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Review

Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review

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Abstract

The bacterium *Piscirickettsia salmonis* is the aetiological agent of piscirickettsiosis a severe disease that has caused major economic losses in the aquaculture industry since its appearance in 1989. Recent reports of *P. salmonis* or *P. salmonis*-like organisms in new fish hosts and geographical regions have increased interest in the bacterium. Because this gram-negative bacterium is still poorly understood, many relevant aspects of its life cycle, virulence and pathogenesis must be investigated before prophylactic procedures can be properly designed. The development of effective control strategies for the disease has been limited due to a lack of knowledge about the biology, intracellular growth, transmission and virulence of the organism. Piscirickettsiosis has been difficult to control; the failure of antibiotic treatment is common, and currently used vaccines show variable long-term efficacy. This review summarizes the biology and characteristics of the bacterium, including its virulence; the infective strategy of *P. salmonis* for survival and evasion of the host immune response; the host immune response to invasion by this pathogen; and newly described features of the pathology, pathogenesis, epidemiology and transmission. Current approaches to the prevention of and treatment for piscirickettsiosis are discussed.

Keywords: control, epidemiology, pathogenesis, pathology, *Piscirickettsia salmonis*, piscirickettsiosis, transmission.

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Introduction

Piscirickettsia salmonis was the first rickettsia-like bacterium to be known as a fish pathogen (Fryer *et al.* 1992). Since the first reports of piscirickettsiosis in Chile at the end of the 1980s, *Piscirickettsia*-like bacteria have been frequently recognized in various fish species farmed in fresh water and sea water and have significantly affected the productivity of aquaculture worldwide (Mauel & Miller 2002). The first record of a fish rickettsia-like organism (RLO) was described in fahaka pufferfish, *Tetraodon fahaka* (Hasselquist 1762), that originated from the Nile River in Egypt (Mohamed 1939).

A similar rickettsial septicaemia, 'parenthesis disease', has been recognized since 1970 in salmon held in sea water in British Columbia, Canada (Evelyn 1992). This septicaemia was first observed in 1970 and 1978 in pink salmon, *Oncorhynchus gorbuscha* (Walbaum 1792), that were being cultured in seawater tanks for experimental purposes and was later observed in 1983 and 1984 in farmed coho, *Oncorhynchus kisutch* (Walbaum

Journal of Fish Diseases

1792), and chinook salmon, *Oncorhynchus tshawytscha* (Walbaum 1792) (Evelyn 1992). Subsequently, a similar rickettsial septicaemia was described in 1991 in Atlantic salmon, *Salmo salar* (Linnaeus 1758), farmed in sea water in British Columbia (Brocklebank *et al.* 1992). Gross pathology was consistent with the features of piscirickettsiosis described in Chile and was similar to pathological lesions previously noted in 1980 in coho and chinook salmon (Evelyn 1992). Later, a *Piscirickettsia*-like organism (PLO) was isolated from farmed Atlantic salmon from the eastern coast of Canada (Jones *et al.* 1998; Cusack, Groman & Jones 2002).

In addition, RLOs have also been recognized in Atlantic salmon farmed in Ireland and Scotland, United Kingdom. In Ireland, an RLO was observed in connection with low-mortality disease in Atlantic salmon post-smolts farmed along the western coast in 1991 (Rodger & Drinan 1993). No pathogen was isolated, but microorganisms similar to *P. salmonis* were observed in different tissues by microscopy. Between 1995 and 1996, four new outbreaks of the disease were described, and an RLO was isolated using the CHSE-214 (chinook salmon embryo) cell line and confirmed using anti-*P. salmonis* antibodies (Rodger & Drinan 1993). Finally, the association between the isolated agent and the disease was confirmed by experimental inoculation. In the same way, an RLO was isolated from seawater-farmed Atlantic salmon with high mortality in Scotland (Grant *et al.* 1996). The main histopathological lesion was encephalitis associated with vasculitis, and a great number of coccoid and basophilic microorganisms, approximately 1 μm in diameter, were found. The agent was isolated from brain tissue using the CHSE-214 cell line without antibiotics and showed a cytopathic effect (CPE) at 9 days post-inoculation at 15 °C; Koch's postulates were fulfilled. Further outbreaks of disease associated with *P. salmonis* in farmed Atlantic salmon have been described in Scotland (Birrell, Mitchell & Bruno 2003; Reid, Griffen & Birkbeck 2004), although the incidence and impact of piscirickettsiosis in Scotland are very low.

Between 1988 and 1992, an RLO was isolated from 51 Atlantic salmon farms along the western coast of Norway (Olsen *et al.* 1997). In total, 71% of these outbreaks occurred during the fall of 1988. Because of morphological and serological similarities to the type strain, the suggested name for the organism was *P. salmonis* (Olsen *et al.* 1997). The main histopathological finding was hepatic necrosis and the presence of an RLO. The disease was frequently recorded after algal blooms, and the smolt pens tended to be overstocked with fish in poor condition (Olsen *et al.* 1997). Since then, the impact of infection caused by *P. salmonis* on the Norwegian industry has been low, perhaps because the better quality of smolt, better culture conditions and natural environmental conditions are not favourable for the bacterium or its possible vector (Olsen *et al.* 1997).

In Chile, the first outbreaks of piscirickettsiosis appeared by the end of 1989, although it has been suggested that the disease has been present in coho salmon since 1983 (Bravo & Campos 1989). Originally, piscirickettsiosis was described as affecting coho salmon (Bravo & Campos 1989; Cvitanich, Gárate & Smith 1990, 1991; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991), causing up to 90% of mortalities on certain farms (Cvitanich *et al.* 1990). Economic estimates conducted in 1989 determined losses of approximately US $10 million, representing the mortality of approximately 1.5 million coho salmon (Cvitanich *et al.* 1990). Subsequently, piscirickettsiosis was also detected in Atlantic salmon and rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (Cvitanich *et al.* 1991; Cvitanich, Gárate & Smith 1995). The disease has been mainly described in sea water and brackish waters (Bravo & Campos 1989; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991) and, very occasionally, in fresh water (Bravo 1994; Gaggero, Castro & Sandino 1995).

In 2006, before the infectious salmon anaemia (ISA) crisis in Chile, the Technological Institute of Salmon (INTESAL) estimated that the direct economic losses caused by piscirickettsiosis during the ongrowing phase in sea water were approximately US $100 million and that losses of the potential concept of harvest were worth approximately US $400 million (Cabezas 2006). These figures represented approximately 25% of total economic revenues from salmon exports during the same year. After the ISA crisis, piscirickettsiosis lost its key role in the health scene; today, however, together with the productive reactivation of the Chilean salmon industry, the disease has re-emerged as the main health challenge in the sector, thereby repositioning issues that are not yet

solved and are associated with diagnosis, treatment, prevention and control (Ibieta *et al.* 2011).

Currently, piscirickettsiosis is one of the most important threats to the sustainability of the Chilean salmon industry (Ibieta *et al.* 2011). Piscirickettsiosis has evolved over time; each new outbreak is increasingly insidious and refractory to treatments, and each has shown increased bacterial virulence, clinical and pathological severity and variable presentation under similar conditions of species, age and management measures (Leal & Woywood 2007; Marshall *et al.* 2007). In general, the salmon industry has focused its control strategy for the disease on antimicrobial therapies and vaccines. The use of antibiotics, both prophylactically and during early infection, may inhibit the growth of the pathogen, but such treatments have been largely unsuccessful in stopping disease outbreaks (Cabello 2006). Similarly, commercial vaccines against *P. salmonis* have not proven to be of high efficacy (Leal & Woywood 2007; Marshall *et al.* 2007).

A bacterium isolated from hatchery-reared juvenile white seabass, *Atractoscion nobilis* (Ayres 1860), in southern California, USA, was originally described as a PLO (Chen *et al.* 1994) and was identified as *P. salmonis* by the sequences of its small- and large-subunit ribosomal (16S and 23S) DNA and the internal-transcribed spacer (ITS) (WSB-98) (Arkush *et al.* 2005). The 16S rDNA homology with the type strain LF-89 was 99%. Additionally, the isolate WSB-98 of *P. salmonis* was recognized by the polymerase chain reaction (PCR) test designed by Mauel, Giovannoni and Fryer (1996). WSB-98 induced both mortality and disease similar to those of the piscirickettsiosis described in salmon in experimentally infected juvenile white seabass. In preliminary experiments, Arkush *et al.* (2005) also demonstrated that WSB-98 can be re-isolated from both chinook and coho salmon following cohabitation with experimentally infected white seabass in sea water at 16 °C.

An RLO in the brains of young seabass, *Dicentrarchus labrax* (Linnaeus 1758), cultured in the Mediterranean, near France (Comps, Raymond & Plassiart 1996), and an RLO from seabass farmed in Greece have been identified and described (Athanassopoulou *et al.* 2004). In addition, the DNA sequences of the 16S rDNA gene and the ITS region were compared with those published for *P. salmonis* strains, and it was found that the seabass PLO (SBPLO) was another strain of *P. salmonis* that was closely related to the salmonid pathogens (McCarthy *et al.* 2005). Two ITS regions were observed in the seabass PLO, one of which contained tRNA genes.

An RLO was observed in farmed Atlantic salmon located in south-east Tasmania, Australia (Corbeil, Hyatt & Crane 2005). Whereas 16S rDNA sequence and phylogenetic analyses demonstrated that the Tasmanian RLO is related to exotic *P. salmonis* isolates, and particularly to the Chilean isolate EM-90, the Tasmanian RLO exhibits an ITS sequence divergence from other *P. salmonis* isolates. This divergence is principally due to a 19-bp deletion, which suggests genetic divergence from *P. salmonis*. In addition, the weak immunohistochemical staining obtained for Tasmanian RLO-infected tissues suggests antigenic similarity to the LF-89 isolate of *P. salmonis*.

Systemic infections in fish caused by gram-negative intracellular bacteria have been commonly referred to as either RLOs, due to morphological similarities with the true *Rickettsia*, or PLOs, following the description of *P. salmonis* (Fryer *et al.* 1992). Currently, many RLOs have been unequivocally shown to be *Francisella* spp., but it should not be assumed that all RLOs/PLOs that are not proven to be *P. salmonis* are in fact *Francisella* spp. (Colquhoun & Duodu 2011).

The agent

Taxonomy

Piscirickettsia salmonis was named to accommodate isolates from diseased salmon in Chile, of which LF-89 was studied in detail (Fryer *et al.* 1992), with 16S rRNA conforming to the gamma subdivision of the Proteobacteria, as for the genera *Coxiella* and *Francisella*. The bacteria of the genera *Neorickettsia, Rickettsia, Cowdria, Anaplasma* and *Ehrlichia* are members of the alpha subdivision of the Proteobacteria. Despite morphological similarities, the genera *Francisella* and *Piscirickettsia* belong to the Gammaproteobacteria and are therefore only distantly related to the true *Rickettsia* (Alphaproteobacteria; Mauel, Giovannoni & Fryer 1999). *Piscirickettsia salmonis* contains many genes that are absent from the *Francisella* genus and is thus likely to occupy distinct ecological niches (Sjödin *et al.* 2012).

The diversity within the 16S, ITS and 23S rDNAs reported by Mauel *et al.* (1999) is not

sufficient to split the genus *Piscirickettsia* into several species. More efforts are necessary to infer phylogenies by considering *P. salmonis* at the genome level, rather than only individual genes. Currently, the genus *Piscirickettsia* contains only *P. salmonis*, but Thomas and Faisal (2009) have described the apparent emergence of a novel *Piscirickettsia* species that causes disease in muskellunge, *Esox masquinongy* (Mitchill 1824), and yellow perch, *Perca flavescens* (Mitchill 1814). Phylogenetic analyses involving sequences of the 16S, ITS and 23S rDNA genes confirmed that muskellunge and yellow perch isolates were identical to each other, but not identical to *P. salmonis*, which devastates cultured salmonids, suggesting that the causative agent was likely a new species of *Piscirickettsia.*

Morphology and culture characteristics

Piscirickettsia salmonis is a gram-negative bacterium that is generally non-motile, aerobic, not encapsulated, pleomorphic, highly fastidious, usually coccoid and found in pairs or ring-shaped structures with an approximate diameter of 0.5–1.5 μm (Fryer *et al.* 1990, 1992). The bacterium replicates by binary fission within membrane-bound cytoplasmic vacuoles in the cells of susceptible fish hosts or fish cell lines. *In vitro* replication is optimal at 15–18 °C, is retarded above 20 °C and below 10 °C and does not occur above 25 °C (Fryer *et al.* 1990). Giemsa-stained tissue smears or imprints from infected organs exhibit darkly stained, pleomorphic *P. salmonis*, commonly in coccoid or ring form, inside cytoplasmic vacuoles surrounded by a membrane. The bacteria occur either singularly or in groups, giving the vacuole the appearance of a morula (Fryer *et al.* 1992). When *P. salmonis* is examined by electron microscopy, the bacterium displays the typical protoplasmic structure of a prokaryote and the cell wall of a gram-negative bacterium (Cvitanich *et al.* 1990; Fryer *et al.* 1990; Schäfer *et al.* 1990). *Piscirickettsia salmonis* has two surface membranes: an external undulated membrane and an internal cytoplasmic membrane (Fryer *et al.* 1990). The bacterium also has structures similar to ribosomes near the plasma membrane, fibrillar DNA in the central region and electrodense spherical structures (Fryer *et al.* 1990, 1992).

Piscirickettsia salmonis was previously considered to be cultivable only in eukaryotic cell lines (Fryer *et al.* 1990; Lannan & Fryer 1991; Birkbeck *et al.* 2004a), but recent reports show that the bacterium may in fact be cultured on cysteine-enriched agar media, verifying the facultative intracellular nature of this fish pathogen (Mauel, Ware & Smith 2008; Mikalsen *et al.* 2008; Gómez, Henríquez & Marshall 2009; Yañez *et al.* 2012, 2013a). AUSTRAL-SRS broth allowed the production of bacteria with a 1.8 optical density when absorbance was measured at 600 nm after 6 days of incubation at 18 °C, and various passages did not alter the culture kinetics (Yañez *et al.* 2012). Recently, Yañez *et al.* (2013a) also described blood-free agar media (Austral-TSHem or Austral-TSFe) for use in the laboratory for the routine culture of *P. salmonis.* Artificial cell-free media provide a proper base to simplify the preparation of *P. salmonis* at a low cost for genetic and serological analyses, for the development of vaccines (Mauel *et al.* 2008; Mikalsen *et al.* 2008; Gómez *et al.* 2009; Yañez *et al.* 2012, 2013a) and for *in vitro* drug susceptibility testing (Yañez *et al.* 2013b).

A novel, genetically different, small infective variant of *P. salmonis* (sP.s) was characterized (Rojas *et al.* 2008) by analysing the sequences of the ITSs located between the genes encoding 16S rDNA and 23S rDNA. This sP.s variant was recovered from infected CHSE-214 cells and from naturally infected fish. The ITS region of the small variant is different from the ITS of the LF-89 strain from which the variant originates. Thus, the sP.s variant can be selectively amplified with a discriminatory set of PCR primers. Transcriptionally, sP.s is fully active and is specifically recognized by antibodies against the standard bacterium. Structurally, sP.s is smaller (<0.2 μm) than the size range of the standard bacterium (0.5–1.5 μm), and the variant's *in vitro* progeny appear to be identical to the progeny of the LF-89 strain. The sP.s is an infective variant of the reference strain and not a new strain, likely resulting from a survival strategy of the bacterium in response to limiting growth conditions. This variant may be responsible for horizontal infection in sea water (Rojas *et al.* 2008).

Genetic characteristics

A previous study based on an analysis of the rRNA operon of five isolates of *P. salmonis* (LF-89, ATL-4-91, NOR-92, SLGO-94 and EM-90)

suggested that sequencing data from three regions (16S-ITS-23S) provided similar phylogenetic information (>99.4% 16S rDNA, 99.1–99.7% ITS and 99.3–99.8% 23S rDNA similarities; Mauel *et al.* 1999). The five isolates under investigation were then found to be closely related to each other. However, the EM-90 Chilean isolate was described as unique, showing a slightly lower percentage sequence identity in its rRNA sequences (>98.5–98.9% 16S rDNA, 95.2–96.9% ITS and 97.6–98.5% 23S rDNA similarities). Nine of the base differences between EM-90 and the other isolates were found between bases 1003 and 1020. It was also found that *EcoRI* and *PstI* restriction sites located in the variable stem-loop region allowed the use of restriction fragment length polymorphism to differentiate EM-90 from the other strains (Mauel *et al.* 1999). The authors concluded that this isolate had diverged genetically from the others.

Piscirickettsia salmonis has two ITS regions: ITS A and ITS B (Casanova *et al.* 2001). Thus, more than one rRNA operon may exist. In both isolates, the smaller region (ITS B) corresponded to ITS sequences previously described for each isolate, and the larger region (ITS A) was nearly the same as the respective ITS B sequences, interrupted by an insert that contained two tRNA genes: tRNA-Ile and tRNA-Ala. It would be very interesting to determine whether the ITSs of *P. salmonis* isolated from other countries have similar characteristics. An ITS sequence analysis of 11 isolates of *P. salmonis* obtained from different salmon species and geographical regions in Chile demonstrated the existence of two different groups: ITSs with higher and lower electrophoretic mobility, including the LF-89 and EM-90 isolates, respectively (Casanova *et al.* 2003).

Only minor genetic variation has been observed between *P. salmonis* isolates from different salmonid host species or from diverse geographical locations (Mauel *et al.* 1999). A phylogenetic analysis of *P. salmonis* based on the sequences of the ITS and the 16S rRNA gene showed that the Scottish isolates conform to a genetic group, together with the Norwegian and Canadian isolates, whereas the Irish isolate conforms to a new group (Reid *et al.* 2004). Differences in the virulence of *P. salmonis* obtained from Chile (LF-89), Canada (ATL-4-91) and Norway (NOR-92) have been shown (House *et al.* 1999). Piscirickettsiosis was observed in fish injected with each of the three isolates, and for each isolate, the cumulative mortality was directly related to the concentration of bacterial cells administered (House *et al.* 1999). The LF-89 isolate was the most virulent, with losses reaching 97% in the three replicates injected with 10^5 $TCID_{50}$, 91% in the replicates injected with 10^4 $TCID_{50}$ and 57% in the fish injected with 10^3 $TCID_{50}$. The ATL-4-91 isolate caused losses of 92% in the three replicates injected with 10^5 $TCID_{50}$, 76% in the fish injected with 10^4 $TCID_{50}$ and 32% in the fish injected with 10^3 $TCID_{50}$. The NOR-92 isolate was the least virulent, causing 41% mortality in the replicates injected with $10^{4.6}$ $TCID_{50}$.

Functional genomics

Analyses of gene content and genetic relationships may help to improve our understanding of the biology and evolution of *P. salmonis*. One concern is the comparative fluidity with which genes may be exchanged, such as by horizontal gene transfer, and the impact of this movement on the outcome of the taxonomic/phylogenetic process. The genomes of relatively few strains of *P. salmonis* have been sequenced. The complete genome of *P. salmonis* (LF-89 strain) was described in Chile (Valenzuela *et al.* 2001). The draft (95% of the genome) comprised an approximately 2 000 000-bp circular genome and a 1500-bp open reading frame (ORF), but currently, there are few whole-genome sequences available for *P. salmonis*. In addition, the genome sequencing data generated by Sjödin *et al.* (2012) represent a considerable advancement in our knowledge of the genome sequences of *Francisella* strains and the two distant but genetically related species *Fangia hongkongensis* and *P. salmonis*.

The presence of a toxin–antitoxin (TA) locus in the genome of *P. salmonis* has been described (Gómez *et al.* 2011). The *P. salmonis* TA locus, named Ps-Tox-Antox, includes its respective regulatory sequences. By *in silico* comparative genomics analysis of the Ps-Tox-Antox locus, the authors determined that this TA is homologous to the VapBC TA system of *Rickettsia felis* and to other chromosomal TA operons. Considering that the expression of the ps-Tox gene has been demonstrated to be highly toxic to *Escherichia coli* cells, the newly described module appears to be a potential innovative tool for pathogen control via peptide interference.

Piscirickettsia salmonis behaviour when exposed to stress conditions, which cause the bacterium to produce large cell aggregates that closely resemble typical biofilm structures, was described in a previous study (Marshall *et al.* 2012). The bacteria appeared to be embedded within a matrix that disappeared when exposed to cellulase, suggesting a polysaccharide composition that is typical of biofilm formation. Two lectins (ConA and WGA) showed a strong reaction with the structure, validating the exopolysaccharide composition of the matrix. The TA mazEF operon of *P. salmonis* exhibited induction of these genes at early stages of biofilm formation, suggesting that this formation might be an adaptive strategy for survival and persistence under stress conditions in marine environments.

On the other hand, the virulence factors of this pathogen are poorly known. *Piscirickettsia salmonis* secretes extracellular products (ECPs), and at least one of their components has cytotoxic effects *in vitro* and probably mediates some tissue damage *in vivo* in salmonid fish infected with this microorganism (Rojas *et al.* 2013). The almost complete inhibition of the *in vitro* effect of the *P. salmonis* ECPs by proteinase K treatment indicates their peptidic nature, and therefore, they can be categorized as exotoxins. In addition, several of these ECPs are thermolabile exotoxins that likely play a role in the pathogenesis of piscirickettsiosis (Rojas *et al.* 2013). *Francisella tularensis*, the most well-known species of this genus, is phylogenetically related to *P. salmonis*, there is no consensus opinion on the synthesis of exotoxins, and genes encoding toxins have not been found in this species. However, in *Francisella novicida*-like isolates, genes coding for putative RTX exotoxins have been recently found (Siddaramappa *et al.* 2011).

A novel and complete 863-bp insertion sequence in the *P. salmonis* genome has been recently characterized and named ISPsa2 (Marshall *et al.* 2011). This sequence has a novel 16/16 bp, perfectly inverted terminal repeat flanking a 726-bp ORF that encodes a putative transposase (Tnp-Psa). The putative transposase encoded within ISPsa2 (Tnp-Psa) carries conserved motifs that are also found in other transposases. The presence of a putative promoter region in frame with Tnp-Psa in ISPsa2 strongly suggests regulated self-expression for the IS and may represent a preliminary indication of the high genomic plasticity of this bacterial fish pathogen. *Piscirickettsia salmonis* contains completely different sets of IS elements compared with *Francisella* (Sjödin *et al.* 2012), and the genome of *P. salmonis* is enriched with ISPsa1 and ISPsa2 (Marshall *et al.* 2011). These insertion sequences and, putatively, other mobile genetic elements in *P. salmonis* represent solid evidence that the adaptive potential of the bacterium resides in its versatile genome. In this context, the description of a TA locus in *P. salmonis* appears to be a natural consequence of this versatility (Gómez *et al.* 2011).

Evidence of the functional presence of four genes that are homologous to those described for Dot/Icm type IV secretion systems (SSTIVs) has been described for *P. salmonis* (Gómez *et al.* 2013). The Dot/Icm system, which is the major virulence mechanism of the related intracellular pathogens *Legionella pneumophila* and *Coxiella burnetii*, is responsible for these pathogens' intracellular survival and multiplication and may also apply to *P. salmonis*. The four *P. salmonis dot/icm* homologs (*dotB, dotA, icmK* and *icmE*) are expressed during both *in vitro* infection of cultured cells and growth in cell-free media, supporting the genes' putative constitutive expression. Additionally, as occurs for other bacterial systems, the acidification of cell-free media results in the overexpression of all four *P. salmonis* genes. Finally, researchers have also demonstrated that *P. salmonis*-containing vacuoles do not fuse with lysosomes, indicating that there is bacterium-driven interference in the endosomal maturation process that ensures bacterial survival, for which the Dot/Icm secretion system is responsible by delivering effector proteins within the host cell.

Although these works has provided some information about the virulence factors of *P. salmonis*, it is evident that further knowledge is required to fully understand the mechanisms of pathogenicity of this bacterium.

The disease

Clinical signs and gross pathology

A range of signs of infection may be present in salmonids infected with *P. salmonis* (Bravo & Campos 1989; Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Severely affected fish are dark in colour, show inappetence and lethargy and swim near the surface or edges of cages (Bravo & Campos 1989;

Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). The most consistent external signs observed during *P. salmonis* infections are pale gills resulting from significant anaemia, abdominal swelling and petechial and ecchymotic haemorrhages on the base of fins (Fig. 1a) and in the periocular and perianal zones. Infected fish often have skin lesions that range from small areas of raised scales to nodules (Fig. 1b) and shallow haemorrhagic ulcers (Fig. 1c, d; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Low levels of hematocrit indicate severe anaemia (Branson & Nieto Díaz-Muñoz 1991). However, during acute infections, mortality may occur without gross signs of disease.

Internally, *P. salmonis* infections spread systemically, resulting in serosanguinous ascites and swollen kidneys, livers and spleens (Fig. 2a). The most diagnostic lesions occur in the liver as subcapsular, gray-to-yellow mottled areas or as ring-shaped foci that are approximately 1 mm in diameter (Fig. 2b; Cvitanich *et al.* 1990, 1991, 1995; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Fibrinous pseudomembranes on the heart and petechial haemorrhages in the visceral organs, swim bladder wall and skeletal muscle are often observed. In general, the intestine shows yellowish mucous content, and the stomach has transparent seromucous content (Almendras *et al.* 1997). *Piscirickettsia salmonis* was isolated from the brain (Fig. 2c) of coho salmon farmed in Chile (Skarmeta *et al.* 2000), and it has been proposed that the erratic swimming behaviour observed in fish with piscirickettsiosis may be due to a pre-existing brain infection with more virulent isolates of the bacterium (Skarmeta *et al.* 2000). *Piscirickettsia salmonis* was also isolated from the brains of Atlantic salmon farmed in Scotland (Grant *et al.* 1996; Reid *et al.* 2004).

However, in recent years, the gross pathology of piscirickettsiosis has evolved towards the presentation of multiple diffuse skin ulcers all over the body. This pathology also includes opercula and peduncles, in addition to many caverns inside the skeletal muscle (Fig. 2d), regardless of the species of salmon and the geographical area of the sea water. The caverns inside the muscle can be observed as a clear area or with exudates. The pathological lesion is observed in both Atlantic salmon and rainbow trout; however, the lesion is more frequent in trout and is generally associated with the small variant of *P. salmonis*. Internally, classic pathological changes due to piscirickettsiosis, such as whitish nodules in the liver, renomegaly and splenomegaly, are not necessarily observed.

Histopathology

The most prominent microscopic lesions are found in the liver, kidney, spleen and intestine, but pathological changes in the brain, heart, skeletal muscle, ovary and gill can also be observed

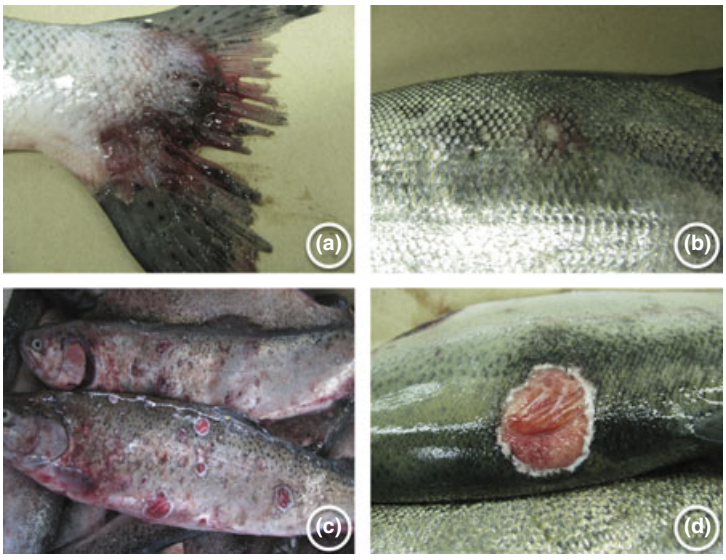

Figure 1 External signs associated with piscirickettsiosis in salmon species. (a) Petechial and ecchymotic haemorrhages on the base of fins. (b) Small areas of raised scales. (c) Multiple and diffuse shallow haemorrhagic skin ulcers in different areas of the body. (d) Focal skin ulcers and a loss of continuity.

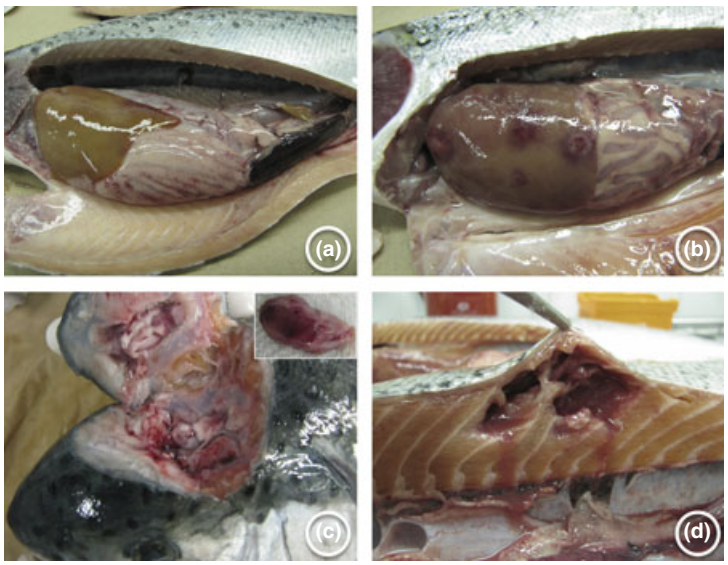

Figure 2 Gross pathology associated with piscirickettsiosis in salmon species. (a) Serosanguinous ascites, splenomegaly, pale liver and diffuse petechial haemorrhages are observed in the pyloric caeca and visceral fat. (b) Pale liver with subcapsular and circular gray-to-yellow mottled areas of approximately 1 mm in diameter and hepatic pseudomembranes. (c) Hyperaemia and diffuse haemorrhages in the meninges and petechiae in the encephalon. (d) Caverns with serosanguinous exudate inside the skeletal muscle.

(Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Cvitanich *et al.* 1991). *Piscirickettsia salmonis* is commonly observed inside macrophages, within cytoplasmic or free vacuoles in the cytoplasm of the host cells. In certain fish, the bacteria are dark-coloured basophilic cells of approximately 0.5–1.5 μm, with a large nucleus and small cytoplasm, and are arranged in small groups inside the haematopoietic tissue (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991).

Multifocal necrosis of the haematopoietic cells in the parenchyma of the kidney is a feature of the more acute phase of piscirickettsiosis, which is followed by granulomatous inflammation (Fig. 3a). This necrosis results in a loss of haematopoietic cells that in turn leads to the observed anaemia that is characteristic of piscirickettsiosis. Glomerulonephritis with vacuolization and capsule oedema are also evident in the kidneys (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993). Vascular and perivascular necrosis and intravascular coagulation, resulting in fibrin thrombi within major vessels, are common findings in the liver (Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997). Multifocal necrosis of hepatocytes and diffuse infiltration of inflammatory cells are also observed in the liver (Fig. 3b; Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997). In more acute infections, the coalescence of areas of necrosis results in a more mottled appearance of the organ, rather than discrete nodules. Within the areas of necrosis, macrophages can be found harbouring intracellular aggregates of *P. salmonis* (Fig. 3b). Focal areas of necrosis underlie the pale, circular lesions observed in more chronically infected fish (Cvitanich *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Olsen *et al.* 1997).

Endocarditis, pericarditis and focal necrosis of the myocardium may also be observed, along with accompanying vascular changes similar to those in the liver and haematopoietic organs (Fig. 3c; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993; Olsen *et al.* 1997). Necrosis and diffuse chronic inflammation of the lamina propria and detachment of the mucosa are observed in the intestine. The pancreas, ovaries, mesentery, testicles, eyes, skeletal muscle, pseudo-gills, nasal capsule and adipose tissue have also been reported to be involved in certain *P. salmonis* infections (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Rodger & Drinan 1993). Epithelial multifocal hyperplasia results in fusion of the lamellae, and focal necrosis and fibrin thrombi within lamellar capillaries may also be observed in the gills (Schäfer *et al.* 1990). Degeneration and necrosis of myocytes, a loss of striation, slight-to-moderate diffuse focal myositis and infiltration by polymorphonuclear cells (PMNs) (Fig. 3d) are often observed in the skeletal muscle (Schäfer *et al.* 1990; Rodger & Drinan 1993). The meninges show moderate granulomatous inflammation and thrombosis (Grant *et al.*

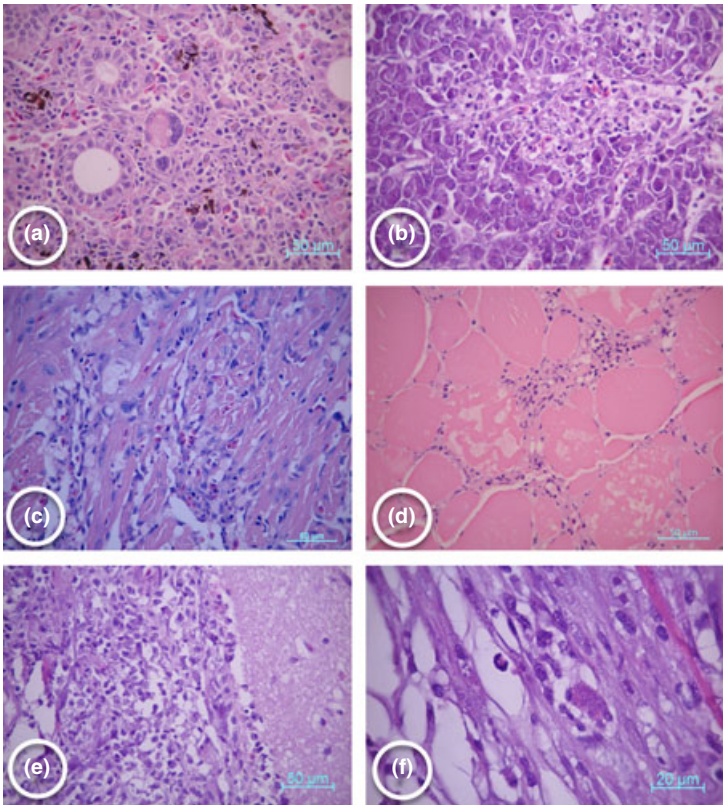

Figure 3 Microscopic lesions associated with piscirickettsiosis infection. (a) Degeneration and focal coagulative necrosis of interstitial and haematopoietic cells in the anterior kidney and diffuse granulomatous interstitial nephritis. (b) Focal coagulative necrosis of hepatocytes, diffuse multifocal hepatitis and intracellular aggregates of bacterial cells inside hepatocytes and/or macrophages. (c) Degeneration and necrosis of cardiomyocytes, moderate-to-severe diffuse focal myocarditis and infiltration by PMNs. (d) Degeneration and necrosis of myocytes, a loss of striation, slight-to-moderate diffuse focal myositis and PMN infiltration. (e) Degeneration and focal necrosis and moderate-to-severe diffuse focal meningoencephalitis. (f) Diffuse fibrohistiocytic infiltration of the leptomeninges, with the presence of macrophages with intracytoplasmic basophilic corpuscles.

1996; Skarmeta *et al.* 2000; McCarthy *et al.* 2005) and severe haemorrhagic meningoencephalitis (Fig. 3e,f).

Diagnosis

A diagnosis of piscirickettsiosis is based on the presence of the characteristic external and internal signs and microscopic signs of the disease in a salmonid host, combined with a demonstration of the presence of *P. salmonis* by one of several procedures (Almendras & Fuentealba 1997; Fryer & Hedrick 2003). Smears or impressions of the kidney, the liver, the spleen or infected cell cultures on glass or plastic substrates can be fixed and then stained with Gram, Giemsa, acridine orange or methylene blue stain for the direct observation of *P. salmonis* within host cells (Fryer *et al.* 1990; Lannan & Fryer 1991). In Giemsa-stained sections, the intracellular bacteria will appear as darkly stained pleomorphic organisms occurring in coccoid or ring form, often within host cell cytoplasmic vacuoles and frequently in pairs, with a diameter of 0.5–1.5 μm.

Piscirickettsia salmonis can be grown in cultured cells (Fryer *et al.* 1990; Lannan & Fryer 1991) and in bacteriological media (Mauel *et al.* 2008; Mikalsen *et al.* 2008; Gómez *et al.* 2009; Yañez *et al.* 2012, 2013a,b). The tissues of choice for isolation of the agent include the kidney, liver and blood during active infection (Lannan & Fryer 1991), but the brain also represents an important tissue for detecting *P. salmonis* (Skarmeta *et al.* 2000). However, following initial detection in stained tissue smears, cultured cells or bacteriological media, the identity of *P. salmonis* must be confirmed by serological methods, for example, immunofluorescence (IFAT) (Lannan, Ewing & Fryer 1991), an enzyme-linked immunosorbent assay (ELISA) (Aguayo *et al.* 2002), immunohistochemistry (Alday-Sanz *et al.* 1994) or a recently developed single-dilution filtration-assisted chemiluminometric immunoassay (SD FAL-ELISA) that can be applied to measure anti-*P. salmonis* IgM in individual or pooled serum and mucus samples (Wilda *et al.* 2012), or molecular techniques, such as PCR (Mauel *et al.* 1996; Marshall *et al.* 1998; Corbeil, McColl & Crane 2003).

Both nested (Mauel *et al.* 1996) and single-step (Marshall *et al.* 1998) PCR assays were developed during the 1990s to facilitate the detection and

characterization of *P. salmonis*. The targeted ITS region of the rRNA operon is more variable than the 16S region exploited in the nested PCR, therefore allowing finer discrimination in the description of new *P. salmonis* isolates (Marshall *et al.* 1998). A real-time PCR assay was developed, and this assay demonstrated higher sensitivity than did the nested PCR (Corbeil *et al.* 2003). The real-time PCR assay has the advantage of being faster and minimizes the risk of cross-contamination that is inherent to nested PCR. Additionally, the assay allows the quantification of bacteria in samples. A real-time PCR assay has also been implemented to detect bacteria in fixed paraffin sections (Karatas *et al.* 2008). Recently, a multiplex PCR-based protocol was designed for the simultaneous detection of *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *P. salmonis* (Tapia-Cammas *et al.* 2011). Finally, an *in situ* hybridization assay for *P. salmonis* using two pairs of primers (PS2S-PS2AS and PS2S-PS3AS) as specific DNA probes against the bacterium has been implemented (Venegas *et al.* 2004).

Pathogenesis

Progression of pathological changes

Microscopic lesions caused by *P. salmonis* during naturally acquired infections can be found in numerous organs and tissues, which is characteristic of a systemic or septicaemic condition (Cvitanich *et al.* 1990, 1991; Fryer *et al.* 1990; Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991). Although the sequential development of naturally acquired infections has not been described, initial infections likely commence when the physical barriers of the skin and/or gills are breached by the bacterium (Almendras *et al.* 1997; Smith *et al.* 1999). Replication of the bacterium results in raised, discoloured areas of the skin that may then progress to shallow ulcers, as observed under field conditions and following experimental application of the bacterium onto the skin with filter-paper patches (Smith *et al.* 1999). The direct application of a bacterial suspension onto the gills also initiates local infections that spread via the blood and then via major vessels to the parenchyma of numerous organs (Almendras *et al.* 1997).

Serosal spread is more characteristic of the infections induced by intraperitoneal (IP) injections of the bacterium (Almendras *et al.* 1997). In injected fish, the capsules of the major organs in the peritoneum are sites of replication prior to invasion of the parenchyma. In the later stages of infection, the internal and microscopic pathological changes observed in fish exposed via different routes become similar, most likely because septicaemia eventually occurs, even in cases of serosal spread of the bacterium (Almendras *et al.* 1997). The septic nature of infections is demonstrated by the presence of infected macrophages, which are visible in blood smears from heavily infected fish (Branson & Nieto Díaz-Muñoz 1991; Almendras *et al.* 1997). There is no recent scientific literature concerning the pathogenesis of the disease in terms of the progression of pathological changes and the load of microorganisms in different challenged fish organs and tissues. However, the pathogenesis of *P. salmonis* in challenged Atlantic salmon via IP, oral or gill infection has been described, in accordance with the progression of microscopic lesions in the liver (Almendras *et al.* 2000).

Interaction with the fish immune system and molecular pathogenesis

Piscirickettsia salmonis is capable of infecting, surviving and replicating in and disseminating from macrophage/monocyte (RTS-11) cell lines derived from the rainbow trout spleen with a similar phenotype as uninfected control cells, without inducing CPE (Rojas *et al.* 2009). Infected macrophages stop proliferating, and a fraction detaches from the plate and transforms into cells similar to non-adherent monocytes with proliferative activity. These results suggest that the infection of salmonid innate immune cells without inducing the important response of cell death facilitates the persistence of the bacterium and its subsequent dissemination into other tissues, favouring evasion of the first line of defence against pathogens. For the first time, the apoptosis of rainbow trout macrophages infected by *P. salmonis* has been characterized using techniques based on morphological changes and cellular DNA fragmentation in the host (Rojas *et al.* 2010). The findings show that bacterial survival and evasion of the host immune response play an important role in the establishment of infection.

In recent years, microarrays have been used to examine immune and physiological responses and

developmental processes in Atlantic salmon (Rise *et al.* 2004; Tacchi *et al.* 2011). Experiments based on microarrays and validated through qPCR to identify genes that are differentially transcribed in response to infection with *P. salmonis* in the macrophages and haematopoietic kidney of Atlantic salmon have been performed (Rise *et al.* 2004). In the macrophages of infected fish, 71 transcripts registered with increased regulation and 31 transcripts registered with decreased regulation. In the haematopoietic kidney of infected fish, 30 transcripts with increased regulation and 39 transcripts with decreased regulation were observed (Rise *et al.* 2004). Ten antioxidant genes, including glutathione S-transferase, glutathione reductase, glutathione peroxidase and cytochrome b558 subunits α and β, showed increased expression in the macrophages of infected fish, but not in the haematopoietic kidney. Genes with decreased expression were those associated with the immune response in the infected haematopoietic kidney, but not in infected macrophages (Rise *et al.* 2004).

Recently, an infection model of *P. salmonis* was used to evaluate the differential transcriptomic responses using a microarray analysis of the anterior kidney, the liver and the skeletal muscle in post-smolts of Atlantic salmon at 48 h post-infection (pi) (Tacchi *et al.* 2011). The obtained results show how the fish respond to the infection and how the pathogen potentially modulates the host immune response. The infection caused a significant alteration of transcriptional activity in all evaluated tissues. In infected fish, 886, 207 and 153 transcripts were differentially expressed in the liver, anterior kidney and muscle, respectively.

Transcripts related to immune responses were modified in all three studied tissues (Tacchi *et al.* 2011). The head kidney had the greatest increase in expression of immune-related transcripts. Overall, the data indicate that *P. salmonis* affects the immune system of the host, activating the innate immune response in the head kidney, the muscle and the liver and potentially inducing inflammatory responses in the head kidney and an interferon-mediated response in the liver. However, this pathogen may compromise the adaptive immune response in infected fish as a mechanism to escape host defences (Rise *et al.* 2004; Tacchi *et al.* 2011).

Transcripts involved in G-protein signalling pathways were down-regulated in all three examined tissues in the challenged fish, and in particular, the levels of regulators of G-protein signalling (RGS) were found to be decreased in all tissues (Tacchi *et al.* 2011). These results suggest that suppression of G-protein signalling may be part of the mechanism used by *P. salmonis* to evade host antimicrobial defences (Tacchi *et al.* 2011).

The host cell protects itself from oxidative damage by synthesizing the strong antioxidant glutathione. The expression of genes involved in the response to oxidative stress was found to be up-regulated in all examined tissues (Tacchi *et al.* 2011). NADPH oxidase exhibited increased expression in the liver, indicating that the fish were likely experiencing oxidative stress. Interestingly, glutathione S-transferase activity was down-regulated in the liver, which resulted in the up-regulation of catalase in the head kidney. Genes encoding heat-shock proteins (HSPs) were also induced following infection by the pathogen. It is well known that these proteins can induce cellular and humoral immune responses to infectious diseases. This family of proteins functions as co-chaperones in stimulating the ATP-dependent activity of HSP70, which is up-regulated in this tissue and protects cells during lipopolysaccharide (LPS) infection by up-regulating the expression of sphingosine kinase 1 (SK1). Together, these results suggest that *P. salmonis* infection may affect the host antioxidant system, eventually causing cell death and necrosis, as observed in several tissues of moribund fish infected by this bacterium (Almendras *et al.* 2000).

Following infection, there was an increase in the expression of genes related to protein metabolism in the liver, with genes involved in both protein synthesis and degradation being up-regulated (Tacchi *et al.* 2011). Genes involved in energy metabolism in the liver increased in expression in challenged fish, whereas the expression of genes related to gluconeogenesis markedly decreased. In the muscle, the expression of genes involved in both protein synthesis and protein degradation was found to be decreased in diseased fish. This result suggests that *P. salmonis* may cause decreased protein turnover in this tissue, reflecting a down-regulation of both anabolic and catabolic pathways in the muscle following infection.

During the complex interaction between a pathogen and its host organism, the induction or prevention of apoptosis may play a critical role in the

outcome of infection. *Piscirickettsia salmonis* may also inhibit apoptosis (Rojas *et al.* 2010) by down-regulating genes encoding proapoptotic proteins and by inducing cell proliferation-related genes in both the liver and the head kidney (Tacchi *et al.* 2011). This phenomenon is in agreement with the findings of Rise *et al.* (2004), who also found a decrease in the expression of apoptosis-related genes, whereas genes related to cell proliferation and the cell cycle were up-regulated. Modulation of the apoptotic response and the proliferation of the host cell may be a mechanism that *P. salmonis* evolved to ensure the maintenance of host cells as the site of infection.

Epidemiology and transmission

Environmental factors

Both temperature and salinity affect the survival of *P. salmonis* outside the host (Lannan & Fryer 1994). *Piscirickettsia salmonis* can survive for extended periods in sea water but is rapidly inactivated in fresh water (Lannan & Fryer 1994). The period of extracellular survival is greater at cooler temperatures (5 °C) and decreases as the temperature increases. In one study, under experimental conditions, *P. salmonis* survived in sea water for at least 21 days at 5–10 °C, 14 days at 15 °C and 7 days at 20 °C. The pathogen did not persist at temperatures above 25 °C (Lannan & Fryer 1994). The highest incidence of outbreaks is observed in the fall and spring, likely due to the water temperature (9 and 16 °C, respectively) (Bravo & Campos 1989; Cvitanich *et al.* 1990). Generally, outbreaks appear after a period of high variation in the environmental conditions of the water, such as fluctuating temperature or an increase in the concentration of non-toxic algae (Branson & Nieto Díaz-Muñoz 1991). In addition, it has been suggested that feeding to satiation, improper nutrition and/or stress could be predisposing factors (Garcías, Mendoza & Carvajal 2005). Piscirickettsiosis has often been described in sea water and estuarine waters but has been occasionally reported in rainbow trout farmed in Llanquihue Lake, which originated from eggs imported from the USA that were always maintained in fresh water (Bravo 1994), and in rainbow trout farmed in fresh water at Chiloé Island (Gaggero *et al.* 1995). In the second study, the gross pathology was similar to that previously described in outbreaks in sea water, and the characteristics of the growth of the pathogen *in vitro* corresponded to the characteristics of *P. salmonis* (Gaggero *et al.* 1995). However, the reduced natural onset of piscirickettsiosis in fresh water suggests that the source could be the sea (Lannan & Fryer 1994). The rareness of outbreaks in fresh water may result from the instability of the bacterium in this environment (Lannan & Fryer 1994).

Horizontal transmission and experimental trials

Although there is still conjecture about the major mode of transmission of *P. salmonis* under natural conditions, direct horizontal transmission has been experimentally demonstrated in sea water and fresh water (Cvitanich *et al.* 1991; Almendras *et al.* 1997; Smith *et al.* 1999). The virulence of *P. salmonis* was evaluated via the IP injection of fish with three different isolates of *P. salmonis* (LF-89, SLGO-94 and SLGO-95; Smith *et al.* 1999). Exposed fish inoculated with the SLGO-95 strain showed a higher and earlier accumulated mortality rate than those fish inoculated with LF-89. Although all salmonid species farmed in Chile are susceptible to piscirickettsiosis, experiments have shown that coho salmon are more susceptible than rainbow trout (Smith *et al.* 1996a). A 50% lethal dose (DL_{50}) and a 50% infective dose (DI_{50}) of $10^{2.8}$ and $10^{1.9}$, respectively, were described in coho salmon (Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991).

Despite the low *in vitro* persistence of *P. salmonis* in fresh water, horizontal transmission has been shown to be enhanced by direct contact among fish (Almendras *et al.* 1997) and by an increase in culture density (Larenas, Contreras & Smith 1997a). A higher accumulated mortality rate (24%) in salmon farmed at a higher density (20 kg m^{-3}) and a higher water temperature (14 °C) has been described (Larenas *et al.* 1997a). The pathogen enters the host through the oral route, gills or skin (Smith *et al.* 1999). Although intact skin and gills can be penetrated by *P. salmonis,* there is an increased risk of infection following injury to these organs (Smith *et al.* 1999). The bacterium may be excreted in bile, faeces and urine from live fish, making coprophagy another viable route of infection (Salinas *et al.* 1997; Smith *et al.* 2004).

Fish with IP and gill infections show significantly higher mortality than fish challenged orally

(Almendras *et al.* 1997). In general, the most effective transmission route is via the skin, followed by the intestine (Almendras *et al.* 1997; Smith *et al.* 1999, 2004). These mechanisms have been observed in both sea water and fresh water, although the low survival of the bacterium in fresh water minimizes the probability of disease onset (Almendras *et al.* 1997; Smith *et al.* 1999). Infection is considered to occur primarily through horizontal transmission because the bacterium is capable of surviving in marine waters for extended periods (Lannan & Fryer 1994). However, in fresh water, unless the bacterium is protected within host cells or other biological material, rapid inactivation makes successful horizontal transmission unlikely (Lannan & Fryer 1994).

The incubation period for piscirickettsiosis depends on the bacterial isolate, the dose at which the isolate is administered to the host, the route of infection, environmental factors and host factors such as immune status, physiological status, species and age (Lannan & Fryer 1994). Death from piscirickettsiosis has been reported as early as 2 days following the IP inoculation of rainbow trout with *P. salmonis* (LF-89) (Smith *et al.* 1999). Other studies have reported deaths 8–29 days after a similar IP inoculation (Garcés *et al.* 1991; Smith *et al.* 1996a), and fish infected via the skin, gills or oral route died 10–14 days after first infection (Smith *et al.* 2004). Piscirickettsiosis-related mortalities have been noted in salmon as early as 2 weeks following their introduction into the areas of infected sea water in Chile (Cvitanich *et al.* 1990). Based on the above information, the incubation period for piscirickettsiosis under natural conditions has been estimated to be 10–14 days.

A real-time PCR technique was developed to detect and quantify *P. salmonis* in samples of sea water to estimate the rest period before new fish can be introduced into seawater farms (Olivares & Marshall 2010). Water samples were collected at 10-day intervals over a 50-day period at both the surface level and a depth of 5 m. The bacterial load decreased to zero at day 50, which indicates that a 50-day rest period after the removal of fish from cages appears to be appropriate before seeding a new stock of fish. Determining the minimal effective bacterial load in the water column of seawater farms may be an important procedure to complement health management for disease control.

Bath or cohabitation challenge models accurately represent natural exposure and provide predictable results for mortality (Strand & Midtlyng 2007). A standardized cohabitation challenge model for *P. salmonis* in Atlantic salmon has been described (Strand & Midtlyng 2007). The first study aimed to evaluate the loss of titres of cultured *P. salmonis* after freezing and showed that both fresh and frozen inocula induced mortality, with curves following a typical dose–response pattern (Strand & Midtlyng 2007). In both cases, the saline-injected control group started to die after 3–4 weeks, reaching 95% cumulative mortality after 6–7 weeks. The group receiving the highest dilution of the frozen material followed the mortality pattern of the saline controls. Similar mortality patterns were observed in a parallel experiment with seawater-adapted smolts. A second trial aimed to investigate how the course of mortality was influenced by the ratio of inoculated (challenger) to non-inoculated (cohabitant) fish and showed that waterborne infection readily occurs in only 30% of challengers (Strand & Midtlyng 2007). The onset of the mortality of inoculated fish occurred on day 16, and cohabitant fish reached an accumulated mortality rate above 95% on days 34–35. A third trial aimed to investigate the temporal patterns of establishing waterborne infection using cohabitant fish and suggested that inoculation was ineffective in establishing lethal infection in all groups receiving diluted inoculum. However, waterborne infection was apparently established in all cohabitant groups at approximately day 21, and shedding may have been needed to reach a threshold before waterborne infection with *P. salmonis* occurred (Strand & Midtlyng 2007). In summary, cohabitant challenge can be achieved in both fresh water and sea water, with an incubation period of 3–5 weeks.

Reservoirs and vectors

The source and reservoir of *P. salmonis* in the natural environment and the bacterium's mode of transmission are unknown. There are many parasitic crustaceans in the marine environment that may serve as vectors for *P. salmonis* (Fryer *et al.* 1990), although no vector/reservoir has yet been identified (Olivares & Marshall 2010). One important role of a vector in the life cycle of rickettsiae is prevention of the desiccation of these fragile bacteria during transmission from host to

host. Because the bacterial cell is protected from desiccation in an aquatic environment, it is possible that no vector is required for *P. salmonis* and that fish-to-fish transmission may occur (Fryer *et al.* 1990).

Piscirickettsia salmonis can replicate in insect- and frog-derived cell lines, suggesting that the bacterium has the potential to persist in invertebrates and non-fish poikilotherms (Birkbeck *et al.* 2004a). The parasitic isopod *Ceratothoa gaudichaudii* (Milne Edwards 1840), commonly associated with farmed salmon in Chile, was identified as a host for *P. salmonis* using an indirect fluorescent antibody test (Garcés *et al.* 1994). *Piscirickettsia salmonis* may penetrate the skin without injury in the absence of vectors, and subcutaneous inoculation is capable of inducing high mortality (Smith *et al.* 2004), suggesting that ectoparasites may perform an important role in transmission [e.g. Caligus rogercresseyi Boxshall and Bravo (2000)].

Reservoirs of infection in marine finfish species have been suspected, but testing of non-salmonid species in Chile failed to demonstrate reservoirs of infection in non-salmonid finfish (Garcés *et al.* 1994; Olivares & Marshall 2010). However, the presence of *P. salmonis* was detected by PCR in Patagonian blenny, *Eleginops maclovinus* (Cuvier 1830), in Huillinco Lake's surrounding salmon farms, which was likely associated with the lake's high salinity (Rozas *et al.* 2009). This finding suggests that a reservoir may exist, but its role in the disease's epidemiology has not been fully explained. In addition, infections with *P. salmonis* are not restricted to salmonid fish, as the bacterium can cause a disease similar to piscirickettsiosis in non-salmonid hosts (Arkush *et al.* 2005; McCarthy *et al.* 2005; Thomas & Faisal 2009).

Vertical transmission

Piscirickettsia salmonis has been described in the ovaries, coelomic fluid and testicles of naturally infected salmon (Schäfer *et al.* 1990; Branson & Nieto Díaz-Muñoz 1991; Cvitanich *et al.* 1991). The infection of ovary tissue in brood stocks that were experimentally infected with *P. salmonis* was observed in the stroma, the cells of the theca externa, the follicular epithelium and the cytoplasmic vacuoles of oocytes at different developmental stages (Larenas *et al.* 1997b). In a sequential study of infection by optical microscopy, *P. salmonis* was observed from day 7 pi until day 20 pi, which indicates that fish eggs are infected from an early developmental phase in the ovary tissue and that the tissue can generate gametes that are viable carriers of the bacteria (Larenas *et al.* 2003). Experimentally, *P. salmonis* was detected in a moderate amount in the eggs, coelomic fluid and seminal fluid in approximately 10% of fertilized eggs from male and/or female rainbow trout brood stocks that were inoculated intraperitoneally (Larenas *et al.* 2003). In another study, all groups of fertilized eggs from male and/or female brood stocks that were inoculated intraperitoneally with *P. salmonis* were capable of generating infected but viable sac fry (Larenas, Zamorano & Smith 2005). Infected fry did not show signs of the disease but were capable of excreting the agent through the faeces (Larenas *et al.* 2005). Piscirickettsial adhesion complex (PAC) was observed in *P. salmonis* by scanning electron microscopy; these extensions allow the bacterium to adhere to the fish egg wall and to enter after 5 min of contact (Larenas *et al.* 2003). Piscirickettsiosis is less frequently observed in salmonid fish during the fresh water stage of their life cycle than after entry into the marine environment. This feature suggests that vertical transmission may not be common for *P. salmonis* (Fryer & Hedrick 2003).

Prevention and control

The efficient control of and treatment for piscirickettsiosis have been difficult to achieve because there are no efficient commercial vaccines (Leal & Woywood 2007; Marshall *et al.* 2007; Tobar *et al.* 2011), and antibiotics (Cabello 2006) have a limited effect on the disease. Thus, the prophylactic control of infections depends on good management practices; periods of fallowing have been one approach to limit the spread of the pathogen (Olivares & Marshall 2010).

Vaccination

Piscirickettsia salmonis antigens that are potentially important for the development of vaccines have been identified based on their reaction with convalescent salmon sera and have been detected using polyclonal and monoclonal antibodies against salmon immunoglobulins (Kuzyk, Thorton & Kay 1996; Barnes *et al.* 1998). Four protein antigens and two carbohydrate antigens with relative molecular sizes of 65, 60, 54, 51, 16 and

approximately 11 kDa have been described (Kuzyk *et al.* 1996). The carbohydrate antigens of 11 and 16 kDa appear to be mainly core-region lipooligosaccharides with lesser amounts of LPS (Kuzyk *et al.* 1996). Eight protein antigens that are likely specific to *P. salmonis* (108, 95, 60, 56, 40, 36, 32 and 20 kDa) were also reported, of which only three had molecular weights similar to those previously described (Barnes *et al.* 1998). Thus, the main antigens are likely those with molecular weights of 56, 36 and 20 kDa.

Structurally, *P. salmonis* lipid A strongly resembles endotoxic enterobacterial lipid A (Vadovic, Fodorova & Toman 2007). The major *P. salmonis* lipid A species represents the hexaacyl form, resembling the classical lipid A found in the *Enterobacteriaceae* family (Vadovic *et al.* 2007; Vadovic, Ihnatko & Toman 2011). This fact might be one of the reasons for the high endotoxic potency of *P. salmonis* (Vadovic *et al.* 2007, 2011), which could explain the cause of the disseminated intravascular coagulation described in cultured coho salmon infected with *P. salmonis* (Cvitanich *et al.* 1991). It appears that the endotoxicity of LPS molecules is primarily determined by the number, nature and arrangement of acyl chains and phosphate groups on the lipid A part of the molecule (Vadovic *et al.* 2011). In addition, both the composition and the structure of major molecular species of *Rickettsia typhi* lipid A resemble those found for the classical forms of enterobacterial lipid A, which have high endotoxicity (Fodorova *et al.* 2005).

Recently, the carbohydrate backbone structure of LPS from *P. salmonis* has been described (Vinogradov, Frimmelova & Toman 2013). The presence of two consecutive residues of diacetylated pseudaminic acid (Pse 5,7 Ac, 5,7-diacetamido-3, 5, 7, 9-tetradeoxy-l-glycero-l-manno-non-2-ulosonic acid) in the LPS appears to be unique among polysaccharides containing this acidic sugar. Similarly, the presence of 4-aminoarabinose (Ara4N, 4-amino-4-deoxy-l-arabinopyranose) on O-4 of the alpha-GlcN1P of the lipid A moiety is a unique feature of this LPS.

There is a lack of field information regarding the immune response and protection generated by vaccines available in Chile, but there are several results derived from experimental clinical trials. In trials with a non-concentrated bacterin, significant protection against natural challenges with *P. salmonis* was demonstrated (Smith *et al.* 1997), whereas fish receiving adjuvant and concentrated bacterin in the same trial showed no protection or greater susceptibility to infection. Additionally, a vaccine composed of live *Arthrobacter davidanieli* has proven effective in reducing mortality from an experimental challenge of coho salmon with *P. salmonis* (Salonius *et al.* 2005). Under field conditions in Chile, use of the vaccine led to a significant reduction in piscirickettsiosis mortality in coho salmon for over 10 months following sea transfer (Salonius *et al.* 2005).

In other trials, an increased relative percentage survival (RPS) was observed when coho salmon were injected with whole-cell bacterins in oil and water adjuvants (Kuzyk *et al.* 2001). Improved protection was observed for a vaccine prepared from recombinant OspA lipoprotein (17 kDa), resulting in RPS values of up to 83% (Kuzyk *et al.* 2001). However, there is a need for further improvement, especially regarding the creation of multivalency, to ensure wider protection against emerging isolates (Wilhelm *et al.* 2006).

In one study, a Scottish isolate of *P. salmonis* (SCO-95A) isolated from the CHSE-214 cell line was inactivated by heat (100 °C for 30 min) and formalin (Birkbeck *et al.* 2004b). Post-smolt Atlantic salmon were intraperitoneally administered vaccines containing 10^9 heat-inactivated or 10^9 formalin-inactivated *P. salmonis* in adjuvant, with a control group receiving phosphate-buffered saline (PBS) in adjuvant. After an induction period of 194 days at 10 °C, the fish were challenged by injection with 0.1 mL of *P. salmonis* (2×10^4 TCID/fish) into the dorsal median sinus and maintained in sea water at 7.5 °C or fresh water at 16 °C. Mortality in the control group reached 81.8%, whereas the heat- and formalin-inactivated vaccines conferred significant protection against *P. salmonis*, with RPS values of 70.7% and 49.6%, respectively. The nature of the protective antigen is unknown but may consist of LPS or a heat-stable outer membrane protein. Fish that survived a dorsal median sinus challenge of *P. salmonis* or were cohabitants showed a strong immune response to the bacteria.

In Chile, expression library immunization technology was used to study the protection of the coho salmon *Oncorhynchus kisutch* against infection by *P. salmonis* (Miquel *et al.* 2003). Purified DNA from *P. salmonis* was sonicated, and the fragments were cloned into the expression vector pCMV-Bios. The plasmid DNA was isolated and

purified, and 20 μg was injected intramuscularly into 60 fish, followed by a second dose of 10 μg that was applied 40 days later. As a control, fish were injected with the same amount of DNA in the vector pCMV-Bios, without an insert. Sixty days post-injection, the vaccinated fish showed a significantly higher titre of specific antibodies against *P. salmonis* than did the control group. The vaccinated and control fish were challenged 60 days after the second dose of DNA with 2.5×10^7 *P. salmonis,* corresponding to 7.5 times the LD_{50}. At 30 days post-challenge, 100% mortality was observed for the control fish, whereas 20% of the vaccinated animals survived. All surviving fish exhibited a lower bacterial load in the kidney than did control fish.

It has been suggested that HSP10 and HSP16 of *P. salmonis* are highly immunogenic in salmon and are thus *bona fide* antigens for inclusion in an experimental vaccine to control piscirickettsiosis (Wilhelm *et al.* 2003). Subsequently, recombinant HSP60 and HSP70 from *P. salmonis* were shown to elicit a humoral response when injected intraperitoneally into Atlantic salmon and conferred protection on fish challenged with *P. salmonis* (Wilhelm *et al.* 2005). In addition, a strong humoral response was reported against membrane-bound transglycosylase B (MltB) and transferrin-binding protein B (TbpB) when these proteins were injected intraperitoneally into Atlantic salmon (Wilhelm *et al.* 2004).

Furthermore, a protective effect against piscirickettsiosis was described as being elicited by a mixture of recombinant proteins in fish (Wilhelm *et al.* 2006). Fifteen *P. salmonis* ORFs encoding HSPs and other surface-exposed antigens were isolated and expressed. Seven of the most promising antigens were formulated into three mixtures, containing HSP60, HSP70 and FlgG (V1); the amino-terminal part of the protein TbpB and the amino- and carboxy-terminal amino acids of the protein MltB (V2); or a recombinant carboxyl region of the proteins Omp27 and FlaA (V3). All three were injected into Atlantic salmon with an average weight of 18 g to test the mixtures' protective efficacy. V1 and V2 elicited a strong protective response in a challenge with the pathogen and achieved the highest level of protection, with RPS values of 95% and 84%, respectively. The humoral response of formulation V1 persisted for 8 months after vaccination (2800 degree-days).

A highly immunogenic protein, ChaPs, was cloned as a unique ORF encoding 545 amino acids, with a 57.3 kDa molecular mass, and its immunological potential was determined using naturally infected fish serum (Marshall *et al.* 2007). Interestingly, a sequence analysis of ChaPs has demonstrated that the protein is an HSP, which is a type of molecule that has already been identified and exploited in recombinant vaccine development (Wilhelm *et al.* 2003).

At present, 33 injectable vaccines against piscirickettsiosis are commercially available in Chile (containing the *P. salmonis* fraction in monovalent and polyvalent formulations; Table 1) (Agricultural and Livestock Service, Servicio Agrícola y Ganadero, S.A.G 2013a). Four are subunit vaccines, and 29 are inactivated vaccines. In total, 31 vaccines are injectable, and two are oral. The number of injected vaccine doses for the control of piscirickettsiosis in Chile has increased ninefold between 2005 and 2010, but the vaccines show variable long-term efficacy (Leal & Woywood 2007; Marshall *et al.* 2007; Tobar *et al.* 2011). Regardless of their nature and administration route, the vaccines' field results have not been sufficiently documented and have shown relatively limited protection, particularly up to 1800 degree-days after injected primo-vaccination in fresh water.

In general, groups of vaccinated fish show lower accumulated mortality rates compared with unvaccinated fish until winter of each year, but the vaccine efficacy decreases significantly and mortality rates increase in both vaccinated and unvaccinated fish at the beginning of spring (Leal & Woywood 2007). All injectable vaccines are relatively effective at preventing the initial piscirickettsiosis outbreaks that occur after the transfer of fish from fresh water to sea water for the ongrowing stage (Tobar *et al.* 2011), but after this initial outbreak, the fish are susceptible to new, more aggressive piscirickettsiosis outbreaks that correlate with weakening of the specific immune response elicited by the first immunization event (Tobar *et al.* 2011). These outbreaks usually affect large fish and occur 10–12 months after transfer, resulting in greater economic losses. Although protecting those fish by means of an injectable revaccination is an interesting solution, the method is extremely difficult, mainly due to economic, practical and operational issues.

Oral immunization presents an attractive alternative to injectable vaccinations (Tobar *et al.* 2011). The vaccine, consisting of an antigen

Table 1 Vaccines with provisional registration for use in salmonids in Chile

Disease	Vaccine type	Presentation	Laboratory
Piscirickettsiosis	Inactivated	Injectable	Agrovet Ltda. FAV Veterquímica S.A. Recalcine S.A. Centrovet Ltda. Tecnovax Chile S.A.
	Inactivated	Oral	Centrovet Ltda.
	Subunit	Injectable	Pfizer Chile S.A.
Piscirickettsiosis-IPN	Inactivated	Injectable	Agrovet Ltda. Centrovet Ltda. FAV Novartis Chile S.A. Tecnovax Chile S.A. Pharmaq AS Chile Ltda.
Piscirickettsiosis-ISA	Subunit	Oral	Centrovet Ltda.
Piscirickettsiosis-IPN-ISA	Inactivated	Injectable	Agrovet Ltda. Centrovet Ltda.
Piscirickettsiosis-IPN-Vibriosis	Inactivated	Injectable	Centrovet Ltda. Tecnovax Chile S.A. FAV Novartis Chile S.A. Pharmaq AS Chile Ltda.
	Subunit	Injectable	Pfizer Chile S.A.
Piscirickettsiosis-IPN-Vibriosis-Furunculosis	Inactivated	Injectable	Agrovet Ltda. Centrovet Ltda. Pharmaq AS Chile Ltda.
Piscirickettsiosis-IPN-Vibriosis-ISA	Inactivated	Injectable	FAV Novartis Chile S.A.
Piscirickettsiosis-IPN-Vibriosis-Furunculosis-ISA	Inactivated	Injectable	Centrovet Ltda. Agrovet Ltda. FAV Novartis Chile S.A. Pharmaq AS Chile Ltda.

Source: Agricultural and Livestock Service (S.A.G), Chile.

solution of injectable vaccine incorporated into an adhesive vehicle and supplied with the daily feed ration, induces a specific immune response at the local and systemic levels. Specific anti-*P. salmonis* antibodies are detected as soon as 300 degree-days after vaccination. Oral vaccination was able to protect fish against *P. salmonis* challenge when administered either as a primary vaccination or as a booster for an injected vaccine. The results show that oral vaccination is an efficacious method for the prevention of piscirickettsiosis outbreaks in sea water during the ongrowing stage.

Despite all of these positive results for piscirickettsiosis vaccines in experimental trials and although more than 10 years have passed since the first vaccine against *P. salmonis* was launched in the Chilean market, most commercially available vaccines in Chile have no apparent effect on reducing mortalities under field conditions. Meanwhile, piscirickettsiosis remains a major health threat to salmon farming in Chile. In our opinion, the variable efficacy could most likely be attributed to the interplay of several factors related to the pathogen (virulence factors, pathogenicity, infectious pressure), host (genetic resistance, smolt quality, immune system and immunity) and environment (water temperature, algal bloom), including husbandry management (biomass, stress, zoning). In addition, several recent studies have shown that *P. salmonis* affects the immune system of the host, activating the innate immune response and potentially inducing inflammatory responses in the head kidney and an interferon-mediated response in the liver. However, *P. salmonis* may compromise the adaptive immune response in infected fish as a mechanism to escape host defences (Rise *et al.* 2004; Tacchi *et al.* 2011). Therefore, the infective strategy of *P. salmonis* for intracellular survival (Rojas *et al.* 2009, 2010) evasion of the host immune response (Rise *et al.* 2004; Tacchi *et al.* 2011; Gómez *et al.* 2013) and the nature of the fish immune system would

activate the host immune response only for a short period of time. However, work contributing to a better understanding of immunological activity and bacterial factors involved in the disease is as yet limited.

In comparison, the tularaemia vaccine, consisting of *F. tularensis* live vaccine strain (LVS), which is phylogenetically related to *P. salmonis*, does not elicit complete protection against lethal challenge with a virulent-type A *Francisella* strain (Schmitt *et al.* 2012). One factor that may contribute to this poor performance is the limited stimulation of antigen-presenting cells (APCs; Schmitt *et al.* 2012). This possibility suggests that insufficient activation of dendritic cells and macrophages could contribute to the incomplete protection engendered by LVS. Schmitt *et al.* (2012) examined whether the interaction of genetically modified LVSs with human APCs correlated with the effectiveness of tularaemia vaccine candidates. Stimulation of APCs *in vitro* was improved by genetic modification of an LVS, but did not correlate with efficacy against challenge *in vivo* (Schmitt *et al.* 2012). Because IFN-γ is a critical mediator of protective immunity against tularaemia, a diminished IFN-γ response likely contributed to the lack of protection after vaccination (Schmitt *et al.* 2012). Several recent studies have shown that IL-17 is also required for control of *F. tularensis* growth and the generation of an effective Th1 response following pulmonary challenge (Schmitt *et al.* 2012). Similarly, several trial vaccines against francisellosis in cod, *Gadus morhua* (Linneaeus 1758), based on simple whole-cell-based preparations have been tested both in experimental challenges and in the field in Norway. None have yet awarded a significant or satisfactory degree of protection (Colquhoun & Duodu 2011). Recently, a defined live attenuated strain providing protection against *F. asiatica* in fish has been reported (Soto *et al.* 2011).

Human rickettsial infection induces long-term immunity, but killed rickettsial vaccines stimulate incomplete protection. Additionally, a live attenuated mutant stimulates strong immunity but reverts to virulence (Walker 2009). Experimental studies in an inbred mouse model of *Rickettsia conorii* infection demonstrated the greater importance of CD8+ T cells than of CD4+ T cells and the crucial role of cytotoxic CD8+ T cell activity in the clearance of infection (Walker, Olano & Feng 2001). Humoral immunity did not play an important role in recovery from infection, as antibodies to OmpA and OmpB did not appear until after the animal was well (Feng *et al.* 2004). Among the challenges is the identification of appropriate CD4+ and CD8+ T cell antigens and a means to stimulate long-lasting immunity (Walker 2009). Regarding host defence against *Coxiella burnetii* infection, specific anti-Phase I Abs play an important role in protection against the development of clinical disease at an early stage after *C. burnetii* challenge, whereas the T cell-mediated Th1 immune response is critical for the clearance and complete elimination of the organisms at the late stage of the infection (Zhang *et al.* 2007).

Chemotherapy

Although *P. salmonis* is sensitive *in vitro* to many antibiotics commonly used to control other infectious diseases in fish (Fryer *et al.* 1990), infected salmonids respond poorly to this treatment, perhaps due to an insufficient concentration of antibiotics to kill the pathogen within the host cell (Almendras & Fuentealba 1997; Mauel & Miller 2002; Fryer & Hedrick 2003). Clarithromycin, chloramphenicol, erythromycin, gentamicin, oxytetracycline and sarafloxacin inhibit bacterial growth in cultured cells (Fryer *et al.* 1990). Additionally, four *P. salmonis* strains (LF-89, EM-90, SLGO-90 and SLGO-95) were used to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of oxytetracycline, oxolinic acid, flumequine, chloramphenicol and gentamicin (Smith *et al.* 1996b). More recent strains of *P. salmonis* (SLGO-94 and SLGO-95) presented a higher resistance level than did older strains (LF-89 and EM-90). Antimicrobial resistance to cotrimoxazole, furazolidone and penicillin and sensitivity to oxolinic acid were also reported in *P. salmonis* isolated from northern Ireland (Rodger & Drinan 1993).

Important variations in the patterns of the *in vitro* antimicrobial sensitivity of *P. salmonis* isolated from different salmon species, types of aquatic environments and geographical areas between 2007 and 2008 (Mora 2010) showed a potential risk of generating antibacterial resistance. Resistance has caused variability in efficacy during field treatment, increasing the number of therapies and adjustments in dosage. These results may indicate the existence of other interesting variables, such as productive and environmental aspects, particularly

temperature, salinity and oxygen concentration. These aspects, in addition to being important stressing agents for the host, may affect the pharmacokinetic properties of antibiotics (Mora 2010).

Similarly, it is possible to identify a potential risk associated with the restricted pharmacologic alternatives for controlling the disease in Chile. As set forth in the Supreme Decree No. 139 from the *Regulation of Pharmaceutical Products Exclusively for Veterinary Use* in 1995, to enable the importation of a veterinary drug and for the drug to be elaborated and commercialized in Chile, the product must be registered with the SAG (S.A.G 2013b). The implementation of any antibacterial therapy in Chile must be supported by a veterinary medical prescription. Table 2 shows the dosage of every active compound according to the SAG register.

According to information compiled by the National Fisheries and Aquaculture Service (Servicio Nacional de Pesca y Acuicultura, Sernapesca) through the General Sanitary Program of Data Record and Laboratories' Information Disclosure during January and December 2012, *P. salmonis* was the most diagnosed pathogen during the ongrowing phase in sea water, with a proportional rate of 54.39% of cases (1041/1916; Sernapesca 2013a). The main infectious cause of the mortality of salmonid species during the ongrowing phase in Chile is piscirickettsiosis (Sernapesca 2013a). In total, 26.6% of mortality on Atlantic salmon ongrowing-phase farms has been classified as having an infectious cause, of which 69.4% is attributed to piscirickettsiosis (Sernapesca 2013a). In coho salmon, 15.5% of mortality on seawater farms has been classified as having an infectious cause, of which 60.3% is attributed to piscirickettsiosis. In rainbow trout, 46.5% of mortality on seawater farms has been classified as having an infectious cause, of which 94.6% is attributed to piscirickettsiosis. This situation could explain why 82% of the total antibiotics used in 2012 in Chile, and particularly florfenicol (62%) and oxytetracycline (37%) (Sernapesca 2013b), were administered to control piscirickettsiosis (Sernapesca 2013b).

The use of florfenicol increased from 36.8% in 2007 to 56.7% in 2008, 61.3% in 2009 (Sernapesca 2011a) and 52% in 2010 (Sernapesca 2011b). Of the total quantity of antibiotics used in 2010, 72% were administered to control piscirickettsiosis, similar to the percentage used between 2005 and 2009 (Sernapesca 2011b). However, a significant decrease in the use of oxolinic acid and flumequine has been noted in Chile (San Martín *et al.* 2010; Sernapesca 2011b), mainly due to approved restrictions in the industry that prohibit the use of quinolones in fish over 1 kg in lots destined for the USA. Among the antimicrobial agents commonly used in aquaculture, for example quinolones, several are classified by the World Health Organization (WHO) as critically important for use in humans and thus should not be used in food-producing animals (Heuer *et al.* 2009). Oxolinic acid has also been removed from fish medicine used in several other salmon-producing countries.

Of the total quantity of antibiotics used in 2007, 66% were administered to Atlantic salmon, a percentage that was significantly reduced in 2008 (to 56.6%), 2009 (to 32.6%) (Sernapesca 2011a) and 2010 (30%) (Sernapesca 2011b). However, the amount used in rainbow trout gradually increased from 22.2% in 2007 to 32.5% in 2008, 44.6% in 2009 (Sernapesca 2011a) and 52% in 2010 (Sernapesca 2011b), which was likely associated with the drop in cultured Atlantic

Table 2 General dosage of the main antibiotics registered in Chile for use in salmonid species (San Martín *et al.* 2010)

Active principle	Presentation and administration routes	Dose	Withdrawal period (degree-days)
Oxolinic acid	Oral suspension at 20%, 50% and 80%	10–30 mg kg^{-1} of biomass per day for 10–15 days	450
Amoxicillin	Oral suspension at 50%	80 mg kg^{-1} of biomass per day for 10 days	300
Erythromycin	Oral suspension at 50% and 80%	75–100 mg kg^{-1} of biomass per day for 10–21 days	500
Florfenicol	Oral suspension at 40% and 50%	10 mg kg^{-1} of biomass per day for 7–10 days	200–300
Flumequine	Oral suspension at 10%, 50% and 80%	10–30 mg kg^{-1} of biomass per day for 10–15 days	300
Oxytetracycline	Oral suspension at 40%, 50% and 80%	55–100 mg kg^{-1} of biomass per day for 10–15 days	600

salmon biomass after the ISA crisis (Sernapesca 2011b, 2013b). However, the use of this antibiotic has increased again with the increase in farmed salmon biomass (Sernapesca 2013b). Thus, the amount of antibiotic used in Atlantic salmon increased to 62% in 2012 (Sernapesca 2013b), and the amount used in rainbow trout increased to 30% in the same period (Sernapesca 2013b). The amount of antibiotic used in 2007 was 642 g pure drug/metric ton of produced salmon (Sernapesca 2011a). Later, as a result of the ISA crisis, the subsequent drop in cultured biomass and the low prevalence of diseases, the amount of antibiotic used decreased to 516 g pure drug/metric ton in 2008, 289 g in 2009, 307 g in 2010, 318 g in 2011 and 413 g in 2012 (Sernapesca 2013b; Table 3).

Thus far, prevention and control strategies for piscirickettsiosis have generated partial results in Chile. Consequently, Sernapesca recently established the Specific Program of Surveillance and Control of Piscirickettsiosis through Exempt Resolution No. 3174, dated 28 December 2012, which has been in force since its publication in the Official Publication on 8 January 2013 (Sernapesca 2013c). Objective of the programme is to decrease the impact of the disease through the early detection and follow-up of cases with the application of timely and gradual control measures. In addition, a monitoring and best practices programme has been established to enhance antibiotic use and to detect antibacterial resistance (San Martín *et al.* 2010).

Conclusions

Much progress has been made in advancing our knowledge of *P. salmonis* over the past 5 years, but there remain certain areas that require further research and development. It is necessary to improve our understanding of genetic and taxonomic relationships within the *Piscirickettsia* genus according to fish species and/or different geographical zones, including the recognition of new species and subspecies. This approach is motivated by the increasing number of whole-genome sequences that are becoming available and by the idea thought that the evolutionary history of the whole genome of *P. salmonis* must be more reliable than the history of one gene, considering the descent of species.

Improved molecular tools for the specific detection and diagnosis of piscirickettsiosis have been developed, but there remain major gaps in our understanding of the epidemiology and pathogenesis of the disease. There are insufficient data on the mechanisms by which *P. salmonis* might spread in the environment. A search for potential vectors and reservoirs of *P. salmonis* would greatly enhance our understanding of the natural history and control of piscirickettsiosis. In addition, work using the cohabitant model of piscirickettsiosis should continue to validate the model's applicability to vaccine efficacy, genetic selection and functional genomics studies.

Surveillance and monitoring programmes for piscirickettsiosis are needed to support prevention and control measures. Surveillance activities are necessary to define the extent of infection, and information from these activities should be used to monitor the progress of ongoing disease response programmes. Tracing investigations are also crucial to support decisions about the most appropriate control measures.

It is likely that only with the development of efficacious *P. salmonis* vaccines and a customized vaccination strategy will significant control of piscirickettsiosis be achieved. Antibody responses rarely provide protection and may promote certain aspects of pathogenesis and intracellular survival. It will be necessary to identify virulence factors and differences between high- and low-virulence isolates using genomics and proteomics tools. Improved knowledge about the progression of infection in fish and about how *P. salmonis* evades the host immune system is essential to developing vaccines with long-term protection. As is the case for tularaemia vaccines, the insufficient activation of APCs may contribute to the incomplete protection engendered by *P. salmonis* vaccines. Among the challenges is the identification of appropriate

Table 3 Antimicrobial volume used in the Chilean salmon industry between 2005 and 2012 according to the volume of harvested salmon

Year	Annual salmon production (thousand/ metric ton)*	Antimicrobial volume (metric ton)*	Grams of pure drug/ metric ton
2005	614	239	389
2006	647	344	531
2007	601	386	642
2008	631	326	517
2009	474	184	288
2010	466	143	307
2011	648	207	318
2012	819	338	413

Source: *National Fisheries and Aquaculture Service, Sernapesca.

CD4+ and CD8+ T cell antigens and a means to stimulate long-lasting immunity. Thus, it is important to characterize the immunological responses following immunization and challenge based on the expression of immune-relevant genes and proteins. Alternatively, it would be an important advance to develop and evaluate a *P. salmonis* live vaccine with avirulent/attenuated strains. Finally, it would be very interesting to perform a clinical field trial based on standardized and accepted protocols and with adequate control groups.

Antibiotic treatment during piscirickettsiosis control programmes must be well coordinated and should only occur once surveillance activities have been completed. The antibiogram of field isolates is exceptionally important for the effective pharmacologic control of *P. salmonis*, but efforts are needed to standardize *in vitro* antimicrobial sensitivity tests and to expand our understanding of the genetic and molecular pathways of antimicrobial resistance in *P. salmonis*. These efforts should be focused on the improvement in management routines, regulatory control of the use of antimicrobial agents, the implementation of prudent use guidelines and monitoring of the use of antimicrobial agents and antimicrobial resistance. All of this information is necessary to establish effective control strategies for the disease.

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Authors' contributions

MR participated in the conception and design of the review, acquired the statistical data for descriptive analysis and wrote the article. RE critically reviewed the article for important intellectual content. All authors read and approved the final article.

Competing interests

The authors declare that they have no competing interests.

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