Turnbull, 1993).

The most marked pathology, necrosis and oedema with a granulomatous response, is found in the haematopoietic tissue of the kidney and spleen and in the hepatocytes in the liver. The haematopoietic tissue displays disseminated intravascular coagulation with necrotic thrombi associated with necrotic changes to vessel endothelium (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Evelyn, 1992; Turnbull, 1993; Rodger and Drinan, 1993; Chern and Chao, 1994; Chen *et al.*, 1994; Palmer *et al.*, 1996). Perivascular cellular inflammation and vascular lesions primarily in the liver, kidney and spleen have also been reported (Branson and Nieto Diaz-Munoz, 1991, Cvitanich *et al.*, 1991, Chen *et al.*, 1994, Olsen *et al.*, 1997). Additionally, some degree of glomerular nephritis with vacuolation, oedema of the capsule and increased level of eosinophilic ground substance is apparent in the kidney (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Rodger and Drinan, 1993; Khoo *et al.*, 1995). Multifocal to diffuse necrosis with oedema, invasion by inflammatory cells, and increased level of eosinophilic ground substance and some fibrosis can be seen in liver (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Rodger and Drinan, 1993; Chern and Chao, 1994; Olsen *et al.*, 1997).

Endocarditis along with variable pericarditis, which is sometimes necrotic, and focal hyaline necrosis of the myocardium is seen in the heart (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Rodger and Drinan, 1993; Chen *et al.*,

1994; Chern and Chao, 1994; Olsen *et al.*, 1997). The intestine displays necrosis and inflammation resulting in diffuse chronic inflammatory lesions in the lamina propria and sloughing of the mucosa (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Chen *et al.*, 1994; Chern and Chao, 1994). Granulomatous inflammation in the meninges and mild inflammatory and thrombotic lesions in brain, pancreas, ovaries, heart, mesentery, testis, eye, muscle, pseudobranch, nasal capsule and adipose tissue are also seen (Cvitanich *et al.*, 1991; Rodger and Drinan, 1993; Chen *et al.*, 1994; Comps *et al.*, 1996; Olsen *et al.*, 1997). Multifocal epithelial hyperplasia resulting in fusion of the gill lamellae and necrotic areas and fibrin thrombi in lamellar capillaries, have been observed. Varying degrees of necrosis of the epidermis, dermis and underlying musculature in the skin lesions and a spreading inflammatory response along the intra-muscular septa have also been reported (Branson and Nieto Diaz-Munoz, 1991; Cvitanich *et al.*, 1991; Chen *et al.*, 1994; Chern and Chao, 1994; Palmer *et al.*, 1996; Olsen *et al.*, 1997).

**Haematology :** The pale coloration of infected fish is because of the presence of a low haematocrit during rickettsiosis. The normal haematocrit value is in the region of 35-50%, while in affected salmonids it is often reduced to between 2 to 35% (Turnbull, 1993). A similar reduction in haematocrit values has been observed in tilapia affected by the disease (Chern and Chao, 1994). Branson and Nieto-Diaz Munoz (1991) reported a simultaneous neutrophilia 10-20 times higher than normal in blood smears from affected fish. Cvitanich *et al.* (1991) demonstrated the presence of large numbers of macrophages which contained either degenerate cellular debris or the organism in peripheral blood smears, and typically normochromic–normocytic erythrocytes but rarely immature red blood cells. These appeared to be the most striking changes observed in the hematology of moribund fish.

### Transmission

Initially rickettsial disease in fish was first associated with increased stress caused by fluctuations in water temperature, severe storms or blooms of non-toxic algae. However, significant fish losses were still observed after the disappearance of the algal bloom and stabilisation of water temperature. There has not been a strong association between disease outbreaks and environmental conditions if outbreaks worldwide are taken into consideration.

Transmission of RLO by an intermediate host also has been considered. The presence of sea lice *Calligus* spp., or isopods and molluscs, which move freely through net pens, may act as possible vectors in regions of disease outbreaks. Intermediate hosts are

involved in terrestrial rickettsial disease with an exception of the genus *Coxiella*, which forms a spore-like structure, transmitted by aerosol dust, infecting the respiratory tract of its host (Weiss and Moulder, 1984). In the aquatic environment an intermediate host or a vector may not be required for delivery of rickettsia to its host since the extracellular survival of *P. salmonis* from Coho salmon has been shown as 14 d at 15°C in sea water (Lannan and Fryer, 1994). The survival of *P. salmonis* in salt water may provide sufficient time to allow horizontal transmission of the organism in the marine environment without the need for an intermediate host or vector. However, rapid inactivation of *P. salmonis* in freshwater may limit the transmission of the organism in this environment and may explain why the disease is rarely observed in freshwater (Lannan and Fryer, 1994).

Cvitanich *et al.* (1991) reported horizontal transmission between injected and uninjected Coho salmon held in static freshwater and seawater aquaria at 15°C, while Garcés *et al.* (1991) did not observe any disease transmission when uninfected coho salmon were held together with infected Coho salmon at 8°C in a tank with flow-through freshwater. Chern and Chao (1994) demonstrated direct horizontal transmission of RLO in tilapia by mixing fish injected intramuscularly with RLO together with uninfected fish in the same tank. Salinas *et al.* (1997) demonstrated horizontal transmission of *P. salmonis* (isolate SLGO-95) experimentally in rainbow trout. They detected the agent in bile, faeces and urine of healthy and/or moribund fish 7 d post-inoculation by using an IFAT. Although horizontal transmission of the rickettsial disease in fish has not been proved under farm conditions, granuloma lesions present on gills, or lesions on the intestines of infected fish suggest that these sites may be a possible route of entry for the RLO, or possibly that RLO can be shed from infected fish through faeces (Turnbull, 1993; Chern and Chao, 1994).

Almendras *et al.* (1997) compared experimental routes of infection by *P. salmonis* using intraperitoneal injection, and oral or gill application. They also examined the importance of physical contact in horizontal transmission in freshwater-raised Atlantic salmon. According to their study, *P. salmonis* is transmitted horizontally to fish without the need for physical contact, and the pathogen appears to enter the fish via the gills, skin and the oral route, although direct contact increased transmission of *P. salmonis* and less mortalities occurred through oral infection. Smith *et al.* (1999) investigated the portal entry of *P. salmonis* using experimental infection of rainbow trout via intraperitoneal injection, subcutaneous injection, patch-contact on the skin, patch-contact on gills, intestinal intubation and gastric intubation. In this study, the cumulative mortalities were 98, 100, 52, 24, 24, and 2% at day 33 post-infection respectively, showing that intact skin and gills may be possible routes of entry for rainbow trout.

Smith *et al.* (2004) examined the portal of entry of *P. salmonis* using *P. salmonis* isolate SLGO-95 using doses of  $10^{4.7}$  and  $10^{3.7}$  TCID<sub>50</sub>. Juvenile Coho salmon were infected with each dose via the skin and gills using calibrated drops of bacterial suspension and through the intestine by intubation through the anus. Cumulative mortalities and survival analysis showed that the most effective portal of entry was the skin, followed by the intestine and then the gills. Results of this study showed that *P. salmonis* can penetrate, and then systematically invade Coho salmon, through the skin and the mucous membranes (Smith *et al.*, 2004).

Cvitanich *et al.* (1991) reported the existence of RLO in the ovaries and testes of immature fish, and in ovarian fluid of mature fish, suggesting the possibility of vertical transmission of the disease in Chile. Gaggero *et al.* (1995) isolated *P. salmonis* from 60-90 d old progeny of Coho salmon, Atlantic salmon and rainbow trout maintained in freshwater. Whether vertical transmission of *P. salmonis* had occurred or the origin of the *P. salmonis* infection came from the freshwater environment, was not clear from this study. Larenas *et al.* (1996) found that approximately 10% of eggs and fry of rainbow trout from experimentally infected male or female brooders were infected with *P. salmonis*. The disease agent was observed inside the yolk of the eggs, again suggesting vertical transmission of the disease. However, the source and mode of transmission of *P. salmonis* still requires further investigation.

Larenas *et al.* (2003) showed vertical transmission of *P. salmonis* by inoculation of male and female rainbow trout broodstock with the bacterium. *P. salmonis* was detected by IFAT in milt and coelomic fluid in the majority of inoculated broodstock and in the fry when one or both parents were inoculated, however the infected fry did not showed signs of the disease. In this study, the bacterium was also detected in progeny obtained through fertilisation ova from non-inoculated females incubated in a medium containing a bacterial suspension. Examination of ova incubated with the bacterium by scanning electron microscopy demonstrated that the bacterium attaches to the ova by means of membrane extensions that probably originate from the external membrane of the bacterium. These extensions allow penetration into the ovum and later termed the 'piscirickettsial attachment complex' (PAC) by the authors. This study supported the hypothesis of vertical transmission for *P. salmonis* and also demonstrated the capacity of the bacterium to adhere to ova. Kidney smears from broodstock used in the study tested negative by IFAT, but their reproductive fluids proved positive when analysed. The result suggesting that diagnosis of *P. salmonis* should be performed on seminal and ovarian fluids as well as kidney tissue (Larenas *et al.*, 2003).

### Detection and Identification of *P. salmonis*

Diagnosis of rickettsial disease is usually based on gross and histological signs with the use of histochemical stains such as H&E, Gram, Giemsa, Acridine orange, Methylene blue, Gimenez, Macchiavello and PAS to detect the pathogen in smears or tissue sections. These techniques are fast and widely used, but they are non-specific (Fryer *et al.*, 1990; Almendras and Fuentealba, 1997). The most specific technique available initially was the isolation of *P. salmonis* in cell lines. This technique is, however, time consuming and difficult since culture has to be performed without antibiotics. This opens the cell cultures to contamination by other bacterial agents (Fryer and Lannan, 1996).

Antibody-based methods are a more specific way of confirming the identity of *P. salmonis* (Fryer and Lannan, 1996). Indirect fluorescent antibody test (IFAT) and immunohistochemistry (IHC), using rabbit anti-*P. salmonis* PAb, have been developed to detect *P. salmonis*. (Lannan *et al.*, 1991; Alday-Sanz *et al.*, 1994). IFAT provides a sensitive and specific method for detection of the pathogen and can be successfully performed on blood films, tissue sections and smears. IHC is a rapid diagnostic test which can be used both on formalin-fixed wax-embedded samples. It has the advantage that identification of the pathogen through immunodetection and examination of infected tissue and histopathological lesions can be performed at the same time (Alday-Sanz *et al.*, 1994). Enzyme linked immunosorbent assay (ELISA) which has also been used for the detection of *P. salmonis* in salmonid has certain advantages such as eliminating the uncertainty of microscopic interpretation, and provides an alternative to polymerase chain reaction (PCR) from which a number of false negative results have been reported (Cassigoli, 1994; Carlos *et al.*, 1997).

Nested PCR is a rapid, highly sensitive and specific test and has been described for the detection of genomic DNA of *P. salmonis*. This technique is useful for the detection of the organism in the early stages of infection. It can also be used to confirm the taxonomy and ecological characteristics of the bacterium and to examine modes of transmission, range of natural hosts, and the pathogens reservoir and geographical distribution. It can also be used to characterise other RLO (Mauel *et al.*, 1996).

DNA-hybridization approaches, such as dot-blot hybridisation assay and *in-situ* hybridisation, have also been used in recent years to detect *P. salmonis* in affected fish (Venegas *et al.*, 2004).

### Treatment and Control of Piscirickettsiosis

**General control :** Improved management practices are important in reducing the disease, including early elimination of dead and clinically diseased fish, using low stocking density with a decreased biomass per

site, high standard of the cage environment, separating year classes at each site to prevent transmission of the agent between year classes, appropriate disposal of blood from harvested fish, removal of infected broodstock and their eggs, and the routine disinfection of eggs (Branson and Nieto-Diaz, 1991; Evelyn, 1992; Rodger and Drinan, 1993; Turnbull, 1993; Almendras and Fuentealba, 1997).

**Chemotherapy :** Antibiotic sensitivity tests with *P. salmonis in vitro* have shown isolates to be sensitive to streptomycin, gentamycin, chloramphenicol, erythromycin, oxytetracyclin, tetracyclin, clarithromycin, sarafloxacin and oxolonic acid and resistant to penicillin, penicillin G and spectinomycin (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991; Grant *et al.*, 1996; Palmer *et al.*, 1996). Most species of the *Rickettsiales* are inhibited by penicillin (Weiss and Moulder, 1984). Oral administration of oxytetracycline may have some benefit for control of the disease (Evelyn, 1992; Chern and Chao, 1994), however, reduced sensitivity of *P. salmonis* to oxolinic acid and oxytetracycline has been reported (Smith *et al.*, 1996).

**Vaccines :** Since there is no effective method of treatment for piscirickettsiosis, vaccination potentially is of great value, especially since vaccines have been used in the control of many other fish diseases (Fryer and Lannan, 1996). A variety of information on vaccine trials for piscirickettsiosis has been reported.

Smith *et al.* (1995) vaccinated pre-smolt Coho salmon intraperitoneally with formalin-killed preparations of *P. salmonis* at a freshwater site. Two different bacteria preparations, non-concentrated bacterin and concentrated antigen emulsified with Freund's complete adjuvant were used. After 20 weeks post-immunisation, the fish were transferred to a sea site to be naturally infected with the pathogen. A lower cumulative mortality to that of the control group was observed in fish vaccinated with the non-concentrated bacterin. However, a slightly higher mortality rate to that of the control group occurred when fish were vaccinated with a concentrated antigen emulsified in Freund's complete adjuvant. Smith *et al.* (1995) suggested that the non-concentrated bacterin resulted in a protective response in the fish. Although the results obtained with non-concentrated bacterin are encouraging, they must be evaluated with caution because the natural challenge may have been low and *R. salmoninarum* together with *P. salmonis* was detected in experimental fish. Thus, the trial was not long enough to determine the level of protection in larger fish in which economic losses due to the disease are more significant.

Smith *et al.* (1997) performed a similar trial for Coho salmon and rainbow trout. Rainbow trout showed a low level of infection when bath challenged and lower cumulative mortalities were obtained compare to the non-vaccinated control group when

naturally challenged in sea water. Smith *et al.* (1997) also obtained the highest levels of antibody in the sera of fish vaccinated with concentrated antigen contrary to the previous study by Smith *et al.* (1995). Although the results are encouraging the challenge was not strong enough to form any firm conclusion since only 20% mortalities occurred in the control fish. Kuzyk *et al.* (2001a) cloned a 17 kDa outer surface protein (OspA) with 62% amino acid sequence homology to the genus common 17 kDa outer membrane lipoprotein of *Rickettsia prowazekii*, previously thought to be found in members of the genus *Rickettsia*. The recombinant OspA, produced in *Escherichia coli*, provided a high level of protection in vaccinated Coho salmon challenged with *P. salmonis* with a relative percent survival of 59% (Kuzyk *et al.*, 2001b). The authors increased the efficacy of the OspA vaccine threefold by the addition of T cell epitopes from tetanus toxin and measles virus fusion protein that are universally immunogenic to mammalian immune systems. Further studies under more controlled conditions are required to develop and evaluate the response of fish to these vaccine preparations.

A Scottish isolate of *P. salmonis* (SCO-95A), cultured in Sf21 insect cells, was used in a vaccination trial using Atlantic salmon post-smolts (Birkbeck *et al.*, 2004b). The vaccine was administered to fish by intraperitoneal injection using either  $1 \times 10^9$  heat-inactivated ( $100^\circ\text{C}$  for 30 min) or  $1 \times 10^9$  formalin-inactivated bacteria in adjuvant, while the control group received phosphate-buffered saline in adjuvant. Groups of fish were maintained in ambient seawater at a mean temperature of approximately  $10^\circ\text{C}$ . However, due to space limitations, each group was divided into 2 tanks, one held in seawater at an ambient temperature of  $7.5^\circ\text{C}$ , and the other group transferred to freshwater gradually acclimatising to  $16^\circ\text{C}$ . Six months after vaccination, fish were challenged with 0.1 ml of *P. salmonis* grown in CHSE cells ( $2 \times 10^5$  TCID  $\text{ml}^{-1}$ ) or ( $2 \times 10^4$  TCID  $\text{fish}^{-1}$ ) by injecting the bacterium into the dorsal median sinus of the fish. The challenged fish were held at  $7.5$  to  $8.5^\circ\text{C}$  in seawater or  $16^\circ\text{C}$  in freshwater for 54 days. Temperature was an important factor in the development of SRS, with no deaths occurring in fish held in the ambient seawater at  $7.5$  to  $8.5^\circ\text{C}$ . In freshwater significant protection for the heat-inactivated and formalin-inactivated vaccines with relative percentage survival of 70.7 and 49.6% obtained respectively. Mortalities obtained in the control group reached 81.8%. The authors of the study did not report the nature of the protective antigen, but indicate that it could be lipopolysaccharide or a heat-stable outer membrane protein.

Miquel *et al.* (2003) used expression library immunisation technology to examine the protection of Coho salmon to infection with *P. salmonis*. Fragments of purified DNA of *P. salmonis* were obtained by

sonication, and then cloned into an expression vector pCMV-Bios. Two libraries were obtained resulting in 22 000 and 28 000 colonies and corresponding to app. 8 and 10 times the genome of the pathogen, respectively. The size of the inserts ranged from between 300 and 1000 bp. The plasmid DNA isolated from one of these libraries was purified and  $20 \mu\text{g}$  injected intramuscularly into 60 fish followed by a second dose of  $10 \mu\text{g}$  40 days later. Control fish were injected with the same amount of DNA of the vector pCMV-Bios without the inserts. All fish were maintained at approximately  $12^\circ\text{C}$ . Sixty days after applying the second dose of DNA, fish were challenged with  $2.5 \times 10^7$  *P. salmonis* corresponding to 7.5 times the  $\text{LD}_{50}$  and at 30 days post-challenge. The level of mortality was 100% for control fish while 20% of the vaccinated fish survived. Protection in vaccinated fish was demonstrated by a decrease in level of mortality, a decreased bacterial load within the fish and the presence of specific anti *P. salmonis* antibodies (Miquel *et al.*, 2003).

Wilhelm *et al.* (2003) isolated and sequenced the heat shock proteins HsP10 and HsP16 of *P. salmonis*. Serum from salmon infected with *P. salmonis* reacted with these antigens by ELISA. The genes encoding HsP60 and HsP70 have also been isolated and sequenced (Wilhelm *et al.*, 2005). The recombinant HsPs were shown to elicit a humoral response when injected intraperitoneally in Atlantic salmon and also gave protection to fish challenged with *P. salmonis*. A strong humoral has also been reported against membrane bound transglycosylase B (MltB) and the transferrin binding protein B (TbpB) when these proteins were injected intraperitoneally into both mice and Atlantic salmon (Wilhelm *et al.*, 2004).

In recent studies by Rise *et al.* (2004) molecular biomarkers of Atlantic salmon macrophage and haematopoietic kidney response to *P. salmonis* were identified. Seventy-one transcripts were found to be up regulated and 31 down regulated by salmon macrophages infected with the bacterium. The authors believe changes in the redox status of infected macrophages may enable macrophages to become infected with *P. salmonis*. The identification of molecular biomarkers to *P. salmonis* infection may help in further development of vaccine and therapeutics to combat the disease.

## Conclusion

RLO is an important pathogen known to infect fish, with a significant impact on the health of various fish species worldwide. *P. salmonis* is the first rickettsial pathogen to be isolated and for which it has been demonstrated to be the etiological agent of piscirickettsiosis in salmonids.

Since the isolation of *P. salmonis* in 1989, there have been increasing numbers of reports of rickettsial disease in a diverse range of fish species cultured in a variety of geographic locations. Information in the

literature relating to the pathogenicity of the bacterium, differences between *P. salmonis* isolates, specific and sensitive detection methods and a suitable control method for the disease is still sparse. Such information is necessary to establish effective control strategies for the disease.

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