Characterization of serum and mucosal antibody responses and relative per cent survival in rainbow trout, Oncorhynchus mykiss (Walbaum), following immunization and challenge with Flavobacterium psychrophilum

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Abstract

Serum and mucosal antibody responses of juvenile rainbow trout, Oncorhynchus mykiss, were characterized by enzyme-linked immunosorbent assay (ELISA) following immunization with various preparations of formalin-killed Flavobacterium psychrophilum cells. The protective nature of these preparations was then determined by immunizing rainbow trout fry and challenging with the bacterium. Juvenile rainbow trout immunized intraperitoneally (i.p.) with formalin-killed F. psychrophilum emulsified with Freund’s complete adjuvant (FCA), and i.p. with formalin-killed F. psychrophilum either with or without culture supernatant generated significant serum antibody responses by 6 and 9 weeks, respectively. Significant mucosal antibody responses were detected by 9 weeks only in fish immunized i.p. with killed F. psychrophilum/FCA. Following immunization and bacterial challenge of rainbow trout fry, protective immunity was conferred in F. psychrophilum/FCA and saline/FCA groups with relative per cent survival values of up to 83 and 51, respectively. Significant protection was not observed in treatment groups immunized by immersion or i.p. without adjuvant at the challenge doses tested. Results suggest that stimulation of non-specific immune factors enhances the ability of fish to mount a protective immune response, but specific antibody appears necessary to provide near complete protection. In this study, an ELISA was developed to monitor anti-F. psychrophilum antibody production in trout. The relationship of such responses to protective immunity suggests that future vaccination strategies against coldwater disease may require stimulation of both the innate and adaptive arms of the immune response.

Keywords: antibody production, coldwater disease, ELISA, Flavobacterium psychrophilum, mucosal immunity, rainbow trout.

Introduction

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (CWD) and also referred to as rainbow trout fry syndrome, is a Gram-negative bacterium that causes an acute septicemic infection in salmonids (Wood & Yasutake 1956). In heavy infections, large numbers of the bacteria can be found in the heart, peritoneum and spleen (Wood & Yasutake 1956), as well as in the nuch of infected fish (Cipriano, Schill, Teska & Ford 1996). Externally, the bacteria may cause necrotic lesions of the caudal peduncle (Wood & Yasutake 1956; Holt 1987; Lumsden, Ostland & Ferguson 1996), dark colouration of the skin (Borg 1948; Evenson & Lorenzen 1996), spinal deformities and
spiral swimming behaviour (Kent, Groff, Morrison, Yasutake & Holt 1989).

Coldwater disease is an increasing concern in freshwater salmonid aquaculture worldwide and has been reported in Denmark (Dalsgaard & Madsen 2000), France (Bernardet & Kerouault 1989), Australia (Schmidtke & Carson 1995), Chile (Bustos, Calbuyahue, Montana, Opazo, Entrala & Solervicens 1995), Finland (Wiklund, Lonnstrom & Dalsgaard 1994), Japan (Wakabayashi, Horiuchi, Bunya & Hoshiai 1991; Wakabayashi, Toyama & Iida 1994), UK (Austin & Stobie 1991; Santos, Bunya & Hoshiai 1991; Wakabayashi, Toyama & Iida 1994), Finland (Wiklund, Lonnstrom & Dalsgaard 1994), Japan (Wakabayashi, Horiuchi, Bunya & Hoshiai 1991; Wakabayashi, Toyama & Iida 1994), UK (Austin & Stobie 1991; Santos, Huntly, Turnbull & Hastings 1992) and across the USA (Pacha 1968; Kent et al. 1989). High mortalities are often observed, resulting in large economic impacts on both the commercial aquaculture sector and public salmonid hatcheries. As a result of these impacts and its wide distribution, F. psychrophilum is considered one of the most significant pathogens in freshwater salmonid aquaculture worldwide (Michel, Antonio & Hedrick 1999).

All species of salmonids are thought to be susceptible to CWD, but coho salmon, Oncorhynchus kisutch (Walbaum), and rainbow and steelhead trout, O. mykiss (Walbaum), suffer the greatest losses in the Pacific Northwest. F. psychrophilum was originally isolated from coho salmon in 1948 (Borg 1948). It has also been reported in Atlantic salmon, Salmo salar L., chinook salmon, O. tsawytscha (Walbaum), sockeye salmon, O. nerka (Walbaum), chum salmon, O. keta (Walbaum), cutthroat trout, O. clarki (Richardson), brook trout, Salvelinus fontinalis (Mitchell), brown trout, Salmo trutta (L.), and rainbow and steelhead trout (Rucker, Earp & Ordal 1953; Bullock, Conroy & Snieszko 1971). F. psychrophilum has also been isolated from non-salmonid fish. Lehmann, Mock, Sturenberg & Bernardet (1991) isolated the bacterium from eel, Anguilla anguilla (L.), carp, Cyprinus carpio L., tench, Tinca tinca (L.), and crucian carp, Carassius carassius (L.). In Japan, Wakabayashi et al. (1994) also isolated the bacterium from ayu, Plecoglossus altivelis (Temminck and Schlegel).

Current methods of control for CWD include antibiotics and external treatment of bacteria, but effectiveness has generally not been satisfactory. Therefore, development of vaccination strategies to control CWD is highly desirable. Although there is no commercial vaccine available, it has been reported that significant protection can be achieved under experimental conditions (Holt 1987; Obach & Laurencin 1991). Despite these findings, limited data on immunity to this pathogen exists, and the role that antibody plays in conferring protection is unknown. This basic understanding is considered essential for the future development of an efficacious vaccine.

Preliminary immunization experiments in our laboratory demonstrated that rainbow trout produced a specific antibody response to F. psychrophilum. The present study was designed to confirm this and characterize systemic as well as mucosal antibody responses of juvenile rainbow trout. The relationship of such responses to protective immunity was also evaluated. Therefore, the objectives of this study were to characterize the systemic and mucosal antibody responses of juvenile rainbow trout following immunization with various preparations of formalin-killed F. psychrophilum and to determine if these responses were correlated with protection against a virulent strain of F. psychrophilum.

Materials and Methods

Fish and rearing conditions

Two experiments were conducted in this study. The first utilized juvenile rainbow trout (mean initial weight, 30 g) and the second utilized rainbow trout fry (mean initial weight 3 g) (Clear Springs Foods strain rainbow trout, Buhl, ID, USA). Fish were maintained in 378 L tanks supplied with specific-pathogen-free 15 ºC spring water treated by ultraviolet light. A portion of the fry was transferred to 19 L tanks for use in challenge studies. Fish were maintained by feeding 1% body weight per day of a pelleted trout feed (Clear Springs Foods, Inc.).

Bacterial culture

A virulent strain of F. psychrophilum (CSF-259-93; Crump, Perry, Clouthier & Kay 2001; MacLean, Vinogradov, Crump, Perry & Kay 2001) was used for all immunization trials, enzyme-linked immunosorbent assay (ELISA) testing and pathogen challenges. The isolate was cultured in tryptone yeast extract salt (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, pH 7.2) broth and maintained at 15 ºC.

For immunization trials, F. psychrophilum cultures were grown in 2 L volumes as described...
above. Bacteria were grown for 72 h, killed by adding 1% formalin and incubated overnight on a stir plate at 15 °C. To confirm that bacteria were not viable, samples were plated on TYES agar, incubated at 15 °C for 72 h and analysed for growth. Killed *F. psychrophilum* cells were harvested by centrifugation for 15 min at 4300 g and re-suspended in 0.85% physiological saline as a stock solution. The stock bacterial solution and culture supernatant were stored at 4 °C for 2 days prior to vaccination.

For challenge trials, *F. psychrophilum* cells were grown on TYES agar at 15 °C for 72 h. Bacteria were harvested with sterile cotton-tip applicator sticks and re-suspended in 0.85% saline to ODs of 0.4 and 0.6 at 525 nm. OD of 0.4 and 0.6 have been shown to be equal to approximately $5 \times 10^7$ colony-forming units (cfu) mL$^{-1}$ and $9 \times 10^7$ cfu mL$^{-1}$, respectively (Holt 1987).

For ELISA antigen production, *F. psychrophilum* cells were grown in 500 mL volumes as described above, harvested by centrifugation for 15 min at 4300 g, and re-suspended in approximately 10 mL phosphate-buffered saline (PBS). The bacteria were rapidly frozen in 1.5 mL centrifuge tubes by immersing the tubes in a bath of dry ice and methanol (50:50). Following freezing, samples were removed from the ice bath, thawed and then vortexed. This process was repeated 10 times. The protein concentration of the antigen was determined by using the Micro BCA™ Protein Assay according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). The ELISA antigen was diluted to 1000 µg protein mL$^{-1}$ and stored at −20 °C until needed for analysis.

**Inoculum preparation**

Inocula for immunization via intraperitoneal (i.p.) or waterborne routes were prepared as follows:

**Intraperitoneal delivery**

*F. psychrophilum*: Formalin-killed *F. psychrophilum* cells were diluted in 0.85% physiological saline to an OD of 0.8 at 525 nm, corresponding to approximately $2 \times 10^8$ cfu mL$^{-1}$ (Holt 1987).

*F. psychrophilum* with Freund’s complete adjuvant (FCA): Formalin-killed *F. psychrophilum* cells were diluted in 0.85% saline to an OD of 0.8 at 525 nm.

This inoculum was centrifuged for 15 min at 4300 g, the supernatant removed and the cells re-suspended in 0.85% saline to one-half of the original volume. Immunization treatments were prepared by adding an equal volume of FCA (Sigma, St Louis, MO, USA) and forcing both solutions through two connected syringes until fully emulsified.

0.85 % saline: Physiological saline (0.85%) served as a control.

0.85 % saline with FCA: Physiological saline (0.85%) was combined with an equal volume of FCA by forcing both solutions through two connected syringes until fully emulsified.

Culture supernatant: Formalin-treated supernatant from 72-h cultures from which the bacteria had been removed was used for immunization.

*F. psychrophilum* with culture supernatant: Formalin-killed *F. psychrophilum* cells were diluted in formalin-treated culture supernatant to an OD of 0.8 at 525 nm.

**Immersion delivery**

Bath solutions were prepared by suspending formalin-killed *F. psychrophilum* cells in 2 L of water and adjusting to an OD of 0.6 at 525 nm. For rainbow trout fry immunizations, an additional immersion treatment was included at a concentration of 1:10, or approximately $9 \times 10^6$ cfu mL$^{-1}$.

Control treatments were prepared by adding a volume of 0.85% saline equal to the volume of bacterial inoculum added in the immersion treatments.

**Fish immunizations**

Groups of 15 juvenile rainbow trout and groups of 300 rainbow trout fry per treatment were immunized either by i.p. or immersion routes. Treatments included formalin-killed *F. psychrophilum* cells with or without FCA, formalin-treated culture supernatant with or without killed bacteria, and immersion for 2 min into diluted bacterin suspensions. Juvenile rainbow trout were injected i.p. with 200 µL and rainbow trout fry with 25 µL of each preparation. Control juvenile rainbow trout were injected i.p. with 200 µL of 0.85% saline with or without FCA. Control rainbow trout fry were either i.p. injected with 25 µL 0.85% saline followed by immersion in water containing saline for 2 min, or i.p. injected with 25 µL 0.85% saline containing FCA. Six
weeks following the primary immunization, both size groups of fish were booster immunized with the same dose and treatment as the primary immunization.

**Sampling**

Individual juvenile rainbow trout were identified by tagging fish with passive integrated transponders. Serum and mucus were sampled from individual juvenile fish prior to immunization and every 3 weeks for the duration of the 12-week study.

For serum and mucus sampling, juvenile rainbow trout were anaesthetized by immersion in 100 μg mL⁻¹ tricaine methane sulphonate (MS-222; Argent, Redmond, WA, USA) and approximately 200 μL of blood was collected by caudal puncture using a 22-gauge needle at each designated sampling period. Blood was placed in 1.5 mL Eppendorf tubes and allowed to clot overnight at 4 °C. Serum was collected following centrifugation at 15 000 g for 5 min. Mucus was collected by gentle swabbing with a sterile cotton-tip applicator, beginning posterior to the operculum and extending to the caudal region 10 times to obtain approximately 0.1 g of mucus. Mucus was then diluted approximately 10 times to obtain approximately 0.01 g of mucus. Mucus was then diluted approximately 1:10 by placing the cotton-tip applicator into 5 mL disposable culture tubes with 900 μL PBS containing 0.02 % sodium azide. Mucus samples were then centrifuged at 2250 g for 10 min and supernatant collected. All serum and mucus samples were stored at −20 °C until needed for analysis.

Prior to bacterial challenge, rainbow trout fry were bled to determine antibody titres. Five randomly selected fish per treatment were bled by severing the caudal peduncle and collecting blood in 0.1 mL haematocrit tubes. The five blood samples from each treatment were combined into one sample and serum was collected as previously described.

**Bacterial challenge**

At 12 weeks post-immunization, rainbow trout fry (mean initial weight, 3.0 g) averaged 10 g in weight and were challenged with live *F. psychrophilum* cells by subcutaneous injection. Duplicate groups of 25 fish per treatment were challenged with 50 μL of live *F. psychrophilum* suspended in saline to an OD of 0.4 or 0.6 at 525 nm. Mock-infected controls (25 fish/treatment) were injected with 50 μL 0.85% physiological saline. The bacterial inoculum was injected subcutaneously at the dorsal midline posterior to the dorsal fin with a 30-gauge needle. Mortalities were recorded daily for 28 days and a minimum of 20% of the daily mortalities were re-examined for *F. psychrophilum* by inoculating spleen tissue onto TYES agar. The cumulative percent mortality (CPM) and relative percent survival (RPS) of all replicates were calculated. The RPS was calculated using the following equation (Amend 1981):

\[
RPS = \left[1 - \frac{\% \text{ mortality of vaccinated fish}}{\% \text{ mortality of non-vaccinated fish}}\right] \times 100.
\]

**ELISA**

**Serum**

An ELISA was performed on all serum samples to identify anti-*F. psychrophilum* antibodies. Immulon II high binding 96-well plates (Thermo Labsystems, Franklin, MA, USA) were coated with 100 μL *F. psychrophilum* antigen diluted in carbonate buffer to 10 μg protein mL⁻¹ and allowed to bind overnight at 4 °C. The plates were then washed thrice in PBS containing 0.05% Tween-20 (PBS-T). Serum samples were serially diluted (from 1:6400 to 1:819 200 in doubling dilutions) in PBS containing 0.02% sodium azide, added to the wells (100 μL well⁻¹), and incubated overnight at 15 °C. Positive and negative rainbow trout serum controls were also added to each plate. The positive control serum was from a fish exhibiting a high titre (204 800) and the negative control serum was a pool of equal volumes of serum from all fish in the study prior to immunization (week 0). Following washing of the wells thrice with PBS-T, 100 μL of mouse monoclonal antibody (MAb 1.14; DeLuca, Wilson & Warr 1983) against trout immunoglobulin (Ig) was added (1:400 in PBS-T containing 0.1% non-fat dry milk) and incubated for 1 h at room temperature. After another wash, the plates were incubated for 1 h at room temperature with 100 μL horseradish peroxidase conjugated goat anti-mouse Ig diluted 1:5000 in PBS-T containing 0.1% non-fat dry milk (Calbiochem, San Diego,
CA, USA). Plates were washed again and bound enzyme was visualized by adding 50 μL of ABTS peroxidase substrate and H₂O₂ in the ratio of 1:1 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA). After 15 min at room temperature, the colour reaction was stopped by adding 50 μL of distilled water containing 1% sodium dodecyl sulphate (Bio-Rad, Hercules, CA, USA). The OD of the wells was determined by using a Bio-Tek Model EL 312E microplate autoreader at 405 nm (Bio-Tek Instruments Inc., Winooski, VT, USA). The ELISA titre was defined as the reciprocal of the highest dilution showing an OD at least two times greater than the negative control.

Mucus

To identify anti-<i>Flavobacterium psychrophilum</i> antibodies in the mucus samples, the serum ELISA protocol was modified. Mucus samples were serially diluted (1:10 to 1:5120 in doubling dilutions) in PBS containing 0.02% azide and 50 μL of each dilution were added to 96-well plates coated with <i>F. psychrophilum</i> as described above. The positive control (50 μL well⁻¹) was a pool of equal volumes of all mucus samples collected at week 0 and spiked 1:400 with serum exhibiting a high titre (204 800). The negative control (50 μL well⁻¹) was a pool of all mucus samples taken at week 0. The ELISA titre was defined as the reciprocal of the highest dilution showing an OD at least two times greater than the negative control.

Statistical analysis

Statistical analysis of serum and mucosal antibody responses between treatment groups at each sampling period were performed by one-way ANOVA on log₁₀ transformed titre data using a computer software program (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA). If significant differences were detected, paired comparisons between treatments were analysed by Tukey’s tests. Analysis of CPM and RPS of challenge studies was analysed by one-way ANOVA and pairwise comparisons were made using Tukey’s test. Values were considered significantly different at <i>P</i> values < 0.05.

Results

Immunization study

Serum antibody response

Juvenile rainbow trout immunized i.p. with formalin-killed <i>F. psychrophilum</i> emulsified with FCA generated strong serum antibody responses against <i>F. psychrophilum</i> (Fig. 1). The average log₁₀ serum antibody titre was elevated significantly above the antibody response of fish injected i.p. with saline/FCA at weeks 6, 9 and 12 (<i>P</i> < 0.001). Antibody production increased throughout the study and peaked at week 12, with individual fish exhibiting antibody titres ranging from 51 200 to 819 200. This antibody response at week 12 differed significantly (<i>P</i> < 0.01) from all other treatments (Figs

![Figure 1](https://example.com/figure1.png)
2 & 4) except for fish immunized i.p. with F. psychrophilum and culture supernatant (Fig. 3).

Juvenile rainbow trout immunized i.p. with killed F. psychrophilum with and without culture supernatant showed similar serum antibody responses following immunization (Figs 2 & 3). Antibody production increased above control groups following the primary and booster immunizations and peaked at weeks 9–12, differing significantly (P < 0.05) from fish injected i.p. with saline. Individual fish in these treatments had antibody titers ranging from <6400 to 51,200 at week 12. The antibody response of fish immunized i.p. with formalin-killed F. psychrophilum was also significantly (P < 0.05) elevated above the saline control at week 6.

Fish immunized by i.p. injection of F. psychrophilum culture supernatant and by immersion in a bath of F. psychrophilum did not generate significant serum antibody responses in comparison with controls (Fig. 4). However, seven and four fish in these treatments exhibited elevated antibody responses above the average titre of the saline injected controls, respectively.

**Mucosal antibody response**

Juvenile rainbow trout immunized by i.p. injection with formalin-killed F. psychrophilum emulsified with FCA generated strong mucus antibody responses against F. psychrophilum (Fig. 1). Antibody production in the mucus was minimal during

Figure 2  Serum and mucus antibody responses of juvenile rainbow trout following immunization by i.p. injection of formalin-killed Flavobacterium psychrophilum (■) and by i.p. injection with saline (□). Primary and booster immunizations (+) were administered for each treatment at 0 and 6 weeks. Asterisks indicate a significant difference *(P < 0.05) and **(P < 0.01) from the saline control.

Figure 3  Serum and mucus antibody responses of juvenile rainbow trout following immunization by i.p. injection of formalin-killed Flavobacterium psychrophilum suspended in culture supernatant (■) and by i.p. injection with saline (□). Primary and booster immunizations (+) were administered for each treatment at 0 and 6 weeks. Asterisks indicate a significant difference *(P < 0.05) and **(P < 0.01) from the saline control.
the first 6 weeks, but, following the booster immunization at week 6, antibody production increased and peaked at week 12. Titres for individual fish ranged from 0 to 1280 at week 12. The antibody response generated was significantly ($P < 0.001$) elevated above fish injected i.p. with saline/FCA and all other treatments at weeks 9 and 12 (Figs 1–4).

Fish immunized by i.p. injection with $F.\ psychrophilum$ with and without culture supernatant, by i.p. injection with culture supernatant, and by immersion into a bath of killed $F.\ psychrophilum$, did not generate significant mucosal antibody responses (Figs 2–4).

**Bacterial challenge of rainbow trout fry**

Following immunization of rainbow trout fry, treatment groups were challenged with two bacterial doses at 12 weeks and monitored for mortalities daily for 28 days. The final CPM of each treatment for the challenge doses of live $F.\ psychrophilum$ at an OD of 0.4 and 0.6 is shown in Fig. 5. At the lower challenge dose (OD adjusted to 0.4), control fish (injected i.p. with saline followed by immersion in water containing PBS) exhibited a CPM of 96%. Fry immunized with $F.\ psychrophilum$/FCA showed significantly ($P < 0.001$) lower CPM relative to this control and all other treatments ($P < 0.05$). Fish vaccinated with saline/FCA also showed significantly ($P < 0.01$) lower CPM relative to the saline control. The RPS of these groups was 83 and 51%, respectively. Immunization by i.p. injection with killed cells suspended in culture supernatant and immersion into a bath of diluted killed cells (1:10) resulted in RPS values of 15 and 13%, respectively. All other treatments resulted in lower RPS values ranging from 0–6%. At a higher bacterial challenge (OD adjusted to 0.6), similar CPM was observed for all treatments (Fig. 5). Fish immunized with formalin-killed $F.\ psychrophilum$/ FCA and FCA alone at this challenge dose exhibited RPS values of 71 and 39%, respectively, and all other treatments resulted in minimal protection. There was no significant protection in any of the other treatment groups at the challenge doses tested.

*Flavobacterium psychrophilum* was re-isolated from the spleen in 93% (115 of 125) of the mortalities examined for the 0.4 OD challenge dose, and in 95% (118 of 124) of the mortalities from the 0.6 OD challenge dose. Mortality in the mock infected controls (25 fish/treatment) was negligible.

Prior to bacterial challenge, antibody titres were determined for pooled samples of blood from fry ($n = 5$) in each treatment group. Fry immunized i.p. with $F.\ psychrophilum$/FCA generated an antibody response similar to that observed in the juvenile fish, with an average titre of 204 800. All other treatments and controls had much lower antibody responses, ranging from < 100 to 6400.

**Discussion**

Vaccination strategies are generally targeted to increase antibody levels in fish to provide protection from pathogen challenge. Antibody response is known to be an important component of the fish immune system and the ability to monitor such a response is essential to understanding adaptive
immunity. The ELISA developed here provided a means to detect serum and skin-derived antibody specific for the fish pathogen *F. psychrophilum*. Passive immunization studies have shown that injection of high titre anti-pathogen serum into fish prior to or soon after pathogen challenge can confer significant protection (Spence, Fryer & Pilcher 1965; Harrell, Etlinger & Hodgins 1975; Marquis & Lallier 1989; LaPatra, Lauda, Jones, Walker & Shewmaker 1994; Akhlaghi, Munday & Whittington 1996). However, the role antibody-mediated protection plays in prevention of CWD has been unclear.

In the present study, juvenile rainbow trout were immunized with formalin-killed *F. psychrophilum* preparations and the development of an adaptive immune response was characterized by measuring antibody production in serum and cutaneous mucus. It was found that fish generated significant serum antibody responses at weeks 9 and 12 when immunized by i.p. injection of formalin-killed *F. psychrophilum* with or without FCA or by killed *F. psychrophilum* suspended in culture supernatant. Fish immunized i.p. with *F. psychrophilum*/FCA generated the strongest response. Titres among these fish remained highly elevated at 12 weeks indicating that the response would have persisted for longer periods. Fish immunized with killed cells also had elevated titres at 12 weeks, but levels were reduced from those of fish receiving adjuvant. Fletcher & White (1973) reported detectable titres in plaice, *Pleuronectes platessa* (L.), 1 year following i.p. injection of *Vibro anguillarum* antigens emulsified with FCA, but did not detect titres in fish injected i.p. with antigen alone. In the present study, treatment groups receiving whole cells suspended in saline or culture supernatant did produce significant responses, but the use of adjuvant enhanced this response. Similar results have been shown when incorporating FCA into an emulsion with various antigens (Fletcher & White 1973; Harrell et al. 1975; Whittington, Munday, Akhlaghi, Reddacliff & Carson 1994; Cain, Jones & Raison 2000).

One component that is often overlooked when evaluating immune responsiveness in fish is the ability to stimulate antibody at mucosal surfaces. The exact mechanism of mucosal antibody production and the role it may play in disease protection is not fully understood in fish. There is evidence that antibody may be produced locally or disseminated from the circulation to mucosal sites. Loghothetis & Austin (1994) reported that mucosal antibody production may be local due to the differential responses between the serum and mucus following immunization with *Aeromonas hydrophila*. Lobb
Coho salmon (mean weight, 0.5 and 27.0 g) immunized i.p. with saline exhibited RPS values of up to 100%. Results presented here suggest that vaccinated in this manner exhibited RPS values of 80% for rainbow trout (mean weight, 2.2 g) immunized i.p. with a heat inactivated

(1987) reported similar results when channel catfish, *Ictalurus punctatus* (Rafinesque), were immunized by immersion in a bath containing dinitrophenylated-horse serum albumin. Cain *et al.* (2000) reported a similar pattern but when adjuvant was incorporated, serum and mucus antibody responses mapped closely following immunization with a hapten-carrier antigen consisting of fluorescein isothiocyanate conjugated to keyhole limpet haemocyanin. The results suggested a high proportion of mucosal antibody was similar to serum IgM and that elevated serum antibodies may have disseminated to mucosal surfaces. This possibility is supported by the results of the present study, as fish immunized i.p. with killed *F. psychrophilum*/FCA exhibited significant serum antibody titres at week 6, but significant antibody responses were not detected in the mucus by ELISA until week 9. At weeks 9 and 12, antibody titres were relatively high in both serum and mucus. This suggests passive transfer of antibody from blood to mucus as a result of enhanced circulating antibody. Further analysis would be needed to determine whether localized production is occurring and the degree of structural homology between serum and mucus Ig.

The characterization of antibody production by juvenile rainbow trout following different immunization treatments gave us a baseline to investigate the role these responses have in eliciting a protective immune response. Rainbow trout fry were immunized in a similar manner, and challenge experiments confirmed that antibody titres were correlated with protection from *F. psychrophilum* challenge. Rainbow trout fry immunized i.p. with *F. psychrophilum*/FCA had significantly lower CPM than saline injected fish and exhibited an RPS of 83% when challenged with the pathogen at an OD of 0.4. A pool of serum from five fish immunized with *F. psychrophilum*/FCA showed a highly elevated antibody titre (204 800) compared with saline injected fish (<100). When fry were challenged with a higher bacterial concentration (OD 0.6), fish immunized with killed bacteria and FCA also exhibited significant protection. Holt (1987) obtained similar results by vaccinating coho salmon with formalin-killed *F. psychrophilum* with FCA. Coho salmon (mean weight, 0.5 and 27.0 g) vaccinated in this manner exhibited RPS values of up to 100%. Results presented here suggest that antibody plays a role in conferring significant protection; however, non-specific stimulation may also be important. Fry immunized i.p. with saline emulsified with FCA exhibited an RPS of 51% following challenge with the pathogen. This is consistent with Holt (1987) in that an RPS of up to 78% was reported in 0.5 g coho salmon injected with FCA alone and challenged at different live *F. psychrophilum* doses. Stimulation by FCA of non-specific components of the immune system, with resulting protection from pathogen challenge is well documented (Olivier, Evelyn & Lallier 1985; Holt 1987; Adams, Auchenachie, Bundy, Tatner & Horne 1988), and macrophage stimulation may be responsible for the enhanced protection (Olivier *et al.* 1985). Another possibility is that an increase in total antibody induced by FCA resulted in enhanced protection. Fish injected with FCA alone have been shown to have total Ig concentrations in serum elevated above normal trout serum (Cain 1997). Western blot analysis of serum from fish immunized with FCA alone in this study did not show reactivity to specific antigens of *F. psychrophilum*. However, some level of non-specific binding was apparent as ELISA titres increased by week 9 in juvenile rainbow trout immunized with FCA alone (Fig. 1). This elevated response indicates that non-specific stimulation may be very important and that this response, combined with a specific antibody response, leads to near complete protection.

Juvenile fish immunized i.p. with formalin-killed *F. psychrophilum* with and without culture supernatant developed significant serum antibody responses above the controls. However, these immunization treatments did not confer significant protection from pathogen challenge when repeated in rainbow trout fry. This may be due to the fact that fry immunized with these formalin-killed *F. psychrophilum* preparations generated considerably lower antibody titres. In addition, non-specific stimulation by adjuvant was absent. Average antibody titres prior to bacterial challenge in fry immunized i.p. with formalin-killed cells with and without culture supernatant were 1600 and 3200, respectively. Significant protection may have been seen in these treatment groups at a lower bacterial challenge dose; the relatively high challenge doses administered to fry resulted in up to 96% CPM in saline injected controls. The antibody responses generated in fry immunized without adjuvant may not have been elevated enough to provide protection from the high challenge doses administered. Obach & Laurencin (1991) did report an RPS value of 80% for rainbow trout (mean weight, 2.2 g) immunized i.p. with a heat inactivated
F. psychrophilum bacterin. However, in that study, the challenge dose administered to fry resulted in CPM for immunized fry and saline injected controls of only 11 and 55%, respectively. It should be noted that these fish were immunized with a higher concentration of a different strain of F. psychrophilum and the process of heat inactivation may affect antigenicity in a different manner than formalin treatment. It appears that highly elevated antibody responses are critical for protecting rainbow trout fry, but the importance of non-specific immune responses should not be overlooked.

This study reports the first description of an ELISA-based technique for detecting significant levels of F. psychrophilum specific antibodies in the serum and mucus of rainbow trout and the first description of an adaptive immune response generated by juvenile rainbow trout to F. psychrophilum. Furthermore, the relationship of such a response to protective immunity has been established and suggests that antibody production and stimulation of non-specific components of the immune system are important. These results are promising for the future development of an efficacious vaccine. Future studies should focus on identification and characterization of antigens important for protection.

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