Routes of entry of *Piscirickettsia salmonis* in rainbow trout *Oncorhynchus mykiss*

P. A. Smith*, P. Pizarro, P. Ojeda, J. Contreras, S. Oyanedel, J. Larenas

Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile

ABSTRACT: Since 1989, *Piscirickettsia salmonis*, the causal agent of piscirickettsiosis, has killed millions of farmed salmonids each year in southern Chile. The portal of entry for the pathogen was investigated by use of selected experimental infections in juvenile rainbow trout (12 g). The methods used were intraperitoneal injection, subcutaneous injection, patch contact on skin, patch contact on gills, intestinal intubation and gastric intubation. Cumulative mortalities at Day 33 post-inoculation were 98, 100, 52, 24, 24, and 2%, respectively. It was shown that intact skin and gills could be penetrated by *P. salmonis*. The high mortality obtained in subcutaneously injected fish indicated that skin injuries could facilitate the invasion of this pathogen. Results suggested that the main entry sites are through the skin and gills and that the oral route may not be the normal method by which *P. salmonis* initiates infection of salmonids.

KEY WORDS: *Piscirickettsia salmonis* · Pathogenesis · Fish disease · Salmonid

INTRODUCTION

Gram-negative, obligated intracellular, fastidious bacteria have been increasingly detected in a wide range of fish species in different geographic locations, and are currently recognised as an important group of fish pathogens (Fryer & Mauel 1997). *Piscirickettsia salmonis* is the etiological agent of a devastating disease of maricultured salmonids in Chile (Fryer et al. 1990, Cvitanich et al. 1991, Garcés et al. 1991) known at present as 'salmon rickettsial septicaemia' (Cvitanich et al. 1991) or 'piscirickettsiosis' (Fryer et al. 1992). Losses are not limited to fish mortality, but also include other costs such as those for antibacterial drugs. Approximately US $7.5 million was spent on antimicrobial treatment of bacterial diseases of salmonid species in 1997 in Chile, and most of this was used for therapeutics against piscirickettsiosis (J. Cassigoli, Chilean Salmon Growers Association, pers. comm. 1998). In spite of the extensive use of drugs, the control of this disease has not been successful and losses have increased progressively (Almendras & Fuentealba

1997). Protective vaccines are needed to prevent piscirickettsiosis and diminish the use of antimicrobials. Administration of experimental *P. salmonis* bacterins has provided inconsistent results to date (Smith et al. 1997). Improved understanding of the disease pathogenesis of piscirickettsiosis will be critical to our ability to design more rational and efficient therapeutic and/or immunoprophylactic strategies. As a first step to determine the mechanisms used by the pathogen to gain access to the fish host, we assessed the efficiency of different entry sites of *P. salmonis* in juvenile rainbow trout *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Fish. Rainbow trout (ca 12 g) were obtained from a freshwater commercial farm located in an area where piscirickettsiosis has never been reported (Metropolitan Region of Chile). To confirm the absence of *Piscirickettsia salmonis*, kidney smears were tested by an indirect fluorescence antibody test (IFAT) according to the method of Lannan et al. (1991), as modified by Larenas et al. (1996a). A polyclonal *P. salmonis* antiserum, kindly provided by Mrs C. N. Lannan, from

*E-mail: psmith@abello.dic.uchile.cl

Oregon State University (USA) was used. Fish were kept at a research facility that used an inflow of filtrated and ultraviolet (UV) disinfected fresh water. All the experimental trouts, infected and sham-inoculated, were held in 100 l fibreglass tanks (25 fish in each tank) that had aeration (ca 8 mg of oxygen l^{-1}) and were supplied with a flow-through fresh water system (1 l min^{-1}). Fish were fed twice a day at a daily rate of 1% body weight on dry commercial pellets. Water temperature was 16.1°C (SD = 0.6°C). Effluent was UV disinfected and treated with 5 ppm sodium hypochlorite.

Bacterium. The SLGO-95 strain of *Piscirickettsia salmonis* (Mauel et al. 1996, Smith et al. 1996b) at the 9th culture passage was used. The organism was cultured using monolayers of the CHSE-214 cell line (ATCC CRL 1681) (Lannan et al. 1984). Cells were grown in Eagle's Minimum Essential Medium with Earle's salts (Automod, Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% foetal calf serum (MEM-10) (Gibco BRL, Grand Island, NY, USA) in the absence of antibiotics and antimycotics. Cell cultures inoculated with *P. salmonis* were incubated at 17°C until the cytopathic effect reached approximately 100%. Infectious supernatants were titrated by end point dilution assay ($TCID_{50}$ ml^{-1}) in 96-well plates with 6 wells per dilution. Dilution end points were calculated using the method of Reed & Muench (1938).

Infectivity study using different entry routes. Fish were infected with *Piscirickettsia salmonis* using 6 different entry sites to evaluate their eventual efficiency in reproducing the disease by means of the comparison of their cumulative mortality and survivability. Experimental fish were distributed into 13 groups, 50 individuals each, which were allotted into two 100 l fibreglass tanks (25 fish in each tank). Each infected fish group had a corresponding sham-inoculated control group and the remaining one consisted of non-inoculated fish. Fish were sedated with tricaine methyl sulfonate (MS-222, Sigma) before being inoculated. Fish were observed until Day 33 post-inoculation (p.i.). No serial samples were taken to avoid distorting the mortality figures. Each dead fish was examined by necropsy and standard microbiology methods to confirm the cause of the death.

Intraperitoneal injection (IP): Fish were injected IP with 100 µl of a suspension containing 10^4 $TCID_{50}$. This group served as a virulence control of inoculum.

Subcutaneous injection (SC): Fish were injected SC in the ventral left side of the body, between the pelvic and the anal fin with 10^4 $TCID_{50}$ contained in a volume of 50 µl.

Skin patch (SP): A modification of the method reported by Kanno et al. (1989) was used. Filter papers (Micro Filtration Systems, No. 2, cat. No. 25, Sierra

Court, CA, USA) measuring 49 mm^2 (square with 7 mm sides) were used. Paper patches were soaked in a rickettsial suspension (10^6 $TCID_{50}$ ml^{-1}), and placed on the skin for 1 min on the left side of the fish, directly on the lateral line, in the region located under the dorsal fin. After taking off the patches, fish were held in a bath containing MEM-10 for 30 min, under permanent oxygenation, and afterward returned to their tanks. A titre of $10^{4.2}$ $TCID_{50}$ ml^{-1} was obtained from a bacterial suspension prepared by means of washing a soaked paper patch in 1 ml of MEM-10. This titre could be an approximation of the actual dose to which the fish were exposed with the paper patches.

Gill patch (GP): Gills were infected using the same technique as described in the skin patch infection method. The paper patch was located in the external surface of the first branchial arch of the left side of the fish.

Gastric intubation (GI): An inoculum containing 10^4 $TCID_{50}$ in a volume of 200 µl was placed in the stomach, in the transitional region between the cardiac and pyloric region, using a plastic cylindrical flexible probe (external diameter 1.7 mm) with a rounded tip (Infant Feeding Tube, Pennine Healthcare Products, UK) connected to a tuberculin syringe. Fish were fasted for 2 d prior to inoculation.

Intestinal intubation (AI): An inoculum containing 10^4 $TCID_{50}$ in a volume of 200 µl was placed inside the descending intestine, through the anal opening, 3 cm from the fish anus by means of the same kind of probe and conditions used in the GI inoculation.

Sequential tissue sampling to study the progression of the bacterium penetration and to evaluate possible cross-infections among entry sites. Another group of 50 fish was inoculated by SP, GP, GI and AI, respectively, to provide serial samples that were examined by IFAT to detect the presence of *Piscirickettsia salmonis*. Examination of the IFAT was performed using 50 microscopic fields at 1000× magnification adopting the method of Elliott & McKibben (1996) developed for quantitative detection of *Renibacterium salmoninarum*. Five fish per group were euthanized by anaesthetic overdose at each sampling time. Samples were taken at 5 min, 15 min, 30 min, 1 h, 2 h, 22 h, 60 h, 7 and 14 d p.i. Skin, gills, stomach and intestine were obtained from the fish of the groups inoculated SP, GP, GI and AI, respectively, at each sampling time. Tissue samples were fixed in 10% buffered formalin (pH = 7.2) embedded in paraffin, and histological cross-sections were processed for IFAT. In addition, kidney smears were taken from all the groups at 60 h and 7 and 14 d p.i. to be used as a marker of systemic infection caused by *P. salmonis*.

In order to determine if cross-infections with *Piscirickettsia salmonis* of other entry sites occurred after

SP and/or GP exposure methods, tissue smears were taken at 5 and 30 min p.i. and examined by IFAT. In the fish inoculated by SP, smears from gill surface and mucosa of stomach and rectum were obtained. In the fish exposed by GP, smears were taken from skin that surrounds the operculum and between the dorsal and pelvic fins, and from the mucosa of stomach and rectum.

RESULTS

Infectivity study

No mortalities or clinical signs of disease were observed in any negative control fish. At Day 33 p.i., cumulative mortalities in experimentally infected fish reached 98% (IP), 100% (SC), 52% (SP), 24% (GP), 2% (GI) and 24% (AI) (Figs. 1 & 2). All diseased fish showed clinical signs and gross pathological features consistent with those described in fish with piscirickettsiosis (Fryer et al. 1990, Cvitanich et al. 1991). Kidney smears from each dead fish was examined by IFAT, and *Piscirickettsia salmonis* was detected in all of them.

All of the fish exposed by SP presented, in addition, skin lesions at the site of inoculation (ca 1 cm^2). Gross pathology in some of the fish infected by SP started with haemorrhagic spots on the skin but most cases exhibited a slight raised area as the earliest detectable change, followed by a whitish decoloration with scale loss and induration. Lesions then progressed to ulceration and darkening (Fig. 3). The underlying subcutaneous tissue and skeletal muscle were also haemorrhagic (Fig. 4). At the histological level, these lesions were characterised as ulcerative, affecting the epidermis, stratum spongiosum, stratum compactum and muscular tissue (Fig. 5). Scale loss, leucocyte infiltration
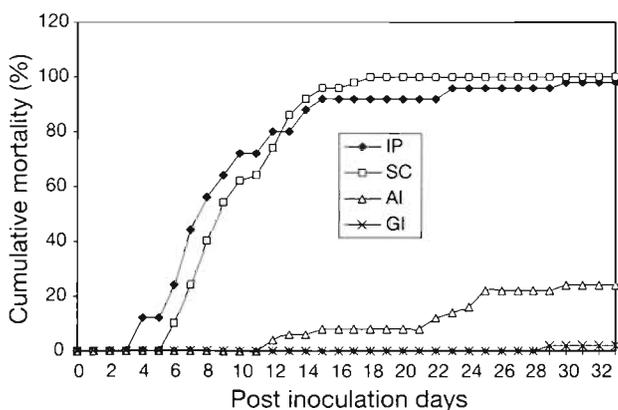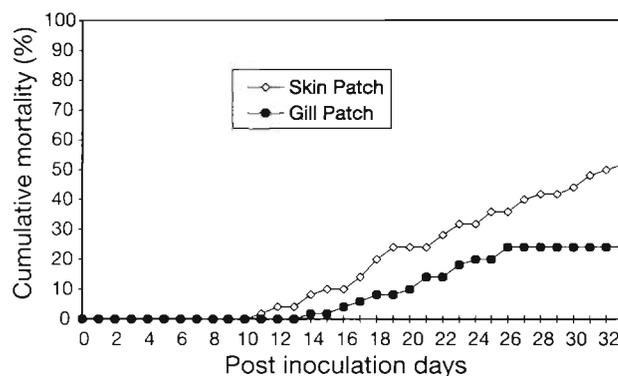

Fig. 2. *Oncorhynchus mykiss*. Cumulative mortality in rainbow trout infected with *Piscirickettsia salmonis* by contact patch in skin and gills

and proliferation of connective tissue was also observed in some areas that presented a nodular appearance.

Statistical comparison of survival probability was done among groups inoculated with the same bacterial doses (i.e. IP, SC, GI and AI, all inoculated with a dose of 10^4 TCID$_{50}$, and SP and GP, which were exposed with soaked paper patches). Survival data were obtained with the non-parametric method of Kaplan & Meier and compared with the Wilcoxon test (Lee 1992). Survival of fish inoculated IP and/or SC was significantly lower than the ones exposed by AI and/or GI methods ($p < 0.05$). No differences were found between IP and SC injection ($p > 0.05$). In contrast, survival probability of GI was higher than AI ($p < 0.05$). Similarly, analysis showed a higher survivability of fish infected by GP compared with the ones exposed by SP inoculation ($p < 0.05$).

Sequential sampling

Results of the detection of *Piscirickettsia salmonis* by IFAT in cross-sections of serial samples of skin, gills, stomach and intestine from fish inoculated by SP, GP, GI and AI, respectively, are shown in Table 1. Kidney smears from fish inoculated by SC, SP, GP and AI were positive at the 3 sampling times (60 h and 7 and 14 d). No positive infection was found in kidney smears from fish inoculated by GI.

The finding of cross-contamination between entry sites was detected in only 1 case. Small amounts of *Piscirickettsia salmonis* were seen (2 organisms) from the smears of the skin close to the gills at 30 min after the GP inoculation. All the other smears were negative.

Fig. 1. *Oncorhynchus mykiss*. Cumulative mortality (%) in rainbow trout infected with a dose of 10^4 TCID$_{50}$ of *Piscirickettsia salmonis* by intraperitoneal injection (IP), subcutaneous (SC), intestinal intubation (AI) and gastric intubation (GI)

DISCUSSION

Rickettsial organisms have diverse morphology, behaviour and pathogenesis. Among their most com-

Fig. 3. *Oncorhynchus mykiss.* Lateral view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact on the skin (scale bar = 0.4 cm). Ulcer at the exposure site (arrow)

mission in the absence of vectors was provided by detecting the bacterium in internal organs after the immersion of fish in *P. salmonis* suspensions. Nevertheless, the disease was not reproduced following these immersion exposures (Pérez et al. 1995, Smith et al. 1997). In a number of experiments, *P. salmonis* has been passed by injection (Cvitanich et al. 1991, Garcés et al. 1991, Smith et al. 1996a), but the normal mode of transmission has not been conclusively demonstrated (Fryer & Mauel 1997).

The infectivity experiments reported here indicate that *Piscirickettsia salmonis* can enter its host through a variety of tissues, with the skin being a highly efficient route of entry for this pathogen. This is supported by the high cumulative mortality obtained (52 %) and the consistent lesions seen at the infection site after inoculation by SP. It is apparent that *P. salmonis* is efficient in attaching and invading through the skin as the exposure time was short (1 min), the contact area small (49 mm^2) in respect to the total skin surface, and these integumentary tissues were intact at least at the macroscopic level. To determine if skin alone, without the lateral line, allows entry of *P. salmonis*, a subsequent SP inoculation was performed (not explained in 'Materials and methods' — this experiment was done only with 10 exposed and 10 control fish) but the

mon characteristics are Gram-negative cell walls, an obligatory existence within host cells and a transmission mechanism usually associated with arthropod vectors (Weiss 1982). Most rickettsial agents of terrestrial vertebrates penetrate their final hosts through the skin or mucous membranes via a puncture wound made by arthropod vectors (Campbell 1994). One exception is Q fever, where the causative organism (*Coxiella burnetti*) is quite resistant to the environment. It appears to develop a sporogenic phase that protects it from drying when it is outside a host or vector, and it can be directly transmitted by aerosol (Weiss & Moulder 1984).

With respect to the aquatic Gram-negative intracellular bacteria, with the exception of *Piscirickettsia salmonis*, the source, reservoir and means of transmission of these pathogens are unknown. In the case of *P. salmonis*, some research has been designed to demonstrate the role of horizontal (Cvitanich et al. 1991, Garcés et al. 1991, Pérez et al. 1995, Almendras et al. 1997, Salinas et al. 1997, Smith et al. 1997) or vertical transmission (Larenas et al. 1996b) and to detect potential vectors or reservoirs (Garcés et al. 1994). Cvitanich et al. (1991), Almendras et al. (1997) and Salinas et al. (1997) reported horizontal transmission in different cohabitation experiments. Further evidence of horizontal trans-
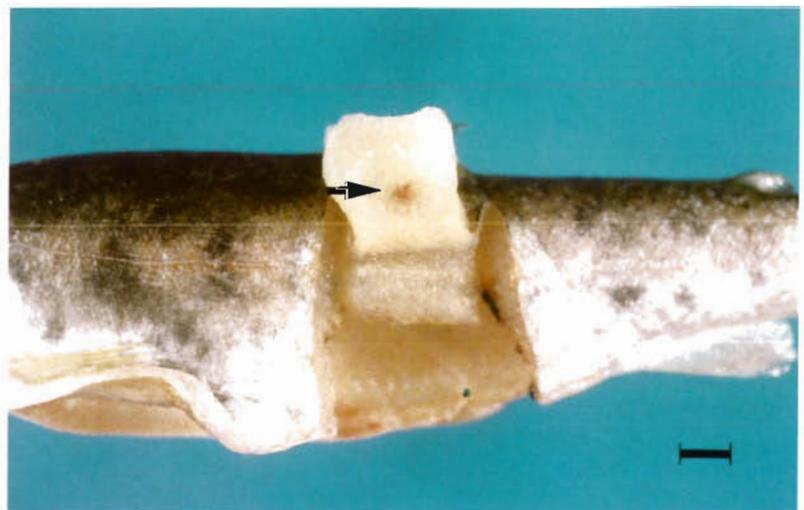

Fig. 4. *Oncorhynchus mykiss.* Lateral view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact on the skin (scale bar = 0.7 cm). Haemorrhage in the skeletal muscle underlying the exposure site (arrow)

Table 1. Detection of *Piscirickettsia salmonis* by IFAT (performed using 50 microscopic fields at 1000× magnification) in histological sections of serial samples of skin, gill, stomach and intestine of rainbow trout following different routes of experimental infection. Values in brackets show the number of positive fish. Five fish were examined, for each route of infection, at a given sampling time. The IFAT interpretation was: + = 0 to 10 bacteria, ++ = 11 to 50 bacteria, +++ >50 bacteria, – = negative

Organ	Sampling time								
	5 min	15 min	30 min	1 h	2 h	22 h	60 h	7 d	14 d
Skin (SP)[a]									
Epithelium surface	+(2)	+(1)	+(2)	–	+(1)	–	–	–	–
Epidermis	++(1)	+(1)	–	+(1)	+(2)	–	+(1)	–	+++(1)
Dermis	–	–	+(4)	+(5)	+(2)	+(3)	–	+(2)	+(2), +++(1)
Skeletal muscle	–	–	–	–	–	–	–	+(1)	+(1), +++(1)
Gill (GP)[b]									
Epithelium surface	+(2), ++(1)	+(3)	+(4)	+(4)	–	+(2)	–	–	–
Epithelium	+(4)	+(3)	+(1)	+(3)	+(1)	+(2)	+(2)	+(2)	+(2)
Capillar vessels	–	–	–	–	–	–	+(1)	+(2)	+(2)
Stomach (GI)[c]									
Lumen	+(2)	+(1)	+(1)	+(1)	–	–	–	–	–
Gastric epithelium	–	–	–	–	+(1)	–	–	+(2)	–
Intestine (AI)[d]									
Lumen	+(1)	+(3)	+(2)	–	–	–	–	–	–
Gut epithelium	–	–	+(2)	+(2)	+(1)	–	+(2)	–	–

[a]Experimental infection with *P. salmonis* by patch contact on skin
[b]Experimental infection with *P. salmonis* by patch contact on gill
[c]Experimental infection with *P. salmonis* by gastric intubation
[d]Experimental infection with *P. salmonis* by intestinal intubation through the anal opening

exposure site was immediately anterior to the dorsal fin. A similar mortality pattern and pathology were observed (Fig. 6). Therefore, although it is not possible to rule out a role of the lateral line in this matter, the skin by itself is an entrance tissue. Skin lesions seen were similar to those described for fish infected with *P. salmonis* in farming conditions (Branson & Nieto-Díaz Muñoz 1991) but were not found when the pathogen is injected intraperitoneally (Garcés et al. 1991, Smith et al. 1996a).

Consistent with the gross pathology and the histological findings described in Fig. 5, skin serial samples examined by IFAT showed a progressive penetration by the bacterium to deeper tissues with time (Table 1). The bacterium was even found in muscular tissues at Days 7 and 14 p.i. Nevertheless the number of bacteria detected, in general, was very small, which was not congruous with the magnitude of the lesions observed.

The ability to penetrate the skin is remarkable, because as Evelyn (1996) hypothesised, in the absence of injury, or without the assistance of another parasite such as a leech or louse, invasion via the skin is much more difficult to accomplish because, structurally, the skin presents a far more formidable barrier to penetration than does the gill or intestine. The only previous evidence for bacterial invasion of fish
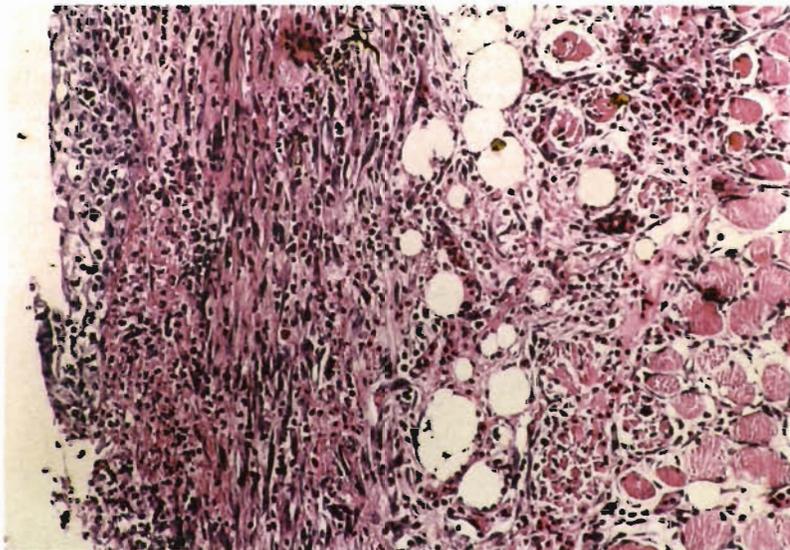

Fig. 5. *Oncorhynchus mykiss*. Skin of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact. Necrosis and infiltration of inflammatory cells in epidermis, dermis and skeletal muscle underlying the exposure site. H&E. 400×
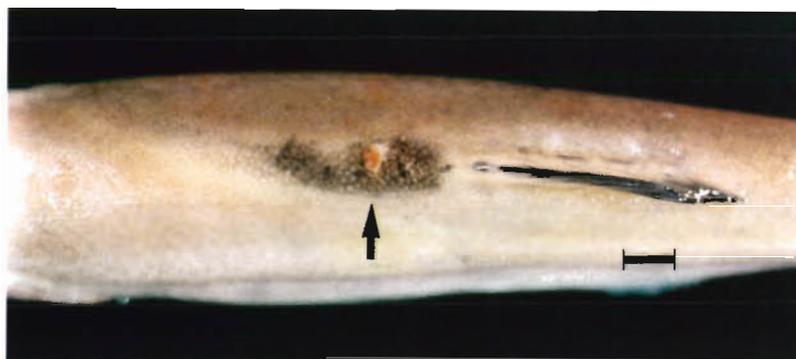
Fig. 6. *Oncorhynchus mykiss*. Dorsal view of a juvenile rainbow trout infected with *Piscirickettsia salmonis* by patch contact placed on the skin anteriorly to the dorsal fin (scale bar = 0.7 cm). Ulcer and darkening on the skin of the exposure site (arrow)

via intact skin was obtained for *Vibrio anguillarum* in experimental infections of ayu *Plecoglossus altivelis* using the paper patch method (Kanno et al. 1989) that was adopted in this work. Results reported by Effendi & Austin (1995) suggests that the skin may be one of the entry sites of *Aeromonas salmonicida*, but a swabbing method of inoculation was used which could have facilitated bacterial penetration.

The pattern of cumulative mortality among fish injected SC was the same as that among fish inoculated by IP injection, reaching 100 and 98%, respectively (Fig. 1). The expected delay in the onset of the mortalities in fish infected SC relative to the IP method was not observed, and the survivability of the 2 groups was also equal (p > 0.05). Although, as discussed previously, *Piscirickettsia salmonis* can enter intact skin, results of the SC injection suggested that skin injures could greatly increase the likelihood of penetration of the pathogen into the host. Mechanical skin damage, frequently observed in cultured salmon, as well as arthropod ectoparasites which cause superficial wounds, could facilitate the transmission of the pathogen. It is worth noting the previous finding by IFAT of *P. salmonis* in *Caligus* sp. and *Ceratothoa gaudichadii*, 2 common parasites of cultured salmon in Chile (Garcés et al. 1994).

Mortality rates following GP exposure indicated that gills are also a route of entry and this site may be important under natural conditions. Sequential examination by IFAT of gills showed that the bacteria can penetrate the epithelium and reach the capillary vessels of the secondary lamellae. The earliest time that the bacteria were found in the capillaries was at 56 h p.i. (Table 1). In a previous report, *Piscirickettsia salmonis* was detected inside gill capillaries as early as 45 min following a 3 min immersion challenge (Smith et al. 1997) although the disease was not reproduced in this experiment. In both works the

number of bacteria observed was small and the finding of the bacteria could have been easily overlooked. In any case it seems that *P. salmonis* can reach the gill capillaries, and probably disseminate through the body, in a relative short period of time ranging from minutes to 56 h. Intact gills have been documented to be the entry site for a number of viral (Ahne 1978, Mulcahy et al. 1983, Evelyn 1996) and bacterial fish pathogens including *Vibrio anguillarum* (Nelson et al. 1985, Baudin Laurencin & Germon 1987) *Aeromonas salmonicida* (Tatner et al. 1984) and *Pasteurella piscicida* (Kawahara et al. 1989).

The low mortality obtained in fish infected by GI (2%) suggests that oral exposure is not an important route of transmission in this disease. No rickettsial organisms were detected subepithelially in the stomach serial samples and in the kidney smears following GP exposure, also indicating a failure of *Piscirickettsia salmonis* to invade the fish using this entry site. It is possible that non-specific factors such as the low pH of the stomach and/or digestive enzymes could inactivate *P. salmonis*. A fluorescent background of heterogeneous material (ca 0.5 μm in diameter) was clearly observed in the gastric lumen, from 5 min to 1 h after IG inoculation. This antigenic material could be degraded *P. salmonis* cells. The fact that the fish were fasted prior to inoculation could have affected the results because *P. salmonis* may be protected inside fish food. Acid degradation of antigens in the stomach is a cause of poor effectiveness of oral vaccination in fish (Gorgetti et al. 1997). Johnson & Amend (1983) reported that the administration of *Yersinia ruckeri* vaccine through anal intubation of rainbow trout produced better protection than oral vaccination. Conversely, Baldwin & Newton (1993) documented penetration of *Edwarsiella ictaluri* after gastric intubation in channel catfish *Ictalurus punctatus*.

Cumulative mortality in fish infected by AI was higher than GI, reaching 24%. In the fish sampled after AI exposure, *Piscirickettsia salmonis* was detected in the kidney smears at the 3 sampling times (60 h and 7 and 14 d) and no degraded antigenic material was observed in the intestine. These findings show that the intestine is a likely entry site rather than the stomach. Therefore, ascending infections through the anal opening and/or infectious material in food that could pass the stomach without inactivation might also lead to infection of fish by the intestine in natural conditions. Nevertheless, it should be noted that both entry routes related to the alimentary canal (GI and AI) were less

efficient than the skin either intact, by SP, or through SC injection for development of piscirickettsiosis.

Recently, Almendras et al. (1997) reported a comparison of experimental infections, with *Piscirickettsia salmonis* via IP, oral (gastric intubation) and gills (instillation) in juvenile Atlantic salmon *Salmo salar*. Their cumulative mortality rates were 57, 41 and 45%, respectively. Those results are somewhat similar to the present work because they showed that gills might be important portals of entry for natural transmission of *P. salmonis*. However, their findings were dramatically different in respect to the gastric intubation. Even though Almendras et al. (1997) used a lower dose of *P. salmonis* (14.8 $TCID_{50}$ contained in a volume of 100 µl compared with 10^4 $TCID_{50}$ contained in a volume of 200 µl used here), the mortality in their study was higher (41 versus 2%). The differences in the bacterial strain and the salmonid species used, along with the fact that the fish used by Almendras et al. (1997) had a concurrent infection with *Aeromonas salmonicida*, may partially explain these contradictory results.

In the present work, *Piscirickettsia salmonis* was detected in one case at an entry site different from the one specifically inoculated. The rickettsia was observed in a smear taken from the skin of the vicinity of the operculum at 30 min after the GP exposure. Given the low bacterial number detected (only 2 organisms were seen), it seems unlikely that this cross-infection was significant in the outcome of the experiments.

In the sequential sampling analysis by IFAT, only small amounts of bacteria were detected in the histological cross-sections of the organs (Table 1). It is possible that a lack of sensitivity of the IFAT and the thin area observed in the cross-sections caused us to overlook the bacterium.

Results of this work suggest that main entry sites of *Piscirickettsia salmonis* are through skin and gills and that the oral route may not be as important in this disease. Invasion through the intact skin is an interesting feature shown by *P. salmonis*. Further studies by electron microscopy and the searching for bacterial attachment factors and cellular receptors are required. As a preventive measure, it would be advisable to avoid ectoparasites and any other cause of skin damage to decrease the risk of piscirickettsiosis in cultured salmon. Finally, research to find drugs that prevent the entry of *P. salmonis* to its host and/or investigation orientated to increasing local immunity at skin and gill levels seems to be interesting to explore.

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