Route of vaccine administration: effects on the specific humoral response in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: The specific humoral response of teleost fish to extracellular bacteria was examined using a rainbow trout - *Vibrio anguillarum* model. Treatment groups were immunized by oral, immersion, and injection routes. All 3 delivery methods conferred full protection in controlled laboratory challenges (p < 0.01). Prior to boosting, serum antibody titers did not correlate with protection in the orally and immersion-vaccinated groups, but, contrary to previous studies, titers measured 10 and 17 d after boosting correlated positively with protection in all 3 vaccinated groups. The route of administration strongly affected the magnitude of the antibody response as measured by enzyme-linked immunosorbent assay (ELISA) and Western blots; however, the antigenic epitopes recognized were not substantially altered by delivery method as evidenced in immunoblot patterns. Given that the primary and booster vaccination protocols were identical, the data suggest that all 3 vaccinated groups may have had a specific humoral response following initial immunization but that specific serum antibody levels before boosting were too low to be detected by ELISA in fish vaccinated by oral and immersion routes. An anamnestic response was evident in all 3 groups. The data support the possibility that teleosts, like higher vertebrates, have a protective immune response to extracellular bacteria that is predominantly humoral. Route of delivery may primarily affect the efficiency with which the immunogenic constituents of the vaccine are presented to the relevant recognition and effector components of the immune system.

KEY WORDS: Vaccine · Route of delivery · *Vibrio anguillarum* · Humoral immune response · Memory

INTRODUCTION

Environmental concerns, regulatory constraints, costs, and pathogen resistance have greatly diminished the appeal of antibiotics and other chemotherapeutants in aquaculture. For these reasons, as well as the early successes of immunization against vibriosis and enteric redmouth disease (Busch 1978, Horne et al. 1984), vaccine development is increasingly viewed as a critical area of aquaculture research. New vaccines are needed to counter an expanding array of pathogens, and improvements must be made in delivery methods.

The route by which a vaccine is administered (i.e. parenteral, immersion, oral) appears to exert different effects on immunological parameters. Understanding the basis for these differences may not only improve vaccine efficacy, but may also provide valuable insight into the nature of the teleost immune system. To gain such an understanding, *in vitro* and *in vivo* methods need to be combined in carefully controlled model systems so that immunological responses evoked by various delivery routes can be correlated with clinical endpoints and measures of protection (e.g. survival following controlled laboratory challenge trials). In the present study, *in vitro* and *in vivo* methods were combined and used as tools to evaluate primary and secondary humoral responses to oral, immersion, and injection vaccination. A rainbow trout - *Vibrio anguillarum* model was utilized in order to take advantage of the extensive literature base that exists for this model, as well as to avoid difficulties that might arise with fastidious species or unusual immune responses.

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Teleost fish are known to be protected by an intricate and well integrated immune system that is functionally comparable to that of higher vertebrates (Gudkovs 1988). It is well established that humoral immunity in higher vertebrates is the principal protective specific immune response against extracellular bacteria (Abbas et al. 1991). For these reasons, enzyme-linked immunosorbent assay (ELISA) and Western blotting techniques were used for the *in vitro* portion of the study to measure specific humoral responses. The *in vivo* portions of the experiments consisted of laboratory bath challenges with the homologous pathogen and were designed to produce mortality rates that approximated those observed during typical epizootics.

In addition to establishing a potentially useful and robust experimental model for future vaccine and immunological research, the purpose of this study was to address 4 key questions. First, can levels of circulating specific antibody be positively correlated with protection in fish immunized by injection, immersion, and oral routes? Second, does route of vaccine administration affect the kinetics of the immune response, both before and after boosting? Third, can an anamnestic response be demonstrated utilizing only immersion or oral vaccination? Fourth, does the pattern of antigen recognition, as shown by Western blotting, fundamentally change depending upon the route of vaccine delivery?

METHODS

Experimental fish. Rainbow trout *Oncorhynchus mykiss* of the McLeary Steelhead strain (Troutlodge, Sumner, WA, USA) were obtained as eyed eggs and were grown to 10 g mean weight under specific pathogen-free (SPF) culture conditions. Treatment groups of 200 fish were randomly assigned to 65 l rectangular tanks receiving 2.5 l min^{-1} of 14 to 16°C dechlorinated city water. Except during periods of oral immunization or fasting, fish were fed daily with a commercial trout diet (Silver Cup) at the rate of 4% body weight d^{-1}.

Bacteria. *Vibrio anguillarum* 775 (serotype 1; obtained from Alpharma NW Inc., Bellevue, WA) was used throughout this study. This strain was chosen due to its minimal antigenic variability (Chart & Trust 1984) and the associated literature base. Strain 775 was isolated and described from coho (*Oncorhynchus kisutch*) and sockeye (*O. nerka*) salmon in Puget Sound, WA (Harrell et al. 1976). All bacterial cultures used for vaccine preparation, antigen preparation, and challenges were grown in 100 to 500 ml batches in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 20°C for approximately 24 h on a shaker platform at 200 rpm. Baffled flasks (Bellco, Inc., Vineland, NJ, USA) were inoculated with one or two 2 ml seed stock aliquots that had been stored at –80°C; final concentrations were approximately 10^9 colony forming units (CFU) ml^{-1}. Standard quality control measures included purity checks, duplicate plate counts at 10^{-7} and 10^{-8} dilutions, and, in the case of vaccines, sterility checks.

Immunization. The same whole cell, formalin-killed (0.3% added to culture before storage at 4°C) bacterin with broth was utilized for all immunizations. Each route of vaccine administration was standardized on the basis of wet pack cell mass. Primary and booster immunizations were carried out using the following identical protocols. Injection-vaccinated fish were first anaesthetized with a 10% ethyl *p*-aminobenzoate (benzocaine, Sigma, St. Louis, MO, USA) solution (10 g in 100 ml ethanol) diluted 1:2000 in water, and then given 0.2 ml (4 mg wet pack cell mass) intraperitoneally via an injection gun. Immersion-vaccinated fish were dipped for 40 s in a 1:4 dilution of the bacterin (4 mg wet pack cell mass ml^{-1}). The oral vaccine was incorporated into Oregon Test Diet (Sinnhuber et al. 1977) at the rate of 6 mg wet pack cell mass g^{-1} diet. This was fed at 4.5% body weight d^{-1} for 5 d, with 2 d of fasting before and after administration. For 5 d during the week preceding immunization, fish were acclimated to the diet without bacterin. All other treatment groups were also given the Oregon Test Diet during the weeks of acclimation and vaccination.

Bleeding and preparation of serum standards. Fish were bled via caudal vein puncture. Blood was immediately diluted 1:4 in 0.15 M NaCl in 1.5 ml polypropylene microcentrifuge tubes and allowed to coagulate and settle overnight at 4°C. The next day, blood samples were centrifuged for 20 min at $2000 \times g$ and serum was stored at –80°C. Thus titers are based upon dilutions of whole blood rather than the serum fraction; this procedure improves serum antibody recovery (Palm 1996). For the ELISA and Western blot analyses, positive and negative serum standards were used as controls for day-to-day variability. The positive standard was a high titer, anti-*Vibrio anguillarum* serum pooled from 90 injection-vaccinated SPF rainbow trout; the negative standard was a pooled serum drawn from 90 unvaccinated SPF rainbow trout.

Challenge protocol. To determine the optimal challenge concentration, an LD$_{50}$ bath challenge was conducted on 3 groups of control fish at 3 dilutions of a 10^9 CFU ml^{-1} culture (1:1000, 1:100, 1:10). Accurate challenge doses were calculated from plate counts of serial dilutions of each challenge culture. Five treatment groups were included in the experimental challenges: negative control (not vaccinated, not challenged), positive control (not vaccinated, challenged),

oral vaccinates, immersion vaccinates, and injection vaccinates. The final static bath challenges lasted 60 min and utilized duplicate 20-fish groups for each treatment. Challenges were conducted at 15°C with aeration; water volumes were adjusted to maintain a density of 100 g fish l^{-1} water. Each replicate group was then randomly assigned and placed in a 18.9 l aerated flow-through tank (37.8 l h^{-1}) and monitored for 14 d. Water temperature was maintained at 15°C and fish were not fed. Dead fish were removed daily and cause of death for each fish was confirmed by reisolation of bacteria from the kidney.

Antigen preparation. For ELISA and Western blot assays, *Vibrio anguillarum* 775 was grown overnight as previously described and centrifuged at 12 000 × *g* for 15 min. Cells were resuspended in phosphate-buffered saline (PBS), pH 7.4, and sonicated 3 times for 30 s with the 13.5 mm probe for the Vibra-Cell™ high intensity ultrasonic processor (Sonics & Materials, Inc., Danbury, CT, USA). Sonicated material was centrifuged at 3000 × *g* for 10 min to remove large cellular debris. The supernatant was drawn off, divided into 1 ml aliquots, and stored at −80°C.

ELISA. Antibody titers were determined by ELISA (Engvall & Perlmann 1972) using the titration method (Tijssen 1985). Immulon I microtiter plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with the *Vibrio anguillarum* antigen preparation in carbonate buffer (pH 9.6) overnight at 4°C at 10 µg protein well^{-1} as determined by the Bradford Method (Hammond & Kruger 1988). After removal of excess antigen, plates were blocked with 200 µl well^{-1} of goat diluting buffer (5 ml heat-inactivated goat serum, 95 ml PBS, 50 µl Tween 20) at 22°C for 15 min. Plate wells were washed 3 times (5.5% NaCl, 0.1% Tween 20), then incubated at 22°C for 60 min with 2-fold serial dilutions of fish sera in PBS (1:10 to 1:1280). Subsequently, the wells were washed 3 times, and incubated at 37°C for 15 min with a 1:320 dilution of the Warr 1-14 (DeLuca et al. 1983) mouse anti-trout monoclonal antibody tissue culture supernatant. Following 3 more washings, the plates were incubated at 37°C for 15 min with a 1:1000 dilution of a goat anti-mouse antibody conjugated to horseradish peroxidase (Kirkegaard and Perry, Gaithersburg, MD, USA). Finally, the 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry) was added, followed by an equal volume of TMB Stop Solution (Kirkegaard and Perry) 4 min later. Optical density (O.D.) of each well was determined with a microplate reader (Molecular Devices, Menlo Park, CA, USA) at 450 nm.

SDS-PAGE, Western blot, and scanning densitometry. *Vibrio anguillarum* 775 antigen (4.5 mg protein ml^{-1}) was diluted 1:1 in a modified Laemmli buffer (Laemmli 1970) containing 1 ml 0.5 M Tris-HCl (pH 6.8), 0.8 ml

glycerol, 1.6 ml 10% sodium dodecyl sulphate (SDS), 0.4 ml 2-b-mercaptoethanol, 0.8 ml 0.05% bromophenol blue, and 4 ml Type 1 (National Committee for Clinical Laboratory Standards) reagent grade water (ZyzaTech, Seattle, WA). The sample buffer was heated at 100°C for 5 min and then, along with prestained low range molecular weight standards (Bio-Rad, Hercules, CA), was separated (135 µg protein gel^{-1}) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel and a 12% separating gel in a Mini Protean II apparatus (BioRad) at 150 V for 55 min at 22°C. Gels were blotted overnight (30 V) onto nitrocellulose (0.45 µm pore size) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using a BioRad Mini Protean II transblot cell and Bio-Ice unit (Towbin et al. 1979). The nitrocellulose was cut into strips and blocked overnight in BLOTTO (Jagus & Pollard 1988). Strips were then incubated 2 h in either BLOTTO-diluted sample (1:20) or standard (1:40) trout sera. This was followed with a 1 h incubation in a goat anti-trout antibody conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:100 in BLOTTO. In between incubations, strips were washed 3 times in PBS-Tween 20 (0.1%); 1 additional PBS wash without detergent was done before addition of substrate. For color development, the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate (Kirkegaard and Perry) was added to blots for approximately 30 min. Strips were then rinsed in Type 1 reagent grade water and dried between sheets of Whatman filter paper. Bands were analyzed for molecular weights (or relative molecular weights in the case of nonprotein antigens) and quantitation of the antibody response using an imaging densitometer (BioRad model GS-670) and Molecular Analyst™ software (BioRad).

Statistical analysis. Due to low mortality in vaccinated groups, survival rates were compared using Fisher's Exact Test; probabilities of less than 1% were considered significant and reported as $p < 0.01$. When appropriate, standard errors were calculated for mean days to death (MDD). Individual ELISA titers are presented for each group (n = 5) to avoid masking key elements of the antibody kinetics; titers were calculated by the titration method (Tijssen 1985) and cut-off values were based upon the mean of 10 control samples in duplicate at each dilution plus 4 standard deviations. Molecular mass determinations for Western blot densitometry utilized a cubic regression analysis (Molecular Analyst™ software, version 2.1/Macintosh, BioRad) of low range prestained protein standards.

Experimental calendar. The schedule for immunizations and challenges, as well as corresponding fish weights, is displayed in Table 1. Primary immuniza-

tions were completed on Day 0. Sera were collected for the first ELISA on Day 3 and again every 7 d thereafter through Day 59. Sera for Western blots were collected on Days 19, 34, 48, and 55.

RESULTS

Protection conferred by vaccines before and after boosting

The *in vivo* immune response of rainbow trout to the *Vibrio anguillarum* vaccines was determined using bath challenge 28 d after completion of primary immunizations and 49 d after completion of booster immunizations (91 d after primary immunizations). The first challenge (Table 2) utilized a concentration of 4.5×10^6 CFU ml^{-1} and demonstrated equally strong protection in all 3 vaccinated groups. RPS (relative percent survival) values ranged from 88.2% for the orally vaccinated group to 100% for the injection-vaccinated group. The proportions of survivors in the 3 groups were not significantly different from each other

Table 1 Experimental schedule for immunizations and challenges, including approximate mean weights of rainbow trout

Day	Fish weight	Activity
–11 to –7		Acclimation to Oregon Test Diet (OTD)
–6 to –5		Fasting
–4 to 0		Oral vaccination with OTD
0	10 g	Immersion and injection vaccination
1 to 2		Fasting
28	20 g	Population sub-sampled and first challenge initiated
29 to 30		Fasting
31 to 35		Acclimation to OTD
36 to 37		Fasting
38 to 42		Oral booster vaccination with OTD
42	25 g	Immersion and injection booster
43 to 44		Fasting
91	55 g	Second challenge initiated

(Fisher's Exact Test, $p > 0.01$). The mortality rate of the positive controls (challenged, not vaccinated) was 42.5%, which corresponds well with the mortality rate of a typical *Vibrio anguillarum* epizootic (Evelyn & Ketcheson 1980). The MDDs for both the positive controls and the oral vaccinates were approximately 5 d, whereas the lone immersion-vaccinated mortality did not occur until 13 d post challenge.

Although the second challenge (Table 3) utilized a higher bacterial concentration (8.6 $\times$ 10^7 CFU ml^{-1}), equally strong protection was observed in all 3 vaccinated groups. No mortalities occurred in the immersion- or injection-vaccinated groups. The RPS of the oral vaccinates was 83.3%. The proportion of oral vaccinates which survived was not significantly different from those of the other 2 vaccinated groups ($p > 0.01$). The mortality rate of the positive controls was representative of a typical epizootic of this pathogen (45%). The MDD of the oral vaccinates (8.8 d) was greater than that of the positive controls (5.5 d).

Table 2. *Oncorhynchus mykiss*. Mortality and mean days to death (MDD) of rainbow trout challenged by 1 h immersion exposure to 4.5 $\times$ 10^6 waterborne *Vibrio anguillarum* cells ml^{-1} 28 d after primary vaccination. Fisher's Exact Test was used to measure the probability that each vaccinated group was different from the positive control. Relative percent survival (RPS) = 1 – (% vaccinate mortality/% positive control mortality) $\times$ 100%. Standard errors of the MDD given in parentheses

Fish group	Mortality (%)	Fisher's Exact Test	RPS (%)	MDD
Negative control	0	–	–	–
Positive control	42.5	–	–	5.1 (0.26)
Oral vaccine	5.0	p < 0.01	88.2	4.5 (0.50)
Immersion vaccine	2.5	p < 0.01	94.1	13.0
Injection vaccine	0	p < 0.01	100.0	–

Table 3. *Oncorhynchus mykiss*. Mortality and mean days to death (MDD) of rainbow trout challenged by 1 h immersion exposure to 8.6 $\times$ 10^7 waterborne *Vibrio anguillarum* cells ml^{-1} 91 d after primary vaccination and 49 d after booster vaccination. Fisher's Exact Test was used to measure the probability that each vaccinated group was different from the positive control. Relative percent survival (RPS) = 1 – (% vaccinate mortality/% positive control mortality) $\times$ 100%. Standard errors of the MDD given in parentheses

Fish group	Mortality (%)	Fisher's Exact Test	RPS (%)	MDD
Negative control	0	–	–	–
Positive control	45.0	–	–	5.5 (0.41)
Oral vaccine	7.5	p < 0.01	83.3	8.8 (2.67)
Immersion vaccine	0	p < 0.01	100.0	–
Injection vaccine	0	p < 0.01	100.0	–

Kinetics of the specific humoral response

Before boosting on Day 42, the oral vaccinates showed almost no serum antibody response as measured by ELISA (Fig. 1). Only 1 fish had detectable antibodies, a titer

Fig. 1 *Oncorhynchus mykiss.* Kinetics of the serum antibody response of rainbow trout following primary and booster vaccinations via the oral route of delivery. Booster doses were completed 42 d post initial vaccination. Bars represent titers of individual fish determined by ELISA, 5 fish being sampled each week
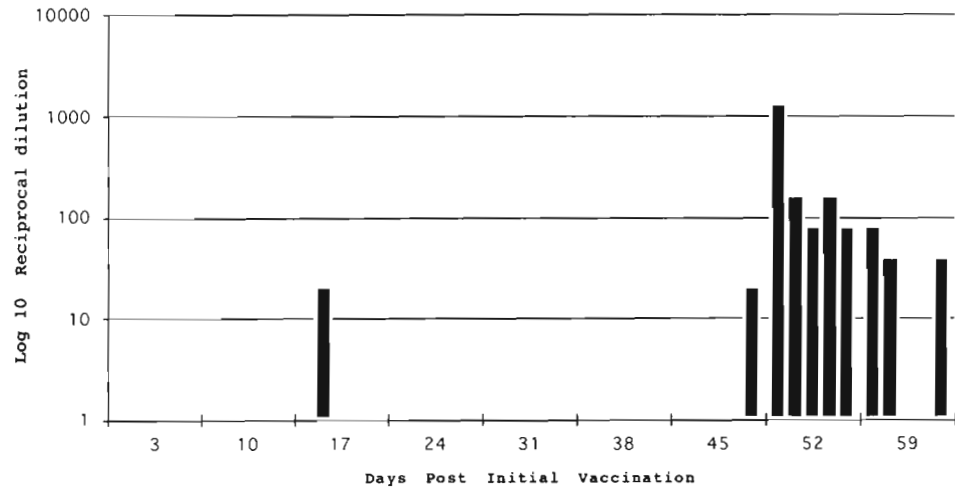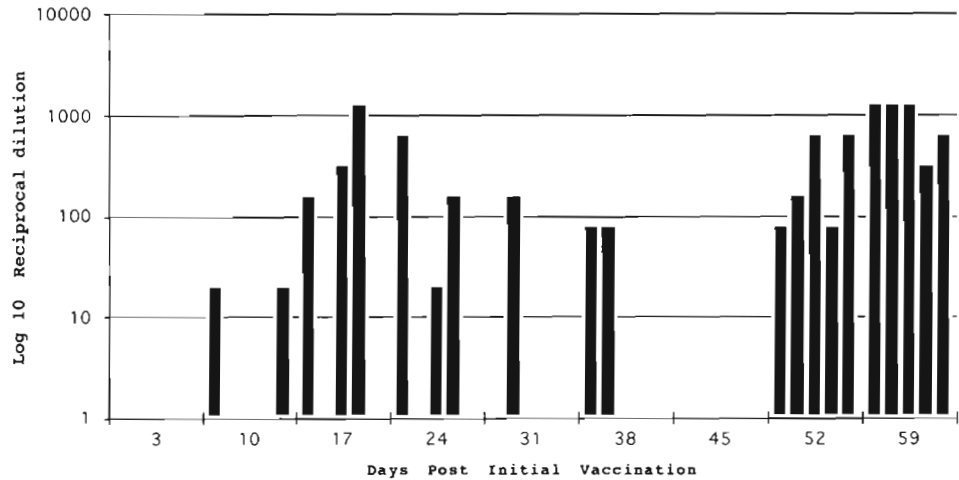
Fig. 2. *Oncorhynchus mykiss.* Kinetics of the serum antibody response of rainbow trout following primary and booster vaccinations via the immersion route of delivery. Booster doses were given 42 d post initial vaccination. Bars represent titers of individual fish determined by ELISA, 5 fish being sampled each week

of 20 on Day 17. After boosting, substantial titers were noted with a peak occurring 10 d after boosting (Day 52) when all 5 fish had detectable levels of antibody (titers ranging from 80 to 1280+).

Before boosting, antibody was detected in 37% (11/30) of the immersion vaccinates (Fig. 2) with titers ranging from 20 to 1280+. Antibody was first detected 10 d after primary vaccination. Three days after boosting (Day 45), no specific antibody was detected in any of the immersion vaccinates sampled. However, all fish sampled 10 and 17 d post booster vaccination had measurable specific antibody levels, with titers from 80 to 1280+.

Before boosting, all injection vaccinates sampled after Day 10 exhibited measurable ELISA titers (Fig. 3). One fish had a titer of 20 on Day 10; by Day 17, all fish had measurable titers. Antibody titers dropped on Day 45, 3 d after the second set of vaccinations, and 1 of the fish sampled had no measurable antibody. Ten and 17 d after boosting, all but 1 fish had titers of 1280 or higher.

Antigenic analysis of vaccinated groups

SDS-PAGE and Western blot assays, coupled with scanning densitometry analysis, revealed the nature and pattern of the antibody response among treatment groups. Information to be derived from such assays includes identification of the antigens (or molecular weight groups of antigens) to which the fish were responding and the relative strength of the response to those antigens or groups of antigens. Pooled sera from 90 unvaccinated and 90 injection-vaccinated fish were used as negative and positive standards, respectively (Fig. 4). The positive standard serum reacted strongly with a group of antigens (probably at least 5) in the 30 to 45 kDa range.

The 4 rainbow trout treatment groups (control, oral vaccinates, immersion vaccinates, injection vaccinates) were sampled on experimental Days 19, 34, 48, and 55 for Western blot analysis. Day 19 blots incubated with vaccinated sera did not show any response beyond that of the control groups. Day 34 blots exhibited a moder-
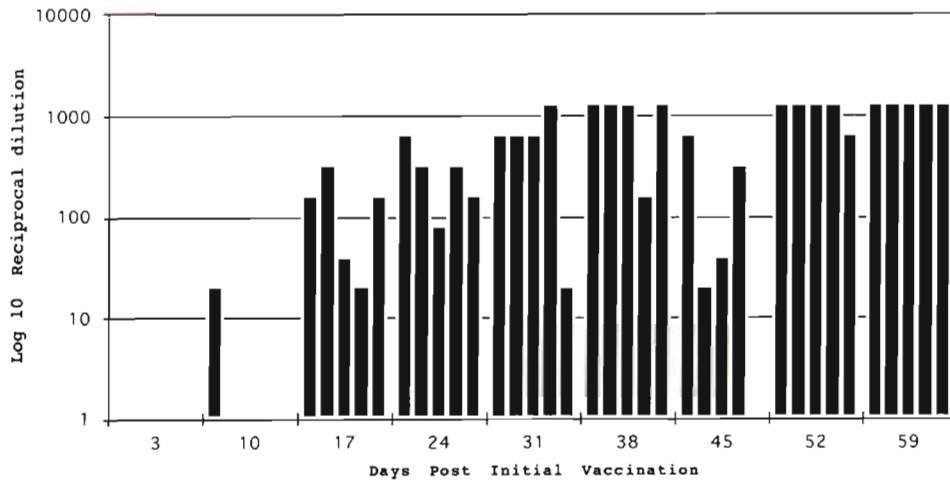
Fig. 3. *Oncorhynchus mykiss.* Kinetics of the serum antibody response of rainbow trout following primary and booster vaccinations via the injection route of delivery. Booster doses were given 42 d post initial vaccination. Bars represent titers of individual fish determined by ELISA, 5 fish being sampled each week

ate response with injection-vaccinated sera producing peaks between 30 and 40 kDa and with immersion-vaccinated sera producing peaks between 30 and 35 kDa. Strong responses were observed on Day 55 with both injection- and immersion-vaccinated sera (between 28 and 44 kDa). Clearest differences among the treatment groups, however, were detectable on Day 48 and only these scanning densitometer tracings are shown (Figs. 4 to 6).

The densitometric tracing of the control serum (not shown) showed almost no response in the 20 to 140 kDa target area, with no bands distinguishable from those of the negative standard tracing. The serum tracing for oral vaccinates (not shown) also did not exhibit any clear bands that were distinguishable from those of the

negative standard or control tracing. Several distinct bands appeared in the 30 to 40 kDa area on the serum tracing of immersion-vaccinated fish (Fig. 5), as well as some response to a couple of high molecular weight bands. Finally, the densitometer tracing of injected fish (Fig. 6) showed a strong response to several bands in a narrow range between 28 and 44 kDa.

DISCUSSION

The use of immunization to successfully protect fish against vibriosis was first reported in 1964 (Hayashi et al. 1964). In that study, the route of administration utilized was intraperitoneal injection. Since that time,
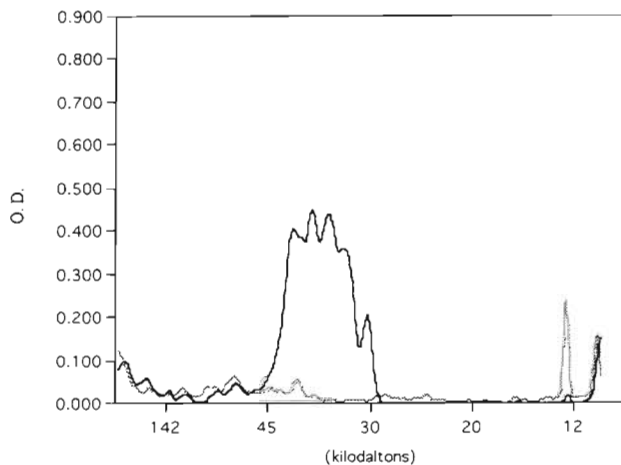

Fig. 4. Scanning densitometer analysis (reflectance mode) of a Western blot of positive standard rainbow trout serum (bold line) reacting to a sonicated *Vibrio anguillarum* whole cell lysate. Positive standard was a collection of pooled sera from 90 rainbow trout vaccinated and boosted by injection (0.2 cc intraperitoneally) with a formalin-killed whole cell *V. anguillarum* bacterin with broth. Negative standard tracing is shown for comparison. O.D.: optical density
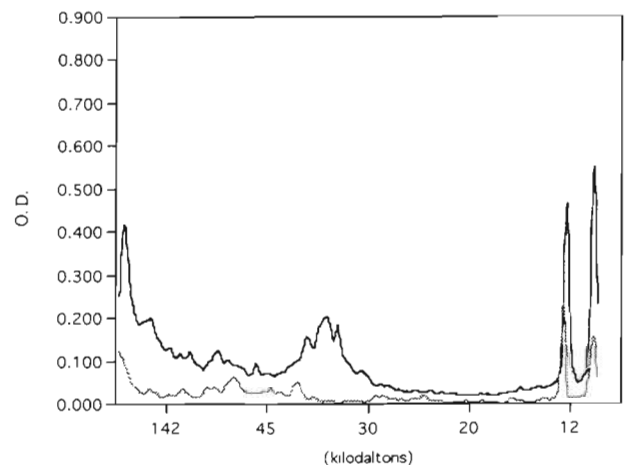

Fig. 5. Scanning densitometer analysis (reflectance mode) of a Western blot of immersion-vaccinated rainbow trout serum (bold line) reacting to a sonicated *Vibrio anguillarum* whole cell lysate. Serum was collected and pooled from 2 rainbow trout 48 d after immersion primary vaccination and 6 d after immersion booster vaccination. Negative standard tracing is shown for comparison. O.D.: optical density
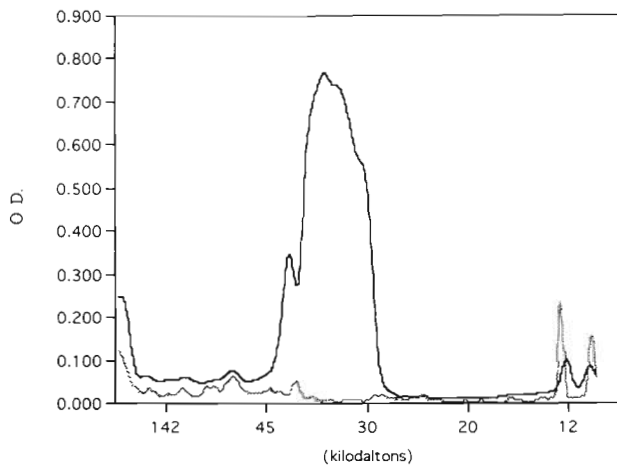
Fig. 6. Scanning densitometer analysis (reflectance mode) of a Western blot of injection-vaccinated rainbow trout serum (bold line) reacting to a sonicated *Vibrio anguillarum* whole cell lysate. Serum was collected and pooled from 2 rainbow trout 48 d after injection primary vaccination and 6 d after injection booster vaccination. Negative standard tracing is shown for comparison. O.D.: optical density

injection vaccination has been shown to provide strong protection, not only against vibriosis, but also against a number of other extracellular bacteria that are pathogenic to fish, including *Yersinia ruckeri* (Ellis 1988b) and *Aeromonas salmonicida* (Hastings 1988). Immersion vaccination was first described by Amend and others for protection against enteric redmouth disease (ERM) and vibriosis (Amend & Fender 1976, Antipa & Amend 1977, Croy & Amend 1977, Busch 1978, Antipa et al. 1980, Amend et al. 1983). Immersion delivery is still used extensively today to combat these diseases. Most investigators consider immersion immunization to provide a level of protection that is less than that conferred by injection but stronger and more consistent than that provided by oral immunization. While oral immunization has not been highly regarded, one should remember that the first report of protection of fish against extracellular bacteria (Duff 1942) involved oral delivery of chloroform-killed *A. salmonicida* cells to cutthroat trout. The first successful test of an ERM vaccine also utilized oral immunization (Ross & Klontz 1965). Despite these early successes and the substantial practical advantages afforded by oral administration, oral vaccines for fish are still largely experimental because of poor or inconsistent protection (Quentel & Vigneulle 1997).

In contrast to most previous reports on fish vaccine efficacy (Lamers 1985, Ellis 1988a, Nakanishi & Ototake 1997, Quentel & Vigneulle 1997), the data presented here indicate that the degree of protection conferred against waterborne challenges of *Vibrio anguillarum* 775 was equal for injection-, immersion-, and orally vaccinated treatment groups with and with-

out boosting (Tables 2 & 3). Lillehaug (1989) achieved similar levels of protection with these routes of vaccine delivery following bath and cohabitation challenges with *V. anguillarum*; however, the oral vaccines were delivered by intubation rather than feeding and 2 *V. anguillarum* serotypes were used in each challenge method. Additionally, the challenge conditions were different from the present study; in particular, fish were challenged 40 d after vaccination (rather than 28 d) and, on the day of the challenge, fish were moved from one facility with 12°C freshwater to another facility with 15°C saltwater (25‰). No information was provided on fish density or challenge culture concentration. The approximate antigenic doses administered orally per fish were 30 mg dry weight of vaccine in the Lillehaug (1989) study versus 13.5 mg wet pack cells in our study. Johnson & Amend (1983) used oral and anal intubation to investigate the efficacy of a commercial *V. anguillarum* bacterin administered to 5 g sockeye salmon. In general, fish vaccinated by anal intubation were better protected than fish vaccinated by oral intubation. In challenges conducted 28 d after primary vaccination, the 2 oral intubation treatment groups in the Johnson & Amend (1983) study were less well protected than the oral vaccinates in our study (35 and 69% versus 5% mortality). In both of these studies, unvaccinated controls experienced similar or lower mortalities during later (Days 59 or 91) versus earlier (Day 28) challenges despite higher challenge levels at the later dates (Tables 2 & 3). These results may reflect stronger pathogen resistance in larger fish. Immunological studies of higher vertebrates have established that circulating antibody is the principal protective immune response against extracellular bacteria (Abbas et al. 1991). For example, human patients with pure B-cell defects are not able to control infections caused by extracellular bacteria (Janeway & Travers 1994). In the fisheries literature, there appears to be confusion regarding the importance of humoral factors. This confusion reflects the inability of investigators to consistently correlate protection with levels of circulating specific antibody.

With *Vibrio anguillarum*, the protective role of antibody has been demonstrated following injection vaccination (Thuvander et al. 1987) and passive immunization (Harrell et al. 1975). However, the correlation has not always been clear after immersion vaccination, and most studies on oral vaccination against vibriosis have shown no correlation (Smith 1988, Quentel & Vigneulle 1997). Thuvander et al. (1987) concluded that the importance of low levels of antibodies is unclear, but that the presence of specific antibodies in a proportion of the fish after vaccination seems to indicate that the whole population is protected. This appears to be the case in the current study, where low ELISA

titers in immersion vaccinates before boosting (Fig. 2) were coupled with a high level of protection in the first challenge (Table 2). Bøgwald et al. (1994) found moderate to high levels of specific circulating antibody after intraperitoneal immunization with formalin-killed, washed whole cells of *V. salmonicida*, *V. anguillarum* (serotype 01), *V. anguillarum* (serotype 02), and *Aeromonas salmonicida*. However, in the same study, no specific antibodies could be demonstrated after oral administration of the same vaccines. In our study, essentially the same results occurred. Little antibody activity was evident in oral vaccinates before boosting but protection was high. Consistent specific serum antibody titers appeared only after boosting (Fig. 1).

The kinetics of the antibody responses in the orally, immersion-, and injection-vaccinated groups before boosting (Figs. 1 to 3) were similar to the results of some previous studies (Smith 1988, Nakanishi & Ototake 1997, Quentel & Vigneulle 1997). Injection titers were high and consistent starting on Day 17; immersion titers were sporadic with 63% of the fish having no detectable levels of antibody; oral titers were almost nonexistent. The dramatic differences between this work and previous work centered on the antibody titers of orally and immersion-vaccinated fish after boosting. Surprisingly strong and consistent antibody titers were measured in all immersion-vaccinated fish sampled 10 and 17 d after boosting (Fig. 2). Eight out of 10 orally vaccinated fish showed moderate to strong titers at the same sampling points (Fig. 1). The titers of the oral vaccinates appear to have peaked about 10 d after boosting while the titers of immersion vaccinates peaked 17 d after boosting or later, as did those of the injection vaccinates. The experimental design did not permit analysis of antibody titers beyond Day 59.

The presence of memory lymphocytes is normally determined by monitoring the secondary response (Lamers 1985). In the present study, an anamnestic response was clearly demonstrated in all 3 vaccinated groups (Figs. 1 to 3) as indicated by the speed, strength, and consistency of the secondary response when compared to the primary response. Before 1985, results of investigations into the piscine anamnestic response were contradictory (Dunier 1985). Since that time, the existence of memory in fish has become generally accepted with the acknowledgment that it can be affected by many factors (fish age, antigenic dose, route of administration, adjuvants, temperature, etc.) and that it differs from immunological memory in mammals. Arkoosh & Kaattari (1991) postulated that memory responses in rainbow trout may be due to a simple expansion of the antigen specific precursor pool without many of the qualitative changes in antibody or B-cell function that are associated with memory in mammals. To our knowledge, Figs. 1 & 2 are the first

demonstration of humoral anamnestic responses in salmonids following oral and immersion vaccinations in which primary and booster immunization protocols were identical. These results also suggest that circulating antibody responses may have been elicited in most or all of the fish after primary oral and immersion immunizations even though the level of antibodies was generally too low to be detected by the ELISA protocol. This failure to measure antibody may explain much of the confusion among previous investigators, particularly with respect to oral immunization. The results presented here do not rule out the possibility that localized or mucosal antibody responses may also contribute to protection, nor can one assume that specific cellular immunity is not involved as well.

The pattern of antibody responses seen in Western blots did not differ significantly among the positive standards, immersion vaccinates, and injection vaccinates (Figs. 4 to 6). However, the magnitude of the responses among these groups did differ. The antigens to which the fish in these treatment groups responded appear to be mainly antigenic outer membrane proteins identified in previous studies (Chart & Trust 1984, Norqvist et al. 1989). This observation, together with the fact that no clear responses were observed in the Day 19 blots, may indicate that our Western blot protocol primarily detected antibody responses to protein antigens, while the ELISA may have detected specific antibody to both lipopolysaccharide (LPS) and protein antigens. This could also explain why the oral and immersion vaccinates had very similar ELISA titers on Day 52, but only the immersion vaccinates had a significant Western blot response on Day 48 (Figs. 1, 2 & 5). Protein antigens in the oral vaccine may have been substantially more degraded in the stomach compared to the LPS antigens. Other investigators have found a similar discrepancy between high ELISA titers and weak immunoblot responses with both human (Wethered et al. 1988) and salmon antisera (Wood & Kaattari 1996). The observed difference was tentatively attributed to recognition of carbohydrate antigens in ELISA assays that were not adequately detected by immunoblots. Bøgwald et al. (1991) did show some reaction of salmon antisera to *Vibrio anguillarum* serotype I LPS antigen in a Western blot. However, almost no bands appeared within the range of the molecular weight standards in the *V. anguillarum* serotype II and *V. salmonicida* LPS preparations, while numerous bands were present in the outer membrane preparations. Alternatively, it is possible that total specific antibody levels may have been insufficient to detect many of the key antigens by the Western blot protocol developed for this study. Nevertheless, since LPS typically does not induce memory B-cells in higher vertebrates (Abbas et al. 1991), fish

vaccine development should probably focus on protein antigens in order to achieve suitable duration of protection.

A by-product of this project was further refinement of the rainbow trout - *Vibrio anguillarum* model system. Gould et al. (1978) concluded that *V. anguillarum* serotype I was an excellent experimental model for testing methods of fish immunization. Their conclusion was based on the antigenicity of bacterins prepared from this organism and the existence of reproducible laboratory challenges that simulate natural exposure. Nelson et al. (1985) agreed and mentioned the additional advantage that vibriosis can be controlled by oral, immersion, and injection routes of vaccine delivery. When this pathogen is combined with rainbow trout, which has been referred to as the aquatic 'white mouse', an extremely versatile model system is created for both applied and basic immunological research. Progress in fish immunology has been hampered to date by the wide variety of pathogens and hosts that have been used.

In summary, injection, immersion, and oral vaccination conferred equal protection to controlled laboratory challenges with *Vibrio anguillarum*. Protection correlated positively with serum antibody levels after, but not prior to, boosting. Correlation before boosting may be possible if extremely sensitive detection methods are developed. The route of vaccine delivery primarily affected the magnitude and kinetics of the antibody response and did not appear to play a significant role in altering the pattern of the antibody response (as shown by Western blotting). An anamnestic response was clearly demonstrated following oral, immersion, and injection vaccination, using identical protocols for primary and booster immunizations. Finally, a rainbow trout - *Vibrio anguillarum* 775 model, incorporating 3 routes of vaccine delivery, can be a valuable tool both for research on fish vaccine development and for increasing our understanding of underlying immune mechanisms.

Additional research is needed to confirm and expand upon the results presented here. In particular, work should be done to clarify the roles played by serum antibody, localized antibody, and the specific cellular response. The effects of route of vaccine administration on the response to specific antigens also deserves more attention.

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