Virulence and electrophoretic profiles of the *Piscirickettsia salmonis* type strain IF-89 in different culture passage numbers

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Abstract

Piscirickettsiosis is a bacterial, septicaemic disease caused by the obligate intracellular bacterium *Piscirickettsia salmonis* that affects maricultured salmonid species. The LF-89 type strain has been reported to be a highly virulent strain. This work presents a comparison of the virulence and electrophoretic profile shown by two passages, 13th (P13) and 42nd (P42) of the LF-89 strain of *P. salmonis* cultured in the CHSE-214 cell line. Two groups of 20 coho salmon (*Oncorhynchus kisutch*) were inoculated with P13 and P42, respectively, both with 10^5 TCID<sub>50</sub>/mL of the bacterium. None of the fish challenged with *P. salmonis* P42 died, while those inoculated with P13 reached a cumulative mortality of 70% at 30 days post-inoculation. By SDS-PAGE, four peptide bands of molecular weights between 70 and 110 kDa were found in P42 but not in P13 of this bacterium. These results suggest that the pathogenic nature of a strain could be reflected in its electrophoretic pattern. It would be worthwhile to consider the changes in protein expression and associated virulence within strains of *P. salmonis* in the research aimed to develop vaccines and new diagnostic tests for this fish pathogen.

Introduction

Piscirickettsiosis is a septicaemic disease caused by *Piscirickettsia salmonis*, an obligate intracellular bacterium that has severely affected the culture of salmonid species in Chile. Losses due to mortality alone are estimated at over 50 million dollars per year (Smith et al., 1995). The first outbreaks of piscirickettsiosis occurred in 1989, although it has been suggested that the infection would have been present since at least 1981 (Bravo & Campos, 1989). It was initially described only in coho salmon, but it soon began to affect all salmonid species cultivated in Chile, causing up to 90% mortality in some facilities (Bravo & Campos, 1989; Fryer et al., 1990; Branson & Nieto Díaz-Muñoz, 1991; Cvitanich et al., 1991). The disease is endemic in brackish and sea water (Bravo & Campos, 1989; Fryer et al., 1990; Branson & Nieto Díaz-Muñoz, 1991; Cvitanich et al., 1991), but has occasionally been described in freshwater (Bravo, 1994; Gaggero et al., 1995).

The causative agent is a Gram-negative, non-motile, non-encapsulated, pleomorphic, microorganism. It is habitually coccoid, forming pairs or rings, and has a variable size from 0.5 – 1.5 µm in diameter (Fryer et al., 1990; Cvitanich et al., 1991). Various strains of *P. salmonis* have been described, the reference strain (LF-89) being the first isolate found in 1989 by Fryer et al. (1990). Small

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changes in the sequence of 16S rRNA allow differentiation of strains (Mauel et al., 1996; Reid et al., 2004). Studies carried out by Smith et al. (1996) and House et al. (1999) have established differences in the virulence of isolates from both Chile and other countries.

It is recognized among some researchers that, for reasons that are not well understood, some virulent strains of *P. salmonis* lose their pathogenicity. The present study describes a case of loss of pathogenicity in strain LF-89, and presents a comparative study of partially purified fractions from virulent and avirulent bacteria by means of electrophoresis.

**Materials and methods**

Seventy coho salmon weighing approximately 20 g obtained from a fish farm in Chile’s Tenth Region were used in this study. Ten of them were sampled to establish their initial sanitary status. These were euthanized by anesthetic overdose (Benzocaina, Veterquimica, Chile) and subjected to necropsy and kidney sampling. Kidney samples were evaluated by means of tissue smears and indirect immunofluorescence techniques for the presence of *Renibacterium salmoninarum* (BKDFluoro Test, BiosChile) and *P. salmonis* (SRS-Fluorotest, Bios Chile), according to the procedure described by Lannan et al. (1991) and modified for microwaves by Larenas et al. (1996). Additionally, samples were obtained for standard bacteriological and virological procedures. The remaining fish were held in six 100 L plastic tanks (with 10 fish each), at the Experimental Unit of Aquatic Animal Diseases of the Animal Pathology Department of the College of Veterinary Sciences, University of Chile (Santiago). The tanks constantly received filtered and dechlorinated potable water (1 L min⁻¹). The effluent was chlorinated (5 ppm) and treated with UV radiation. Air was pumped into each tank by means of an independent pumping system reaching 8 mg L⁻¹ of dissolved oxygen. The fish were fed twice daily with commercial pelleted fish food, at a rate of 1% of body weight.

*P. salmonis*: Strain LF-89 (ATCC(R) VR 1361) (Fryer et al. 1992) was utilized for the inoculation of fish. The bacteria were maintained and multiplied in CHSE-214 (ATCC CRL 1681) cell lines without addition of antibiotics (Lannan et al. 1984), as described by Fryer et al. (1990). Passage numbers 13 (P13) and 42 (P42) of the LF-89 strain were used for the virulence and electrophoretic study. Supernatants were titrated in microplate arrays by means of end-point dilution. Final titers were calculated using the technique of Reed & Muench (1938).

**Experimental Groups:** The fish were divided into three experimental groups (n=20 each), each divided into two tanks of 10 fish each, allowing for duplicate results. Two of the experimental groups were inoculated with *P. salmonis* at either P13 or P42, at a titer of approximately $10^{5.3} \text{ TCID}_{50}/\text{mL}$, while the third group was inoculated with a bacteria-free cell culture medium as a control. All fish were inoculated intraperitoneally as described by Smith et al. (1996). The different groups were maintained in separate 100 L tanks until the end of the study.

**Electrophoretic profile:** Bacterial samples (P13 and P42) were utilized once 50 and 100%
cytopathic effect of cell cultures was observed. Infectious supernatants were subjected to a partial purification protocol as follows: a) suspensions were centrifuged at 1,000 x g for 10 min; b) the supernatant obtained was centrifuged again at 11,000 x g for 1 h in a refrigerated centrifuge; c) the remaining pellet was re-suspended in PBS (pH=7.2) and centrifuged once more at 11,000 x g for 1 h. This final pellet was re-suspended in 200 µL PBS. Protein concentration of this suspension was determined by the method of Lowry et al. (1951). As a negative control, cells from cell line CHSE-214, previously treated with trypsin, were centrifuged and then re-suspended in PBS (pH=7.2). All samples were diluted in a denaturing and reducing solution for 5 min. Protein analysis was carried out by sodium dodecyl sulfate polyacrylamide (8%) gel electrophoresis (SDS-PAGE) following the protocol of Laemmli (1970) at 150 volts during 90 minutes. 1, 1.5 and 2 µg of total protein were loaded into the gel and silver stain was later applied. Each gel was photographed and digitalized in tif format. All of them were scanned and analyzed with UN-SCAN-IT gel Automated Digitizing System v 4.1.

Results and discussion

No deaths were recorded among the fish either in the control group or in the group inoculated with P42 of *P. salmonis* during the study period, which extended for 30 days after inoculation, while the fish inoculated with P13 suffered 70% mortality during the same time period.

The electrophoretic studies demonstrated the presence of four bands of proteins between 70 and 110 kDa in the avirulent passage (P42) that were not present in P13. These bands were observed only with a 100% cytopathic effect in cell culture and when 1.0, 1.5 and 2 µg of total protein were loaded (Figure 1). The software corroborated these differences. Recently, in our laboratory a similar result was obtained with other two virulent passages (P6 and P9) showing an electrophoretic profile similar to P13.

The causes of the loss of virulence in *P. salmonis* are still not understood. It is well known that other microorganisms lose their virulence through serial passages in cell cultures or in species other than their natural hosts. This has permitted the elaboration of attenuated vaccines for animals and man. According to Fux et al. (2005), the extraordinary plasticity of the bacterial
genome casts doubt upon whether reference strains adapted to laboratory conditions actually reflect the true pathogenicity of the “real world”. Some strains have been subcultured for decades since their first isolation and may have lost their original physiopathologic characteristics, suggesting the creation of virtual species containing a supragenome. Important genetic differences have been found in laboratory strains of *E. coli*, *P. aeruginosa* and *S. aureus*. The greater virulence of clinical strains has been associated to the presence of individual genes, clusters of strain specific genes (the pathogenicity islands) and the inactivation of individual genes (Fux et al., 2005).

According to the present study, the virulence of L-89 strain can be determined by its electrophoretic pattern. This variation in the expression of proteins between virulent and avirulent strains should be taken into account in vaccine research, as well as in the development of new diagnostic tests.

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**References**


