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Short Communication

Infectivity of *Piscirickettsia salmonis* in immersion-bath exposed rainbow trout *Oncorhynchus mykiss* (Walbaum) fry

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Piscirickettsia salmonis is an obligate bacterial pathogen which causes piscirickettsiosis, a systemic disease affecting some anadromous and marine teleost fish species. To investigate the pathogenesis of this disease, a time-course study was conducted, using immunohistochemistry, after challenging rainbow trout *Oncorhynchus mykiss* (Walbaum) fry by an immersion bath with *P. salmonis*.

To carry out this assay, fish (total $n = 72$; weight $\approx$ 2.5 g) were allotted to six subgroups (12 fish each) held in individual 50-L tanks supplied with a flow-through freshwater system (25 L h^{-1}) at 15.4 °C (SD 0.8).

The SLGO-95 strain of *P. salmonis* (Smith *et al.* 1996b) was used. Bacteria, after thawing, were cultured and titered, by end-point dilution assay, in the CHSE-214 cell line. Cells were cultured at 18 °C in Eagle's minimal essential medium with Earle's salts (MEM), which is autoclavable (MEM Auto-Mod Sigma-Aldrich), supplemented with 2 mM L-glutamine and 10% of foetal calf serum (both from Gibco, Life Technologies).

For the experimental challenge, four fish subgroups (A_1, A_2, B_1 and B_2) were exposed to *P. salmonis*, each one as an independent batch, by immersion for 15 min in a 50-mL bacterial suspension (in MEM) containing $\approx 10^5$ tissue culture infectious dose 50% per mL. After exposure, each subgroup was kept for 30 min in 1-L sterile MEM, although some fish were sampled within this period, and was then replaced in the 50-L tanks. Fish from subgroups A_1 and A_2 were used to record clinical signs, mortalities and gross and histological lesions (standard haematoxylin and eosin) for 30 days after the immersion challenge, as a bacterial virulence control. Methanol-fixed smears from the kidneys of dead fish were obtained for Gram staining and also for labelling to detect *P. salmonis* by the indirect immunofluorescence test (IFAT) according to Lannan, Ewing & Fryer (1991). Two additional fish subgroups (C_1 and C_2) were included as non-infected controls. Excepting that these fish were sham-exposed, they were treated as subgroups A_1 and A_2. All these immersion procedures were carried out under permanent aeration at 16.5 (±0.5) °C.

Fish of subgroups B_1 and B_2 were sequentially sampled. Three fish were killed by anaesthetic overdose (Tricaine Sigma-Aldrich) at each of the following post-exposure (*post-exp*) times counted from the start of the immersion with *P. salmonis*: 5 min, 15 min, 30 min, 1 h, 3 h, 6 h, 18 h and 3 days. Fish were formalin-fixed and processed by standard histological methods. Five-µm sagittal sections of the whole body, excepting the tail, were examined, using an immunoperoxidase test to detect *P. salmonis* in the tissues.

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No mortalities or clinical signs occurred in the mock-treated fish (subgroups C_1 and C_2). In exposed fish (subgroups A_1 and A_2), cumulative mortalities reached 91.7% at day 23 *post-exp* (Fig. 1), indicating that fry were very susceptible to this *P. salmonis* strain. Clinical signs of diseased fish were anorexia, skin darkness, lethargy and, usually when near to death, uncoordinated swimming behaviour close to the water surface. Gross pathology was characterized by pale gills, petechiae in the wall muscles of the coelomic cavity and liver, spleen and kidney enlargement and muco-haemorrhagic content in the large bowel. Histopathology showed haemorrhagic and necrotic foci mostly in haemopoietic kidney, liver, spleen and intestine. These tissues also exhibited perivascular and endothelial necrosis with associated thrombi. Examination of kidney smears, stained with Gram or labelled with IFAT, showed organisms with the characteristic morphology widely described for *P. salmonis* (Fryer *et al.* 1990; Lannan *et al.* 1991; Fryer & Hedrick 2003; Arkush & Bartholomew 2011). The clinical and pathological findings were consistent with those associated with acute piscirickettsiosis in salmonid fish in the field (Branson & Nieto Díaz-Muñoz 1991) or under experimental conditions (Garcés *et al.* 1991; Smith *et al.* 1996a). This immersion-bath method allowed the experimental reproduction of piscirickettsiosis, which could thus be used as a model to develop challenging tests mimicking the natural way of fish exposure. Appropriate challenging methods are required to test vaccines and therapeutic drugs against fish pathogens in a reliable way (Adams *et al.* 1987).

The outcome of the sequential tissue sample examination is shown in Table 1. In summary, the time course of events, considering the first *post-exp* time that *P. salmonis* was detected in a particular tissue, was as follows: at 5 min, it was found attached to the surface of the external epithelial cell layer of the skin and gills; from 15 to 30 min, it was detected within the epithelial layer of skin, gills, oesophagus, pyloric caeca and intestine; from 3 to 6 h, it was present in the wall muscle layers of oesophagus and stomach and in skin dermis, and from 18 h onward, it was evidenced in the lumen of blood vessels and virtually in all the fish organs.

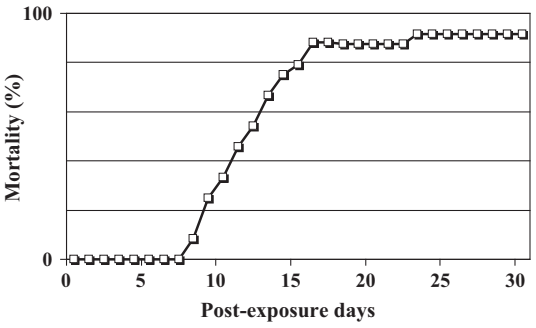

Figure 1 Cumulative mortality (%) of rainbow trout (*Oncorhynchus mykiss*) fry exposed to *Piscirickettsia salmonis* via an immersion bath.

Hence, these results show that there was a relatively steady progression of the bacterial invasion at early stages from skin, respiratory and digestive epithelia to deeper tissues, followed by a rapid spread to probably all the irrigated tissues of the fish body, once the micro-organism reached the blood stream. As already mentioned, the bacterium was found attached to the epithelial cells as early as 5 min *post-exp* (Fig. 2a), which is consistent with the results obtained in an *in vitro* infectivity study of *P. salmonis* in CHSE-214 cells (Smith *et al.* 2010). An efficient attachment ability is expected in successful aquatic pathogens because without it these organisms would run the risk of being washed off the host or being voided from the host's gastrointestinal tract (Evelyn 1996). The mechanisms and/or structures used by *P. salmonis* for attachment to the epithelial cells are unknown, although the membrane extensions that are reported to allow the anchorage of this bacterium to the ova chorion (Larenas *et al.* 2003) may have occurred with a similar function in the present study.

The invasion kinetics from the attachment time to the moment when the blood stream was reached by this bacterium was too rapid to be explained only by cell to cell contiguity spreading. In this respect, although the multiplication rate of *P. salmonis* might be higher *in vivo* than *in vitro*, at least in an axenic culture medium, the generation time of *P. salmonis* (≈12 h) is relatively slow (Vera *et al.* 2012) and the time it takes *in vitro* to be incorporated into the cytoplasm after cell exposure is 1 h in rainbow trout kidney macrophages (McCarthy *et al.* 2008) and is 3–6 h in CHSE-214 cells (Smith *et al.* 2010). The mechanisms that *P. salmonis* uses to invade fish tissues *in vivo* are unknown, but the outer membrane lipopolysaccharides (Vadovič, Fodorová & Toman 2007; Vinogradov, Frimmelova & Toman 2013)

Table 1 Time-course detection of *Piscirickettsia salmonis* by immunoperoxidase in selected tissues obtained from rainbow trout (*Oncorhynchus mykiss*) exposed to this bacterium by an immersion bath[a]

	Post-exposure time							
Tissue	5 min	15 min	30 min	1 h	3 h	6 h	18 h	72 h
Gills								
Epithelium surface	3	3	2	3	1	0	0	0
Epithelium	0	0	1	1	3	2	2	2
Skin								
Epithelium surface	3	3	2	2	1	0	0	0
Epidermis	0	1	1	2	2	2	1	0
Dermis	0	0	0	0	0	1	1	1
Subcutaneous tissue	0	0	0	0	0	0	1	0
Oesophagus								
Epithelium	0	3	2	2	1	0	0	0
Muscle layers	0	0	0	0	1	2	3	3
Stomach								
Epithelium surface	0	0	1	0	0	0	0	0
Muscle layers	0	0	0	0	1	0	1	0
Pyloric caeca								
Epithelium surface	0	0	1	0	1	0	0	0
Epithelium	0	0	1	0	2	1	0	0
Intestine								
Epithelium surface	0	0	1	1	1	0	0	0
Epithelium	0	0	2	1	3	2	0	1
Muscle layers	0	0	0	0	0	0	0	1
Liver	0	0	0	0	0	0	3	3
Spleen	0	0	0	0	0	0	2	3
Skeletal muscle	0	0	0	0	0	0	3	3
Kidney	0	0	0	0	0	0	3	3
Cardiac muscle	0	0	0	0	0	0	3	3
Blood	0	0	0	0	0	0	3	3
Brain	0	0	0	0	0	0	1	1
Spinal cord	0	0	0	0	0	0	1	1

[a]Figures show the number of fish in which *P. salmonis* was detected in a particular tissue. The tissues were obtained from three fish at each sampling time.

and the recently described exotoxins (Rojas *et al.* 2013) of this bacterium might play a role in this phenomenon.

In the digestive tube walls, attachment and invasion were earlier in the epithelial layers of the anterior than in the posterior segments of this

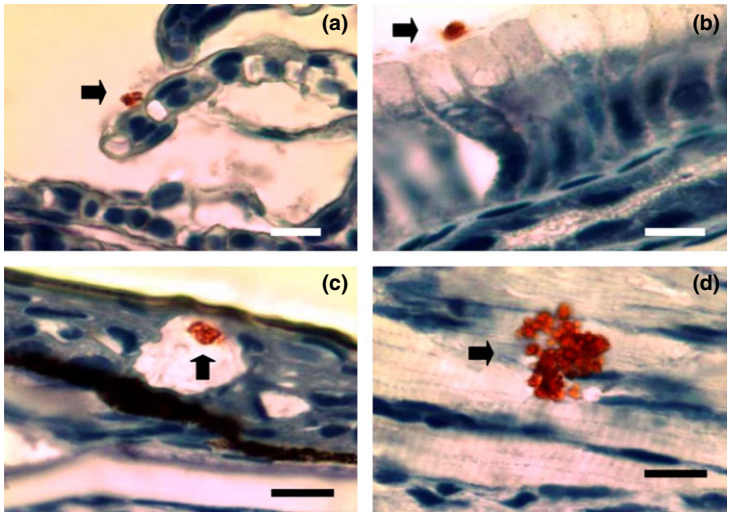

Figure 2 Immunoperoxidase test. Tissue sections of rainbow trout (*Oncorhynchus mykiss*) fry exposed by an immersion bath with *Piscirickettsia salmonis* (*P. salmonis*) at different post-exposure (*post-exp*) times counted from the moment of the initiation of the exposure. *P. salmonis* presence is indicated by black arrows. (a) *P. salmonis* on gill epithelial surface at 5 min *post-exp*. (b) *P. salmonis* on the surface of columnar epithelial cell of the gastric mucosa at 30 min *post-exp*. (c) *P. salmonis* inside a goblet cell cytoplasm at 1 h *post-exp*. (d) *P. salmonis* cluster in skeletal muscle at 3 days *post-exp*. Bars ≈ 10 μm. 1000×.

tract (Table 1), which probably reflects the normal way of transit of the digestive content in the alimentary canal. It is relevant to note that the oesophagus wall was invaded by *P. salmonis* (Table 1), including the muscularis externa layer (image not shown). This site of bacterial entry has not been described in previous infectivity studies of this pathogen in salmonid fish (Almendras *et al.* 1997; Smith *et al.* 1999; Smith *et al.* 2004) and neither has it been reported among the usual routes of entry of fish pathogens in general (Evelyn 1996; Birkbeck & Ringø 2005). Nevertheless, the infectious haematopoietic necrosis virus also uses the oesophagus as an entrance site in rainbow trout fry (Drolet, Rohovec & Leong 1994; Helmick *et al.* 1995), which suggests that this section of the digestive tract might be a more common entry portal for fish infectious agents than what has been reported to date.

Piscirickettsiosis is almost exclusively a seawater disease (Fryer *et al.* 1990; Mauel & Miller 2002; Arkush & Bartholomew 2011) and given the fact that fish in this environment drink large volumes of water (Edwards & Marshall 2013), in which *P. salmonis* can be suspended (Lannan & Fryer 1994; Olivares & Marshall 2010), it is possible that the gut epithelium, especially that of the oesophagus and intestine, plays a role as an attachment point and subsequent route of entry of *P. salmonis* in natural conditions.

Although the bacterium was also found attached to the gastric epithelial lining (Fig. 2b and Table 1) and inside the wall muscle of the stomach (Table 1), it was clearly present in lower amounts than in the oesophagus and intestine (Table 1). It therefore appears that the stomach wall might be a potential entry portal for *P. salmonis*, at least in rainbow trout fry, although probably less efficient than other segments of the digestive tract. In a previous work to assess the routes of entry of *P. salmonis* in juvenile rainbow trout (Smith *et al.* 1999), fish were exposed by patch contact in skin and gills and by intubation in stomach and intestine. Cumulative mortalities were 52, 24, 2 and 24%, respectively. These results are consistent with those obtained here with respect to skin and gills as important entry portals and the gastric environment as a probable hostile medium, although not 100% lethal, to *P. salmonis*.

A final consideration about the gut infectivity results is that the bacterium reappeared in the intestine epithelium at advanced stages of infection (Table 1), which is consistent with the severe enteritis found in fish having piscirickettsiosis in the field (Branson & Nieto Díaz-Muñoz 1991) and in the exposed rainbow trout fry here.

In addition to the variety of cell types that *P. salmonis* has already been described to infect, the bacterium in this study was also found in the cytoplasm of goblet cells of the skin epidermis (Fig. 2c), which occurred from 15 min to 6 h *post-exp*, and in skeletal muscular cells in more advanced stages of infection, from 18 h *post-exp* (Fig. 2d and Table 1). The role of the goblet cells in the establishment of *P. salmonis* infection is unknown, but during differentiation these cells migrate to the superficial epithelial layers and remain there (Roberts & Ellis 2001; Janice & Khan 2013); it could therefore be hypothesized that they do not aid movement of the bacterium into deeper tissues and probably have a defensive function instead. It has been well documented that goblet cell mucus is an important component of non-specific immunity against infectious agents in a wide variety of vertebrates including teleost fish (Ellis 2001; Janice & Khan 2013). However, it should also be taken into account that fish mucus can be beneficial to some pathogens either as a source of nutrients and/or chemoattractants, as it occurs with *Vibrio* (at present, *Aliivibrio*) *anguillarum* (Garcia *et al.* 1997; O'Toole *et al.* 1999).

Non-specific and specific immunity is developed to a large extent in salmonid fry of even smaller size (weight) than those of 2.5 g used here (Tatner 1996; Gadan *et al.* 2013), but fully mature immunocompetence is obtained at later ontogenic stages, which in rainbow trout occurs in fish weighing over 4 g (Johnson, Flynn & Amend 1982). Due to this ontogenic variation in the immune response, together with other physiological changes, it is not possible to directly infer the results obtained here in fry to post-smolts or adult fish, in which the disease normally occurs.

In summary, infectivity of *P. salmonis* in rainbow trout fry would comprise three major stages: (i) a rapid attachment to epithelial cells mainly of skin and gills, but also of the alimentary canal; (ii) a progressive invasion from the sites of entry to deeper underlying tissues until reaching the bloodstream and; (iii) a rapid haematogenous spread to virtually all body tissues. It is evident that *P. salmonis* is very successful in systemically

infecting its hosts, but the mechanisms this bacterium uses to breach, circumvent, or use to its advantage the many physical (starting from the mucus), anatomical and immunological barriers that healthy fish possess to avoid invasion by pathogenic micro-organisms are elusive and intriguing so far. Therefore, further research is necessary to reveal the pathogenesis, specially at a cellular and molecular level, of this significant fish disease.

Conflict of interest

No conflict of interest declared.

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