Isolation of a virulent *Piscirickettsia salmonis* from the brain of naturally infected coho salmon

A.M. Skarmeta[1], V. Henríquez[1], M. Zahr [1,2], C. Orrego[1,3] and S. H. Marshall[1]

[1] Laboratorio de Genética e Inmunología Molecular. Instituto de Biología. Facultad de Ciencias Básicas y Matemáticas. Universidad Católica de Valparaíso. Casilla 4059. Valparaíso, Chile. [2] Present Address: Departamento de Biología. Facultad de Ciencias. Universidad de Valparaíso. Av. Gran Bretaña 1111. Valparaíso, Chile. [3]Conservation Genetics Laboratory, Department of Biology, San Francisco State University, 1600 Holloway Avenue. San Francisco, California 94132, USA

Abstract

A *Piscirickettsia salmonis* was isolated from the brain of a diseased coho salmon during an outbreak in Southern Chile. The isolate, AS-98, showed similar genetic, biochemical, serological and virulence characteristics to the type strain LF-89. The brain might represent an important residence site for *P.salmonis*.

Piscirickettsia salmonis is an endemic and widely distributed pathogen among cultured salmonids in seawater net pens in Southern Chile. Since the initial isolation of this intracelullar bacterium from a naturally infected coho salmon (*Oncorhynchus kisutch*) in Chile in 1989 (Fryer *et al*., 1992), similar pathologies have been described worldwide (Almendras, 1997; House *et al*., 1999). However, massive outbreaks of Piscirickettsiosis or Salmonid Rickettsial Septicemia (SRS) causing high mortalities and serious economic losses have only been reported in Chile (Almendras and Fuentealba, 1997), which suggests a particular aggressiveness of the prevalent local strains. *Piscirickettsia salmonis* causes a systemic infection that targets the kidney, liver, spleen, heart, skeletal muscle, brain, intestine, ovary and gills of salmonids (Almendras and Fuentealba, 1997). Kidney and liver tissue are recommended source for isolation of the rickettsial agent (OIE, 1997). The only report of a virulent rickettsia like organism (RLO) isolated from brain has been from Atlantic salmon (*Salmo salar*) in Scotland (Grant *et al*., 1996).

We describe the first isolation, identification and characterization of a highly virulent *P. salmonis* from the brain of naturally infected, moribund, farmed coho salmon in Southern Chile.

Fish were collected at the corners of a net pen during an epizootic in November 1998 at Chiloé Island (X region in Chile). Detection of *P. salmonis* was determined by PCR assay using specific primers that target the ITS (Internal Transcribed Spacer) region of the ribos-

omal operon (Marshall *et al*., 1998). All individuals sampled were PCR positive with 82% positive for kidney, liver and brain, while 18% were PCR-positive only for kidney and brain samples. Fresh tissues and dilutions thereof, were inoculated into cultured chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line CHSE-214 (Fryer *et al*., 1992). A bacterium was isolated from brain tissue after 40 days of incubation at 17 °C. The titre reached in cell culture was 10^6 to 10^7 (Tissue Culture Infective Dose) $TCID_{50}$/ml (Reed and Meunch, 1938). The isolate, named AS-98, was identified as *P. salmonis* by two complementary procedures: a) by IFAT using polyclonal rabbit antiserum against the type strain LF-89 (Inmunofluorescense Kit, BiosChile); b) by PCR using DNA obtained via Chelex extraction (Marshall *et al*., 1998) from the original brain tissue as well as from cultured cells infected with AS-98, which yielded amplified PCR products of the expected sizes (Marshall *et al*., 1998). A primer set specific (Hernríquez et al., unpublished) for amplification of the ITS locus of the phylogenetically distant *P. salmonis* strain EM-90 did not support DNA amplification from AS-98.

AS-98 was amplified for a section of the ITS region with primers RTS1 and RTS-4 (Marshall *et al*., 98) and cycle sequenced for subsequent automatic display of sequences in an ABI377 instrument (Heath *et al*., 2000). Both strands of the amplified product were sequenced in quadruplicate. The consensus sequence matched the sequence reported for LF-89 by our group (Heath *et al*., 2000; GenBank accession AF212834) corresponding to sequence positions 224 through 459 of an earlier ITS sequence obtained for LF-89 (Mauel *et al*., 1999; GenBank accession U36943.2). Position 451 defined as K (G or T) in GenBank accession U36943.2 was a T in AS-98 as was the case for LF-89 as sequenced by Heath *et al*. (2000), GenBank accession AF212834. The sequence obtained for AS-98 corresponds to a variable section of the ITS that allows distinctions between LF-89 and *P. salmonis* strains EM-90, NOR-92 and ATL-4-91 (Heath *et al*., 2000). Strains C1-95 and SLGO-94 are not distinguished from LF-89 by DNA sequencing nor denaturing gradient gel electrophoresis (Heath *et al*., 2000).

Biochemical analysis of AS-98 – CHSE 214 infected cells showed a polypeptide profile equivalent to those observed from other isolates of *P.salmonis* using protein gel electrophoresis under denaturing conditions (SDS-PAGE, Laemmli, 1970). Moreover, inmunoblotting of these gels (Towbin *et al*., 1979) exposed to polyclonal rabbit antibodies elicited against crude extracts of *in vitro* grown type strain LF-89 (Fryer *et al*., 1992), demonstrated the existence in AS-98 of antigens specific to all isolates of *P. salmonis* tested.

In order to assess virulence potential of the isolate, smolts of coho salmon were challenged with *P. salmonis* AS-98 after two cell culture passages. Two groups of 40 Coho salmon, weighing 80 gr each, were intraperitoneally injected with $10^{4.6}$ and $10^{3.6}$ $TCID_{50}$ of *P. salmonis* per fish, respectively (Reed and Meunch, 1938). The study was performed in tanks with recirculating ultraviolet sterilised seawater. Mortality began after 8 and 14 days post-inoculation with $10^{4.6}$ and $10^{3.6}$ $TCID_{50}$ of *P. salmonis* per fish, respectively. Cumulative mortality approached 50 % after

30 days in both groups and reached 90% and 88% mortality, respectively, when the experiment was terminated at day 41 post-inoculation. Similar levels of virulence (90% mortality with $10^{4.6}$ $TCID_{50}$ per fish) have been described for the type strain *P. salmonis* LF-89 (House *et al*, 1999) in fresh water using younger fish (12-17 g). Nonetheless, other strains of *P. salmonis* (NOR-92 and ATL-4-91) require higher bacterial titres to produce similar mortalities (House *et al*. 1999). These results seem suggest that AS-98 is a particularly virulent strain of *P. salmonis* for coho salmon. Additionally, it should be noted that AS-98 was isolated just from the brain of the target fish, while no rickettsials could be recovered from liver and kidney of the same individual. This result supports the idea that the infective dose of *P. salmonis* was much higher in the brain than that present in the classical target organs (liver and kidney). This observation should be considered in defining the biological behaviour of the agent in infected fish. In agreement with this, molecular evidence from our laboratory (Heath *et al*., 2000) demonstrated that the genomic load of *P. salmonis* in the brain of one individual was 100 times higher than that of either liver or kidney indicating that the brain might be a preferred site for the replication of *P. salmonis*. Consistent with this picture is one behavioural consequence of *P. salmonis* infection namely the propensity of affected individuals to engage in an abnormal mode of surface swimming (Almendras and Fuentealba, 1997). These observations support the idea that colonization of the brain by *P. salmonis* may play an important role in the pathogenesis of Piscirickettsiosis.

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