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Infectivity of a Scottish isolate of *Piscirickettsia salmonis* for Atlantic salmon *Salmo salar* and immune response of salmon to this agent

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ABSTRACT: A Scottish isolate of *Piscirickettsia salmonis* (SCO-95A), previously shown by intraperitoneal injection to have a lethal dose (LD_{50}) of $<2 \times 10^3$ infectious rickettsial units, was tested for virulence by bath challenge, surface application to the skin, or dorsal median sinus injection. Atlantic salmon *Salmo salar* post-smolts were used in all experiments, and exposure to 1×10^5 tissue culture infective doses (TCID) of *P. salmonis* ml^{-1} for 1 h in a bath challenge resulted in only 1 mortality, 18 d later, in 10 exposed fish. Application of 2.5×10^6 TCID of *P. salmonis* SCO-95A to paper discs on the skin failed to induce any mortalities within 42 d. Intraperitoneally, fish were administered vaccines containing 10^9 heat-inactivated (100°C, 30 min) or 10^9 formalin-inactivated *P. salmonis* SCO-95A in adjuvant, with a control group receiving phosphate-buffered saline (PBS) in adjuvant. After an induction period of over 6 mo fish were challenged by injection of *P. salmonis* into the dorsal median sinus. Mortalities in the control group reached 81.8% and the heat-inactivated and formalin-inactivated vaccines gave significant protection from *P. salmonis*, with relative percentage survivals of 70.7 and 49.6%, respectively. The nature of the protective antigen is unknown, but could be lipopolysaccharide 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 *P. salmonis*.

KEY WORDS: *Piscirickettsia salmonis* · Piscirickettiosis · Salmonid rickettsial septicaemia · SRS · Immune response · Vaccine

INTRODUCTION

Piscirickettsiosis, formerly termed salmonid rickettsial septicaemia (SRS), was described as a new syndrome causing heavy losses of coho salmon in Chile in 1989 (Fryer et al. 1990, Branson & Diaz-Munos 1991, Cvitanich et al. 1991) and growth in tissue culture cells revealed the causative agent to be a rickettsia-like organism (Fryer et al. 1990). Analysis of the 16S rDNA sequence defined the organism as a member of the γ proteobacteria rather than the α proteobacteria that contains the *Rickettsia* (Fryer et al. 1992), and the organism was placed in a new genus as *Piscirickettsia salmonis* (Fryer at al. 1992). Since the first outbreaks of SRS in Chile it has been recognised on the Pacific and Atlantic coasts of Canada (Brocklebank et al. 1993, Jones et al. 1998), Norway (Olsen et al. 1993), Ireland (Rodger & Drinan 1993) and Scotland (Grant et al. 1996, Birrell et al. 2003, Reid et al. 2004). Losses in Chile were reported to exceed 10 million fish in 1995 (Smith et al. 1997); in the northern hemisphere, losses have been very low by comparison, possibly reflecting the greater sensitivity of coho salmon *Oncorhynchus kisutch* to *P. salmonis* (Mauel & Miller 2002).

Comparison of 16S rDNA and the internal transcribed spacer (ITS) between the 16S and 23S rDNA

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showed that isolates of *Piscirickettsia salmonis* from Chile (LF-89^T, C1-95 and SLGO-94), Norway (NOR-92) and Canada (ATL-4-91) formed a homogeneous group with 1 outlier, the Chilean isolate EM-90 (Mauel et al. 1999). However, a more recent study by Reid et al. (2004) that included isolates from Scotland and Ireland showed much greater variation than previously recognised, with 2 Irish strains related to EM-90 and a further 2 Irish strains forming a new, distinct group more distant from all other recognised isolates. All 8 Scottish isolates obtained over a 7 yr period were closely related and similar to the NOR-92 and ATL-4-91 isolates from Atlantic salmon (Reid et al. 2004). The incidence of SRS is increasing in Norway and Scotland, with 17 and 7 outbreaks of disease, respectively, recorded in 2002 (Olsen 2003, Reid et al. 2004).

Several studies have reported the effect of Chilean strains of *Piscirickettsia salmonis*, mainly LF-89^T, on coho salmon, Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* (Smith et al. 1996, Almendras et al. 1997, House et al. 1999, Kuzyk et al. 2001), and one study included the effect of Strain NOR-92 on coho salmon (House et al. 1999). Herein we report on the infectivity of a Scottish isolate of *P. salmonis* administered to Atlantic salmon by various routes aimed at developing a challenge model to test the efficacy of experimental vaccines against SRS. We also show that vaccines prepared from inactivated *P. salmonis* give significant protection from *P. salmonis* challenge and that exposure of fish to *P. salmonis* induces a strong immune response to this organism.

MATERIALS AND METHODS

Bacteria. *Piscirickettsia salmonis* was isolated from Atlantic salmon *Salmo salar* during an outbreak of SRS in Scotland in 1995 (Grant et al. 1996) and subsequently identified as *P. salmonis* SCO-95A from its 16S rDNA sequence and its reaction with a latex agglutination identification kit (Latex Agglutination Detection System Rickettsia, Microtek, Canada). Although homogenates of tissues from naturally or experimentally infected fish did not react positively in the latex agglutination test, homogenates of infected tissue cultures reacted with equal intensity to similar concentrations of LF-89^T from tissue cultures. Samples of kidney were removed aseptically from moribund naturally infected fish, homogenised and added to monolayer cultures of CHSE-214 (Chinook salmon embryo) cells in Eagle's minimal essential medium (MEM) containing 10% foetal calf serum and 4 mM glutamine (Invitrogen, UK). The SCO-95A strain was maintained in culture in CHSE-214 cells with subculture at 2 to 3 wk intervals, and bacteria used in the experiments were at Passage Nos. 50 to 60 in CHSE cells. Samples were also stored in liquid nitrogen in foetal calf serum containing 10% dimethyl sulphoxide.

Quantification of *Piscirickettsia salmonis*. The concentration of infectious piscirickettsial units or TCID of *P. salmonis* in cell cultures was determined by a form of plaque assay (Cvitanich et al. 1991) in which serial dilutions (up to 10^{-8}) of homogenates of tissue-culture cells in Eagle's MEM were added to duplicate wells of monolayer cultures of CHSE-214 cells in Costar 24-well tissue-culture plates. After culture for 10 d at 18°C, the medium was removed, cells Giemsa-stained and plaques resulting from *P. salmonis* replication were counted. Each plaque was considered to represent 1 TCID of *P. salmonis* in the original culture fluid and was considered approximately equivalent to 1 $TCID_{50}$ (Smith et al. 1997).

Infectivity of *Piscirickettsia salmonis* by bath challenge. Birkbeck et al. (2004) showed that *P. salmonis* can be cultured in Sf21 insect cells with a yield approximately 60 to 100 times greater than that obtained in CHSE-214 cells and that the virulence for Atlantic salmon was unchanged. *P. salmonis* was cultured in four 175 cm^2 flasks containing confluent monolayers of Sf21 cells in 100 ml TC100 medium + 10% foetal calf serum (Invitrogen). Complete disintegration of the cells had occurred within 12 d and the 400 ml culture fluid (subsequently shown to contain 1×10^7 TCID of *P. salmonis*) was added to a tank containing ten 100 g Atlantic salmon post-smolts lightly anaesthetised with benzocaine in 40 l seawater (final concentration of *P. salmonis* = 10^5 TCID ml^{-1}) at 14°C. After 1 h the fish were transferred to a 1.5 × 1.5 m tank at 14°C with running seawater of 35 cm depth and with a flow rate of 10 to 15 l h^{-1}.

Infectivity of *Piscirickettsia salmonis* by surface application. The method of Smith et al. (1999) was used. Suspensions of *P. salmonis* cultured in CHSE-214 cells and concentrated by centrifugation to contain 5×10^8 TCID ml^{-1} (Birkbeck et al. 2004) were applied (5 µl containing 2.5×10^6, 2.5×10^4 or 2.5×10^2 TCID) to sterile Whatman filter-paper discs of 6 mm diameter. Eagle's MEM was used as a control fluid. A small area of the flank of a 100 g Atlantic salmon post-smolt, anaesthetised with 20 ppm benzocaine was wiped with a tissue and the disc applied face down to the flank of the fish for 1 min. Each group of 5 fish was marked with a pan-jet and fish were transferred to a 1.5 × 1.5 m tank with running seawater of 35 cm depth and with a flow rate of 10 to 15 l h^{-1} at 14°C for the duration of the experiment.

Infectivity of *Piscirickettsia salmonis* by injection into dorsal median sinus. Groups of 5 Atlantic salmon post-smolts (250 to 300 g) were lightly anaesthetised with benzocaine and marked by a pan-jet; 0.1 ml

CHSE-214-cultured *P. salmonis* suspension in Eagle's MEM (3×10^5, 3×10^4 and 3×10^3 TCID per injection dose) was injected into the dorsal median sinus close to the dorsal fin. Fish were transferred to a 1.5 × 1.5 m tank with running seawater of 35 cm depth and with a flow rate of 10 to 15 l h^{-1} at 14°C for the duration of the experiment.

Preparation of *Piscirickettsia salmonis* bacterin vaccines. *P. salmonis* cultured in Sf21 cells was collected by centrifugation at 10 000 × *g* for 10 min at 4°C, washed twice in phosphate-buffered saline (PBS, 8.0 g NaCl, 0.2 g KH_2PO_4, 2.8 g $Na_2HPO_4.12H_2O$, 0.2 g KCl) and resuspended to A600 nm = 1.0. This suspension was later found by titration to contain 5×10^8 TCID *P. salmonis* ml^{-1}. Heat-inactivated bacterin was prepared by heating 90 ml suspension at 100°C for 30 min. Formalin-inactivated bacterin was prepared by treatment of 90 ml of the above suspension of *P. salmonis* with formalin (final concentration of formaldehyde = 1% v/v) for 24 h at 4°C. Cells were collected by centrifugation, washed twice with PBS and resuspended in 0.1% formaldehyde in PBS. To prepare injection mixtures, cells were collected by centrifugation at 10 000 × *g* for 10 min at 4°C, washed once in PBS, adjusted to a concentration of 1.5×10^{10} ml^{-1} in PBS and emulsified with 2 volumes of Montanide 711 adjuvant to provide 10^9 inactivated cells per 0.2 ml vaccine dose.

We anaesthetised 3 groups of 60 Atlantic salmon post-smolts (~100 g weight) with benzocaine (final concentration 20 ppm), and 0.2 ml of PBS emulsified in adjuvant, heat-inactivated cells + adjuvant or formalin-inactivated cells + adjuvant was injected intraperitoneally. Fish were maintained in a separate tank for each group in ambient seawater (mean temperature approximately 10°C) for 194 d before challenge.

Due to tank-space limitations it was not possible to hold all fish at elevated temperature during the challenge with *Piscirickettsia salmonis*, and each group was divided into 2 tanks, one held in seawater at ambient temperature (7.5°C) and the other transferred from seawater to freshwater and gradually acclimatised to 16°C. *P. salmonis* was grown in CHSE cells (Titre 2×10^5 TCID ml^{-1}) and 0.1 ml (i.e. 2×10^4 TCID $fish^{-1}$) was injected into the dorsal median sinus of anaesthetised fish of 500 to 700 g weight. Fish were maintained at 7.5 to 8.5°C in seawater or 16°C in freshwater for 54 d.

Confirmation of the presence of *Piscirickettsia salmonis* in moribund or dead fish. Fish were inspected at least 3 times daily and dead or moribund fish were removed from tanks and frozen at −80°C to await bacteriological examination. In small batches, fish were thawed, sections of kidney removed aseptically, homogenised and serial dilutions in Eagle's MEM applied to monolayer cultures of CHSE-214 cells. Monolayers were examined after 10 to 15 d for cytopathic effects and visible piscirickettsia within inclusions in the cells.

ELISA of anti-*Piscirickettsia salmonis* antibody titres in fish. As many of the serum samples were from fish that had been immunised with *P. salmonis* cultured in Sf21 cells, a single preparation of *P. salmonis* grown in CHSE-214 cells was used as the antigen preparation and stored frozen in small quantities at −20°C. Each well of a Dynatech Immulon 1 96-well plate was treated with 1000 µl *P. salmonis* antigen suspension diluted 1/20 in 0.05 M carbonate buffer, pH 9.6 (coating buffer) for 3 h at 4°C. The antigen solution was removed and replaced with 100 µl 10% dried milk solution overnight at 4°C. Doubling dilutions of each salmon serum (100 µl), beginning at 1/200, were added to each row and the plate was incubated for 2 h at 37°C. The plate was washed twice with PBS-T (PBS + 0.05% Tween 20) in an automated plate-washer (Luminar Technology Swatwash) and blotted dry. Rabbit anti-salmon immunoglobulin antiserum (100 µl diluted 1/3200) was added to each well and the plate incubated for 2 h at 37°C. After removal of the well contents and washing twice with PBS-T, 100 µl horseradish peroxidase-labelled donkey anti-rabbit immunoglobulin (Scottish Antibody Production Unit, diluted 1/1000 in PBS-T) was added followed by incubation for 2 h at 37°C. After twice washing with PBS-T, 100 µl substrate solution (34 mg o-phenylenediamine dihydrochloride dissolved in 0.05 M citric acid/0.1 M Na_2HPO_4, pH 5.0 buffer, with 20 µl hydrogen peroxide solution added 100 ml^{-1} immediately prior to use) was applied to each well. After colour development in the dark for 30 min the reaction was stopped by addition of 50 µl 12.5% sulphuric acid per well and absorbance read at 492 nm in an ELISA plate reader (Dynatech). The end-point in ELISA titrations was determined graphically as the reciprocal of the dilution yielding A492 nm = 0.25. All chemicals were obtained from Sigma-Aldrich.

Serum samples for ELISA. Blood was taken aseptically from 10 fish at a sea site with no history of *Piscirickettsia salmonis* (unexposed); from 10 fish from a sea site at which SRS was confirmed, but from an adjacent pen to that containing infected fish (exposed); from 5 normal fish from an unrelated trial at the Lochailort Trials Unit (normal); from 10 fish which survived dorsal median sinus injection of 3×10^3 to 3×10^5 TCID of *P. salmonis* (challenged); and from 5 cohabitants from the same trial (cohabitants).

Statistical analysis. Statistical analyses were performed with Minitab Version 13. A χ^2 test was used to compare differences in survival in the vaccine trial, and the non-parametric Kruskal-Wallis *H*-test was used to compare differences in median antibody titres

between groups. As it has been reported that antibody titres are usually log-normally distributed rather than normally distributed (Wardlaw 1999), ELISA data was analysed both before and after logarithmic transformation but the same results were obtained in both cases. A significant difference was defined as a p-value of <0.05.

RESULTS

Infectivity of *Piscirickettsia salmonis* by bath challenge

Birkbeck et al. (2004) showed that intraperitoneal injection of *Piscirickettsia salmonis* SCO-95A is virulent for Atlantic salmon, with an LD_{50} of $<2 \times 10^3$ TCID, but that only 1 of 20 cohabitant fish held in the same tank succumbed to SRS. Therefore, we exposed fish to 1×10^5 TCID ml^{-1} *P. salmonis* for 1 h in a bath challenge. Of 10 exposed fish, only 1 died (Day 18) of SRS during the experiment.

Infectivity of *Piscirickettsia salmonis* by surface application

Application of *Piscirickettsia salmonis* to the flank of Atlantic salmon via a filter paper (Smith et al. 1999) failed to induce any mortalities within 42 d, even at the highest dose tested (2.5×10^6 TCID $fish^{-1}$).

Infectivity of *Piscirickettsia salmonis* by injection into dorsal median sinus

Because of the difficulty in inducing SRS by cohabitation, bath challenge, or surface application, the dorsal median sinus was chosen as a route of injection of *Piscirickettsia salmonis* to challenge fish in vaccine studies. This was to avoid introducing organisms to the site of administration of the adjuvanted vaccine in the peritoneal cavity. Mortalities induced were 2/5, 3/5 and 0/5 for groups administered 3×10^5, 3×10^4 and 3×10^3 TCID, respectively. The experiment was terminated on Day 46 after challenge, when serum was taken from surviving fish including all 5 cohabitant fish to assess their immune response to *P. salmonis*.

Vaccination of Atlantic salmon with *Piscirickettsia salmonis* bacterins

In this experiment, 2 vaccines were prepared from the same stock of *Piscirickettsia salmonis* by heat inactivation at 100°C for 30 min or by formaldehyde treatment to investigate the contribution of lipopolysaccharide (LPS), which is heat-stable, and outer membrane proteins, which are generally heat-labile. The vaccines were emulsified in adjuvant, and a control group was administered PBS in adjuvant. After an induction period of 194 d at a mean temperature of 10°C, fish were challenged by injection of 2×10^4 TCID of *P. salmonis* into the dorsal median sinus and maintained either in ambient seawater (7.5°C) or in freshwater at 16°C; 5 fish that died within 2 d of injection with *P. salmonis* were discounted. None of the fish held at 7.5°C died or showed any sign of SRS over 54 d. For fish maintained in fresh water, mortalities confirmed to be associated with SRS occurred from Day 26 onwards. On Day 34, 23 moribund fish were culled on welfare grounds, and *P. salmonis* infection was confirmed in these fish. The experiment was terminated on Day 54, when mortality had reached 24.0 and 47.6% in the heat-inactivated and formolised vaccine groups, respectively (Fig. 1). The mortality in the control group administered PBS + aduvjant was 81.8%;

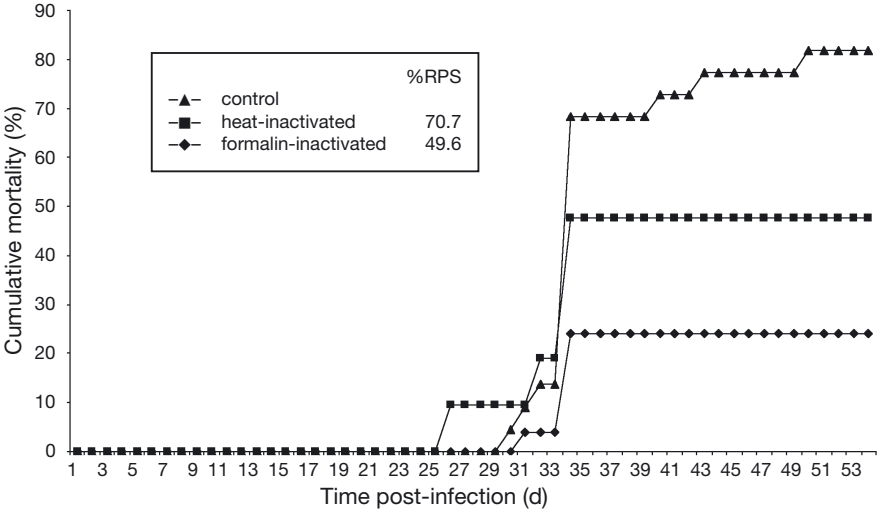

Fig. 1. *Salmo salar* infected with *Piscirickettsia salmonis*. Comparison of 2 bacterin vaccines for protection of Atlantic salmon post-smolts from salmonid rickettsial septicaemia. Fish were challenged by injection of *P. salmonis* SCO-95A ($10^{4.3}$ infectious rickettsial units) into dorsal median sinus 194 d after vaccination and held at 16°C in fresh water. All samples were emulsified in Montanide 711 adjuvant and bacterins contained equivalent of 10^9 infectious rickettsial units. When experiment was terminated, mortalities were PBS control: 18 of 22 fish; heat-inactivated bacterin: 6/25; formalin-inactivated bacterin: 10/21. RPS: relative percentage survical

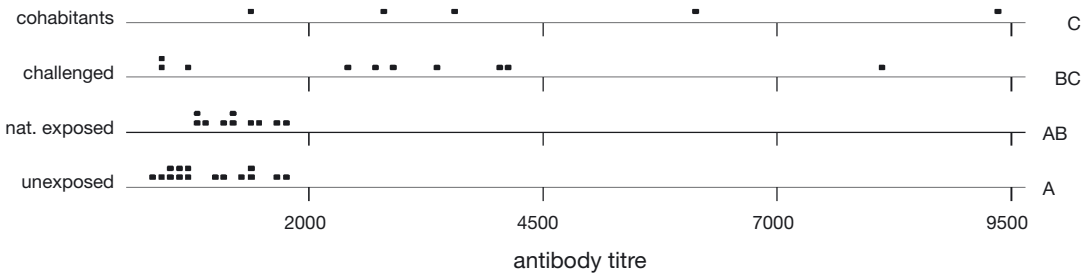

Fig. 2. *Salmo salar* infected with *Piscirickettsia salmonis*. Dot-plots of anti-*P. salmonis* antibody titres in sera of control Atlantic salmon or those exposed to *P. salmonis*. Groups were unexposed: 10 fish from site with no history of salmonid rickettsial septicaemia (SRS) and 5 fish from an unrelated experiment; naturally exposed: 10 fish from neighbouring pen at site experiencing SRS; challenged: 10 fish surviving dorsal median sinus injection of *P. salmonis* 46 d previously; cohabitants: 5 cohabitant fish held in same tank as 'challenged' fish. Significant differences ($p < 0.05$) between groups are indicated by different letters; groups with same letter were not significantly different

the relative percentage survival (RPS) in the group that received heat-inactivated vaccine was 70.7% (χ^2; $p < 0.001$); the RPS in the formolised vaccine group was 49.6% (χ^2; $p < 0.05$).

Immune response of fish to *Piscirickettsia salmonis*

All 15 sera from fish at 2 sites not exposed to SRS had ELISA titres of <1800, as did all 10 fish from a site at which SRS was confirmed in neighbouring pens. Of the 10 fish that survived dorsal median sinus challenge with *Piscirickettsia salmonis*, 7 had ELISA titres >2000, as did 4/5 cohabitants in the same experiment (Fig. 2). The median ELISA titre of fish surviving SRS-challenge (2772) was significantly greater ($p = 0.027$) than that of unexposed fish (943) but not significantly greater than that of exposed fish. For cohabitants, the median titre (3550) was significantly greater than that of unexposed fish (median = 943, $p = 0.005$) and SRS-exposed fish (median = 1192, $p = 0.010$).

DISCUSSION

A reliable challenge model for piscirickettsiosis is essential for the development of effective vaccines against *Piscirickettsia salmonis*. Cohabitation provides the most satisfactory challenge method for vaccine studies, as this mimics natural exposure most closely and requires the organism to overcome external as well as internal host defences (Nordmo 1997). Although injection challenges are regarded as more reliable, if adjuvanted vaccines are delivered by intraperitoneal injection (i.p.) then challenge by this route is not ideal, as adjuvant alone can give some long-term protection (Olivier et al. 1985). Intraperitoneal injection of Type Strain LF-89^T is highly virulent for coho salmon, with an LD_{50} of $10^{1.5}$ $TCID_{50}$, but it is less virulent for rainbow trout, for which an LD_{50} of $10^{4.7}$ $TCID_{50}$ was found by Smith et al. (1997). House et al. (1999) recorded an LD_{50} of $<10^3$ $TCID_{50}$ for LF-89^T injected intraperitoneally into coho salmon and the median times to death of their groups of fish injected with 10^5, 10^4 and 10^3 $TCID_{50}$ were almost identical to those found in this study (16, 26 and 40 d compared with 17, 25 and 40 d, respectively). Therefore, the virulence of Strain SCO-95A by intraperitoneal injection in Atlantic salmon appears to be comparable to that of LF-89^T in coho salmon.

The only study to compare the virulence of strains of *Piscirickettsia salmonis* from different geographic locations is that of House et al. (1999), who found Canadian and Norwegian isolates from Atlantic salmon (ATL-4-91 and NOR-92) to be less virulent for coho salmon (with LD_{50}s of $10^{3.4}$ and $10^{4.6}$ $TCID_{50}$, respectively) than Coho Salmon Strain LF89^T (LD_{50} $<10^3$ $TCID_{50}$).

Our lack of success in generating SRS by bath immersion is similar to the finding of Smith et al. (1997) for rainbow trout. Immersion challenge of rainbow trout for 3 min in a suspension of *Piscirickettsia salmonis* at 5.7×10^4 $TCID_{50}$ ml^{-1} (approximately 1 LD_{50} ml^{-1} by i.p. injection) did not cause clinical disease or mortality, although piscirickettsia were detected in tissues from 45 min post-exposure onward, throughout the trial (Smith et al. 1997). Both the challenge dose and time of exposure employed here were greater than those used by Smith et al. (1997), with fish exposed to ca. 500 LD_{50} ml^{-1} for 1 h still able to generate only 1 mortality in 10 fish.

As no invertebrate vector has been found for piscirickettsia (Mauel & Miller 2002, Fryer & Hedrick 2003, Birkbeck et al. 2004), it is considered to be transmitted via water. Cvitanich et al. (1991) showed that transmission of SRS was possible from injected fish to cohabitants, with the latter dying 31 and 37 d after exposure to injected fish in seawater and freshwater aquaria, respectively, at 15°C. Only 1 study to date has reported high transmissibility of *Piscirickettsia salmonis* by cohabitation. Almendras et al. (1997) reported a

Chilean isolate of *P. salmonis* from Atlantic salmon to be highly virulent for Atlantic salmon by injection and contact or non-contact cohabitation. Intraperitoneal injection of only 14.8 $TCID_{50}$ of Strain NCR1010 led to 57% mortalities between 17 and 23 d post-injection, and 59 and 50%, of contact and non-contact cohabitants, respectively, died between 17 and 28 d at 11°C. Since many of these fish were also found to be infected with *Aeromonas salmonicida*, the high mortalities resulting from such a low infectious dose may have been due to a co-operative interaction of these bacteria on the immune system.

Smith et al. (1999) concluded that the skin and gills represent the natural route of entry of *Piscirickettsia salmonis* into salmonids, and induced 52% mortalities in rainbow trout following application of $10^{4.2}$ $TCID_{50}$ *P. salmonis* via a skin patch for 1 min. In the present study, no mortalities were induced by this method in Atlantic salmon when approximately 1000 LD_{50} SCO-95A were applied to filter-paper discs. Therefore, although the Scottish Strain SCO-95A was virulent by injection (Birkbeck et al. 2004), it was very poorly transmissible by cohabitation, bath challenge or surface application, and dorsal median sinus injection was chosen for a subsequent vaccine challenge.

Good protection against *Piscirickettsia salmonis* challenge was obtained in groups of fish vaccinated with either the heat-inactivated or formalin-inactivated bacterins and challenged at 16°C. Temperature is an important factor in the development of SRS, and no deaths occurred in fish held in ambient seawater at 7.5 to 8.5°C after injection of *P. salmonis*. Also, an outbreak of SRS at a farm site in Scotland was resolved naturally by falling water temperatures (A. Grant pers. comm.). The interval between vaccination and challenge exceeded 6 mo in this study, indicating that prolonged and significant immunity to SRS was induced. As greater protection was obtained with the heat-inactivated bacterin (RPS = 70.7%) than with formalinised bacterin (RPS = 49.6%), it appears that the protective antigen(s) are heat-stable, and these would be present in both preparations. The dominant heat-stable immunogen of the Gram-negative bacterial cell envelope is lipopolysaccharide (LPS) and this is considered the protective antigen in vaccines against *Vibrio anguillarum* (Evelyn 1984) and *Yersinia ruckeri* (Stevenson 1997). Although LPS is a T-independent antigen (Janeway et al. 1999) the protection induced by the above vaccines can exceed 1 yr (Johnson et al. 1982). The vast majority of proteins would be denatured by heating at 100°C for 30 min, but it is possible that a heat-stable protein could be the protective antigen, rather than LPS. Smith et al. (1995, 1997) tested *P. salmonis* bacterin vaccines in 2 field trials in Chile. Fish vaccinated 13 wk prior to transfer to sea with $10^{5.7}$ $TCID_{50}$-formalinised *P. salmonis* suffered approximately 3% mortality compared with approximately 15% loss in control fish that received PBS (χ^2; $p < 0.05$). Interestingly, fish receiving $10^{6.1}$ $TCID_{50}$-formalinised *P. salmonis* in Freund's complete adjuvant suffered greater losses than the control group. In a second trial, fish vaccinated with $10^{5.5}$ $TCID_{50}$-formalinised *P. salmonis* without adjuvant 4 wk prior to transfer to sea experienced significantly greater mortality (40%) than the control group (24%), indicating a possible immunosuppressive action of some component of *P. salmonis*. However, this was not supported by a laboratory trial using rainbow trout, in which similar bacterin vaccines gave significant protection (2 to 4% mortality versus 20% in a control group) from intraperitoneal injection of Strain LF89 (Smith et al. 1997). In a further bacterin vaccine trial, Kuzyk et al. (2001) obtained weak protection of coho salmon challenged with *P. salmonis* LF-89^T after a 3 wk induction period at 10°C. Vaccines containing approximately 10^5 and 10^4 $TCID_{50}$ in adjuvant gave RPS values of 15 and 17.5, respectively, whereas a 1/20 dilution (approximately $10^{3.7}$ $TCID_{50}$) showed enhanced mortality (RPS = –30%). The recombinant *P. salmonis* OspA vaccine developed by Kuzyk et al. (2001) gave good protection, comparable to that of the bacterin vaccine described herein, and if OspA (a lipoprotein of approximately 17 kDa) is heat-stable when in the *P. salmonis* outer membrane, it cannot be ruled out as a possible protective antigen in this study.

The immunising dose applied in this study (10^9 bacteria) was comparable with that used in commercially available fish vaccines but much greater than in the bacterin vaccines of Smith et al. (1995) and Kuzyk et al. (2001). The latter studies were limited by the relatively low yield of *Piscirickettsia salmonis* obtained in CHSE-214 cells, but production of larger quantities of *P. salmonis* in insect cells (Birkbeck et al. 2004) permits a wider range of cell concentrations to be used in vaccines to establish vaccine efficacy.

A significant immune response was detected in 7 of 10 fish that survived challenge with *Piscirickettsia salmonis* and in 4 of 5 cohabitant fish sampled (Fig. 2), indicating that the cohabitants had experienced significant exposure to *P. salmonis* without displaying overt disease.

Using a radio-immunoassay, Smith et al. (1997) detected an immune response in rainbow trout and coho salmon that survived natural SRS infection in Chile, as well as in vaccinated and experimentally challenged coho salmon. Although details were not provided, the magnitude of the response appears similar to that detected in Atlantic salmon in the present study, using ELISA. In this study, no immune response was detected in fish from a site experiencing an outbreak of

SRS in adjacent cages. However, Smith et al. (1995) noted that it is common to find severe piscirickettsiosis in some cages on a site, with fish in neighbouring cages apparently unaffected.

The specificity of the antibodies induced in the present study was not investigated in detail. Native *Piscirickettsia salmonis* SCO-95A was used as target antigen for the ELISA measurements, but similar results were found for most sera tested with heat-inactivated antigen (results not shown). For fish that responded to a *P. salmonis* recombinant OspA vaccine, Kuzyk et al. (2001) found no correlation between antibody titres to OspA and protection from *P. salmonis* infection. Thus, although fish have been shown to respond to many antigens of *P. salmonis* (Kuzyk et al. 1996, 2001), the dominant protective antigen remains to be identified so that effective vaccines can be developed.

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