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Immunological aspects of amoebic gill disease in salmonids

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ABSTRACT: *Paramoeba* sp. antigens emulsified with Freund's complete and incomplete adjuvants were immunogenic in rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* by intraperitoneal (i.p.) injection as well as for sheep and rabbit by subcutaneous and intravenous injections respectively. Atlantic salmon were immunized passively with an i.p. injection (0.1 ml 100 g^{-1} fish body weight) of sheep anti-*Paramoeba* sp. antibodies (APA). Sheep APA were detected in fish sera by enzyme-linked immunosorbent assay for up to 8 wk after i.p. injection. Assessments of passive and active immunization were undertaken concurrently in order to demonstrate their relative efficacies and especially to evaluate the practical potential of passive immunization. Immunized fish were exposed to natural infection by cohabitation with infected Atlantic salmon 45 d post-immunization. Transmission of the disease was successful; however, unequivocal protection was not demonstrated in any of the immunized fish suggesting a minor role for systemic antibodies in protection against amoebic gill disease.

KEY WORDS: *Paramoeba* sp. · Atlantic salmon · Immunization · Vaccine · Passive immunity

INTRODUCTION

Amoebic gill disease (AGD) is an important infectious disease which constrains salmonid mariculture in Australia (Munday et al. 1990). The most prominent features of the disease are excessive mucus on the gills macroscopically, epithelial hyperplasia, fusion of the secondary lamellae and the presence of variable numbers of amoebae *Paramoeba* sp. histologically (Munday et al. 1990). AGD was first diagnosed in sea farmed salmonids on the east coast of Tasmania in summer of 1984-85 (Munday 1985). During this period, mortalities of up to 10% per week occurred in Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* (Foster & Percival 1988a). There can be up to 3 outbreaks of AGD infection during the months of elevated water temperature when there is an absence of freshwater flushing from river outlets.

In Tasmania, disease associated with amoebae usually occurs in the first summer of sea cage farming of salmonids following their transfer from the freshwater hatchery (Langdon 1990). The amoebae become apparent in low numbers on the gills 2 mo after the introduction of fish to the sea (Foster & Percival 1988a). Increased intensity of infection and mortality occurs with the stress of elevated water temperatures (>15°C) and factors such as poor hygiene, crowding, and decreased water exchange through biofouling on nets (Langdon 1990, Munday et al. 1990). However, even fish stocked at low densities may succumb to the disease (Foster & Percival 1988b).

AGD has also been reported in coho salmon *Oncorhynchus kisutch* in Washington State and California, USA (Kent et al. 1988), chinook salmon in New Zealand (Howard & Carson 1993a, C. Anderson pers. comm.) and Atlantic salmon, rainbow and brown trout in France (B. L. Munday unpubl.) farmed in a marine environment.

Control of AGD currently is restricted to treatment with freshwater (by towing sea cages to freshwater)

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which is difficult and expensive. Therefore, immunoprophylaxis is attractive as an alternative.

Observations have suggested that previously exposed salmonids develop immunity to AGD (Munday et al. 1990). The possibility of an immune response to *Paramoeba* sp. (PA) in recovered fish has been suggested based on both epidemiological and histological features of the disease (Munday et al. 1990). Moreover, Bryant et al. (1995) detected antibodies in rainbow trout against numerous sonicated amoeba (but not the *Paramoeba* sp. causing AGD) antigen by both enzyme-linked immunoadsorbent assay (ELISA) and immunoblotting, confirming that amoebic components can be immunogenic in trout.

The aims of the present study were firstly, to assess the humoral responses of fish, rabbit and sheep to AGD antigens; secondly, to determine the longevity of passively transferred anti-*Paramoeba* sp. antibodies (APA) in fish; thirdly, to evaluate the degree of protective immunity in fish immunized actively or passively; and finally, to ascertain if fish develop circulating antibodies to *Paramoeba* sp. as a result of natural AGD.

MATERIALS AND METHODS

***Paramoeba* sp.** *Paramoeba* sp. (PA-016) was obtained from the Fish Health Unit, Mt. Pleasant Laboratories, Department of Primary Industry and Fisheries, Launceston, Tasmania.

***Paramoeba* culture.** *Paramoeba* sp. was cultured according to the method described by Howard & Carson (1993b). Briefly, a medium of malt and yeast extract seawater agar, comprising 750 ml 0.2 µm filtered seawater, 250 ml distilled water, 0.1 g malt extract, 0.1 g yeast extract and 30 g agar (Oxoid No. 1), was prepared and steam autoclaved at 121°C for 20 min. When the agar had cooled to 60°C, 1 ml of 1% pimaricin (Sigma) as antifungal agent was added. The agar was then poured into 23 cm^2 bioassay dishes (Nunc, Denmark).

Plates were left to solidify overnight at room temperature (RT), and were seeded with 2 ml of a *Pseudomonas maltophilia* suspension ($\sim 1.5 \times 10^8$ cells ml^{-1}) in 0.2 µm filtered autoclaved seawater. The plates were inoculated with 5 ml of amoeba suspension (~800 amoebae cells), extracted from a 2 to 6 wk old established culture, 24 h after the addition of bacteria.

Amoeba culture plates were sealed with cellophane tape to prevent evaporation and were maintained in an incubator at 20°C. Subculturing was conducted every 4 to 6 wk.

***Pseudomonas maltophilia* culture.** Pure cultures of *P. maltophilia* obtained from Mt. Pleasant Laboratories were grown in Oxoid nutrient broth No. 2 at 37°C. *P. maltophilia* was recovered from the broth after 48 h incubation by centrifugation. The cells were subsequently washed with phosphate-buffered saline (PBS), aliquoted and stored at 4°C. Fresh cultures of this bacterium were maintained throughout the experimental period by regular subculturing on sheep blood agar plates.

Preparation of amoebic antigen. In order to produce antisera in sheep and a rabbit and immunize fish against *Paramoeba* sp., the following protocol was established. Live amoeba from 3 plates were harvested with 3 ml of 0.2 µm filtered seawater per plate using bent pasteur pipette 'hockey sticks'. The suspension was washed 3 times in 4 to 5 volumes of sterile, filtered seawater by centrifugation for 10 min at 500 × *g*. The supernatant, which contained most of the bacteria, was discarded each time and the pellet was resuspended in the sterile seawater by vortexing. The efficiency of bacterial removal was tested by direct microscopic examination of a wet smear at 400× magnification using phase contrast and by Gram stain. Even so, up to 4 or 5 bacteria per high power field surrounded the *Paramoeba* sp. and some occurred within the amoeba. A dense suspension of washed amoeba were then frozen and thawed several times and sonicated for 3 min. A 3 ml suspension was further inactivated using 0.5% formalin, left at 4°C, and finally 50% v v^{-1} Freund's complete adjuvant (FCA) was added and the solution homogenised. The protein content of the sonicated antigen was determined using a Lancer Microprotein rapid stat diagnostic kit (Oxford Labware, St. Louis, MO, USA).

Preparation of crude amoebic antigen from diseased fish. Mucus from the gills of 40 severely infected Atlantic salmon was collected by scraping the gills. Normal saline was added to the suspension and homogenised. The suspension was filtered through glass wool and inactivated with 0.5% v v^{-1} formalin overnight. This crude vaccine was tested for sterility by culturing on blood agar, homogenised with 50% v v^{-1} FCA and kept frozen (−20°C) until used.

Preparation of bacterial antigen. Soluble antigen was prepared by washing, resuspending in PBS and sonicating *Pseudomonas maltophilia* cells by 3 × 3 min cycles. Bacterial sonicate was filtered (0.2 µm) and its protein concentration was determined prior to storage at −20°C for further use.

Production of antisera. Two sheep were injected subcutaneously in the cervical area with a total of 3 ml of antigen (1.5 ml PA solution containing approximately 1 mg protein plus 1.5 ml FCA per dose per sheep). Four weeks later, sheep were boosted with the same amount of antigen, but in 50% v v^{-1} Freund's incomplete adjuvant. After 6 wk a large volume of blood was collected and the antiserum was separated

and frozen at –20°C until used. The sheep antibody response to PA was later tested by ELISA.

Rabbit antiserum was prepared as described by Howard & Carson (1991) with slight modification. Briefly, sonicated antigen obtained from 300000 to 3000000 *Paramoeba* sp. was injected into the marginal ear vein of a New Zealand white rabbit by several injections (total inoculum: 1 mg protein) at 3 d intervals for 3 wk. Antiserum was collected 21 d post-inoculation by complete exsanguination of the rabbit. Rabbit antiserum was tested for antibody response to PA by ELISA and stored at –20°C for later use.

Serum adsorption. *Paramoeba* sp. injected into the sheep, rabbit and fish had been feeding on *Pseudomonas maltophilia*; therefore it was likely that bacterial contamination existed upon sonication. To ensure that results were not perturbed due to the presence of anti-*Pseudomonas* antibodies, the serum was adsorbed prior to conducting an ELISA. Diluted sheep anti-*Paramoeba* sera in PBS (1:100) was adsorbed with PBS-washed and sonicated *P. maltophilia* (final concentration 10 mg protein ml^{-1} sera) for 1.5 h at room temperature and gently mixed intermittently. Also, washed live *P. maltophilia* cells were diluted to a concentration equivalent to the McFarland standard number 2 to 4 and added to the tubes already containing serum and sonicated *P. maltophilia*. These tubes incubated at room temperature for an additional 1 h. The solution was centrifuged at 1000 × *g* for 10 min and the supernatant collected. The efficacy of adsorption was determined by ELISA using several different dilutions of antigen and antibody.

ELISA. The ELISA used for detecting antibodies against *Paramoeba* sp. was a modification of the ELISA described by Bryant et al. (1995). Briefly, soluble antigen was coated to plates (Linbro Cat no. 76:381:04, ICN Flow) ranging in concentration from 15 to 25 µg protein ml^{-1} in borate coating buffer. Coated plates were incubated at 4°C for 16 to 24 h. Antigen was then flicked off and wells were blocked by 1% gelatin for 30 min at RT. Plates were washed 5 times with distilled water plus Tween 20 (DWT). The adsorbed sera were added to wells and plates were incubated for 90 min. In the sheep and rabbit ELISA, plates were washed and rabbit anti-sheep (KPL®, Kirkegaard Perry Laboratories) or swine anti-rabbit (Dako Patts®) immunoglobulin conjugated to horse radish peroxidase (HRP) at 1:2000 dilution in 1% w v^{-1} gelatin (Oxoid) in PBS+ 0.05% Tween 20 were added. After 90 min incubation and washing with DWT, 2,2'azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) chromogen in 100 mM citrate phosphate pH 4.2, 2.5 mM hydrogen peroxide, was added. The reaction was stopped after 20 min at RT by the addition of 0.01% sodium azide in 0.1 M citric acid and optical density (O.D.) was measured at 405 nm. In the fish ELISA, mouse monoclonal anti-rainbow trout IgM antibody (mAb I-14) (DeLuca et al. 1983) was added after washing the sera and the plate was incubated for 90 min at RT. After washing in DWT, rabbit anti-mouse immunoglobulin conjugated to HRP (Dako®) was added and the assay continued as above. In order to ascertain the reactivity of specific antibody to *Paramoeba* sp., non-adsorbed control sera were also used. In addition, one plate in each assay was coated with sonicated *Paramoeba* sp. while another plate was coated with sonicated *Pseudomonas maltophilia*. A positive *Paramoeba* ELISA result was any with an O.D. greater than the mean O.D. + 3 SD of 50 sera from naive fish.

Trial 1 — rainbow trout humoral responses to PA antigens and rate of elimination of antisera from passively immunised Atlantic salmon. (1) In order to study the effect of the sonicated antigen (prepared from cultured *Paramoeba* sp.) in FCA on the humoral response of rainbow trout, 25 fish (mean weight ± SD, 127 ± 11 g) from the Aquaculture Key Centre (AKC) were immunized with 1 mg (protein concentration) of the sonicated antigen.

(2) In order to study the effect of the crude antigen in FCA on the humoral responses of Atlantic salmon, 15 fish (62 ± 8.2 g) from AKC were used. Fifteen Atlantic salmon were injected with 0.5 ml of the crude antigen in FCA (wild *Paramoeba* sp.) in order to monitor their humoral response to this antigen. Fish were kept in temperature controlled (15°C) tanks and bled 6 wk post-immunization (p.i.).

(3) Because of the high level of APA obtained in sheep, sheep sera were used for passive immunization of fish. For determination of the rate of clearance of sheep anti-*Paramoeba* sp. antibodies (APA) from fish sera, 100 Atlantic salmon, 50 fish from AKC (62 ± 8.2 g) and 50 fish (57 ± 7.0 g) from Salmon Enterprises of Tasmania (SALTAS) were used. Fish were tagged individually with plastic T-bar anchor tags (Hallprint Pty Ltd, South Australia) placed in the flesh just below the base of the dorsal fin for individual identification. Thirty fish (15 from SALTAS and 15 from AKC) for Replicate 1 in one tank and 30 fish (15 from SALTAS and 15 from AKC) for Replicate 2 were allocated for this group. Control group consisted of 20 fish from SALTAS and 20 fish from AKC.

Sheep APA antiserum was used for intrapertoneal (i.p.) injection of fish. Fish were passively immunized (1 ml syringes, 25 G × 19 mm needles) with the hyperimmune serum at a rate of 0.1 ml 100 g^{-1} fish body weight. Control fish were injected with 0.1 ml PBS. The rate of elimination of sheep APA in fish body was monitored at 1, 4 and 8 wk p.i.

(4) Control fish: Blood samples were collected from the caudal vessels of 40 Atlantic salmon each of

SALTAS and AKC origin prior to the commencement of the experiment. Fish were tagged, injected with PBS and kept in the tanks. Blood samples were collected from 20 fish at each interval. Sera were harvested and used as controls in the ELISA.

Trial 2 — evaluation of protection by horizontal infection challenge. ***Trial 2.1:*** Atlantic salmon, comprising 320 Atlantic salmon 'smolt' (brought from Wayatinah, SALTAS freshwater operation) (57 ± 7.0 g) and 320 'smolt' from the AKC (totally 640) (62 ± 8.2 g, the same stock of fish used for Trial 1) were used for evaluation of protection following passive and active immunization. Forty fish (20 from SALTAS and 20 from AKC) for Replicate 1 in one tank and 40 fish (20 from SALTAS and 20 from AKC) for Replicate 2 (total number of fish for any one treatment was 80) were allocated for each group.

Fish were randomly divided in 2 groups. All were weighed and individually tagged before immunization. All fish were starved 12 h before commencement of the experiment, then anaesthetised before tagging and/or any treatment.

Intraperitoneal injections were carried out as follows:

(1) Control group: Fish were injected i.p. with 0.1 PBS.

(2) Passive immunization with sheep and rabbit APA: Fish were injected with the immune serum at a rate of 0.1 ml per 100 g fish body weight.

(3) Live *Paramoeba* sp.: A pellet of *Paramoeba* sp. was resuspended in the solution of 1% carbenicillin disodium salt (Sigma No. C-1389) in sterile seawater by vortexing gently and left to stand for 2 h. The suspension was centrifuged (500 × *g*) and the pellet was resuspended in sterile seawater. *Paramoeba* sp. densities were determined by counting cells using a Neubauer haemocytometer, adjusted and suspended in 0.2 µm filtered seawater so that 3800 amoebae in 0.1 ml of solution were injected intraperitoneally to each fish.

(4) Sonicated *Paramoeba* sp. with adjuvants: A cell count of washed *Paramoeba* sp. was performed then an ultrasonic machine was used to disrupt the cell membrane. The suspension was filtered through an 0.2 µm filter and its protein content was determined. Different protein contents were prepared containing 1 mg and 10 mg with and without 50% v v^{-1} FCA. This solution was administered according to the experimental design by i.p. injection of 0.1 ml into each fish.

Fish were exposed to fish infected with *Paramoeba* sp. 30 d post-immunization.

Trial 2.2: Forty Atlantic salmon were injected i.p. with the crude antigen in the freshwater operation unit of SALTAS (Wayatinah). These fish were cohabited with infested fish with *Paramoeba* sp. at Dover (sea culture operation) 2.5 mo later for determination of the protection efficacy of the crude vaccine. A flow diagram of Trial 2 is shown in Table 1.

Trial 3 — detection of local antibody from mucus using ELISA. Gills of 10 Atlantic salmon (200 ± 14.2 g), experimentally infected with AGD by cohabitation for 6 to 8 wk were perfused and the mucus was extracted according to the method described by Lumsden et al. (1993). Mucus was extracted in the same way from 6 naive fish. Sera were also collected from these fish.

For comparative purposes sera were also collected from naturally infected fish, 8 from Atlantic salmon smolts with a moderate primary infection and 52 from 2 yr old Atlantic salmon from fish which had been exposed to several waves of infection during a sea-summer.

Table 1 Time flow of Trial 2, showing the time of immunization, challenge and blood collection for serology of the experimental Atlantic salmon *Salmo salar*. BC: blood collection and gill samples for histopathology

Immunization (i.p. injection) (Time 0)	1st challenge (unsuccessful) (1 mo)	2nd challenge (successful) (45 d)	Blood collection (60 d)
320 fish (Atlantic salmon) from SALTAS	Challenged	Challenged	BC
320 fish (Atlantic salmon) from the Aquaculture Key Centre	Challenged	Challenged	BC
40 fish (Atlantic salmon) from SALTAS (Wayatinah) (crude antigen)	Challenged 2.5 mo post-immunization		
40 fish (Atlantic salmon) (naive fish, control fish)	Blood collection for control sera in ELISA (60 sera samples from control fish Trial 1)		

Table 2. Humoral response (ELISA O.D.) of sheep, rabbit and fish (rainbow trout) to *Paramoeba* sp. vaccines. Non-immune: non-immune serum; Immune: immune anti-*Paramoeba* sp. serum

Dilution of sera	Sheep (pooled sera)		Rabbit		Fish (pooled sera)	
	Non-immune	Immune	Non-immune	Immune	Non-immune	Immune
1:64	0.174	2.401	0.145	0.821	0.145	0.421
1:128	0.156	2.341	0.153	0.701	0.146	0.354
1:256	0.151	2.000	0.134	0.683	0.123	0.387
1:512	0.145	1.921	0.131	0.434	0.132	0.254
1:1024	0.133	1.653	0.099	0.241	0.111	0.183
1:2048	0.123	1.324	0.098	0.171	0.095	0.101
1:4096	0.121	1.245	0.074	0.149	0.119	0.098

Table 3. Humoral response of Atlantic salmon *Salmo salar* to crude *Paramoeba* sp. vaccine at 6 wk post-immunization. Means with the same superscript are not significantly different ($p < 0.05$)

Group	No. of fish	Percentage seropositive	ELISA O.D. Mean ± SE	Max.	Min.
Wild PA/FCA	15	40	$0.452^{a} \pm 0.096$	1.324	0.075
Control fish (AKC naive fish)	25	0	$0.092^{b} \pm 0.037$	0.142	0.078

Fish husbandry. In each experiment fish were maintained in two 4000 l freshwater temperature control tanks (2 replicates) at 15°C in recirculating biofilter systems at AKC and fed with commercial trout pellets (Gibson) twice daily. Water quality was monitored daily with 20 to 40% water changes.

In challenge experiments fish were acclimatised to seawater 15 d prior to cohabitation with the diseased fish. After 1 mo p.i. fish were cohabited with 50 infected fish (25 for each tank) and water temperature was initially maintained at 18°C. Since *Paramoeba* sp. could not be detected up to 2 wk after cohabitation another batch of infected large fish (5 fish) were placed in each tank. After 1 wk fish started to show clinical signs of infection.

In all challenge experiments mortalities were sampled for gill samples twice a day for histopathological survey. Two weeks after the last (effective) cohabitation, survivors, which in fact were suffering severely from the disease, were euthanised for collection of gill and blood samples.

Analysis of results. A paired *t*-test was employed to compare 2 observations (mean ELISA O.D.) within a group of fish. Comparisons between more than 2 groups of fish (mean ELISA O.D.) in a treatment and between treatments in an experiment were carried out using 1-way analysis of variance (ANOVA). Unless otherwise stated, a probability level of less than 0.05% was considered significant.

RESULTS

Trial 1—active immunization

The results of ELISA for immunized sheep (pooled sera of sheep), rabbit and fish (pooled sera of rainbow trout) are shown in Table 2. Sheep responded well to the sonicated antigen/FCA. Rabbit showed lower anti-*Paramoeba* sp. antibody levels (sonicated antigen) while rainbow trout had a much lower response despite being injected with the same antigens.

Atlantic salmon injected with formalin killed wild PA + FCA developed a significant antibody level at 6 wk p.i. (mean ELISA O.D.: 0.452). Only 40% of fish were seropositive and the rest of fish were seronegative (Table 3).

Passive immunization

The rate of clearance of sheep APA from Atlantic salmon sera [mean ELISA O.D. ± standard error (SE) of SALTAS and AKC fish] is shown in Fig. 1. There was a high level of sheep APA at 1 wk p.i. but these antibodies declined rapidly to 4 wk then more gradually to 8 wk p.i.

Trial 2.1—protection

No diseased fish infested with *Paramoeba* sp. was observed up to 15 d post-cohabitation (first exposure). It was assumed that the donor fish infestation had not persisted. Therefore, another stock of diseased fish was obtained and placed with the experimental fish at 45 d p.i. From 1 wk after cohabitation (second expo-

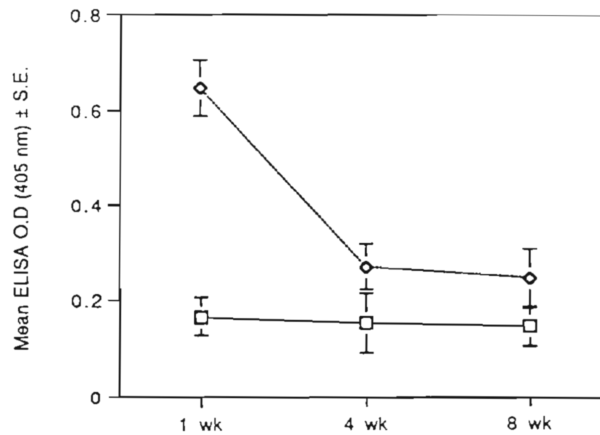

Fig. 1. Rate of clearance of sheep APA from Atlantic salmon *Salmo salar* for up to 8 wk post-immunization. (□) Control fish; (◇) fish immunized i.p. with sheep APA

sure) fish showed considerable mortality. *Paramoeba* sp. were detected on the gills of almost all exposed fish. The temperature was decreased to 16°C. However, amoebic gill disease appeared in an outbreak form (morbidity: 100%) and rendered the evaluation of protection unreliable, although it was notable that no treatment appeared to delay the onset of AGD.

Results of histopathology of gills collected from mortalities are as follows. No *Paramoeba* sp. or lesions were observed in the gill samples taken from the small number of mortalities after the first cohabitation indicating the absence of paramoebiasis. Gill samples collected 7 to 14 d after the second cohabitation (exposure to the second batch of infected fish), showed infestation with *Paramoeba* sp. and had mainly moderate to severe lesions.

The serological results of Atlantic salmon as a result of the experimental infection (after 2 wk exposure to AGD) are shown in Table 4.

Table 4. Humoral response of Atlantic salmon *Salmo salar* to experimental infection with *Paramoeba* sp. Means with the same superscript are not significantly different ($p < 0.05$)

Groups of fish in 2 replicates	No. of fish	Percentage seropositive	ELISA O.D. of responders		
			Mean ± SE	Max.	Min.
Control (exposed)	32	50	$0.298^{abd} \pm 0.032$	0.343	0.072
Sheep immune serum	35	45	$0.345^{bd} \pm 0.111$	0.486	0.086
Rabbit immune serum	40	44	$0.256^{a} \pm 0.057$	0.329	0.088
Live PA injection	26	53	$0.341^{bd} \pm 0.039$	0.421	0.0113
Sonicated (1 mg) PA in PBS	20	58	$0.299^{abd} \pm 0.067$	0.411	0.142
Sonicated (10 mg) PA in PBS	42	46	$0.234^{a} \pm 0.042$	0.501	0.123
Sonicated (1 mg) SPA in FCA	29	38	$0.354^{bd} \pm 0.057$	0.451	0.141
Sonicated (10 mg) SPA in FCA	37	49	0.365[illegible] ± 0.023	0.467	0.086
Control fish (naive) (SALTAS and AKC fish, mean ELISA O.D.)	50	–	$0.101^{c} \pm 0.041$	0.078	0.142

Trial 2.2—protection

Forty Atlantic salmon vaccinated with the crude antigen (wild *Paramoeba* sp.) did not show any significant serological differences in comparison with those injected with laboratory cultured *Paramoeba* sp. These fish were challenged 2.5 mo p.i. in cohabitation with diseased fish. All the fish showed gross lesions of paramoebiasis and *Paramoeba* sp. was detected in almost all samples.

Trial 3—detection of local antibody in gill mucus

The gill mucus antibodies of the fish infected with *Paramoeba* sp. antigens were undetectable by ELISA. Of these fish, 18% showed seropositive in the ELISA (Table 5). Table 5 also provides comparative data for the immunological responses of Atlantic salmon exposed (or not exposed) to *Paramoeba* sp. by a variety of means.

Both naturally and experimentally infected fish had significantly higher antibody levels than control. There was no significant difference in antibody levels between these groups. However, the percentage of seropositive fish in the experimentally infected group was higher than in the naturally infected group (Table 5).

DISCUSSION

Sheep, rabbit and rainbow trout developed humoral antibody when they were immunized with *Paramoeba* sp. vaccines with highest response occurring in sheep (Table 2) confirming the immunogenicity of the amoebic antigens. Rainbow trout immune responses to *Paramoeba* sp. antigen with FCA in this experiment are consistent with the results of

Table 5. Immunological responses of Atlantic salmon *Salmo salar* exposed (or not exposed) to *Paramoeba* sp. by a variety of means. s: serum; m: mucus. Means with the same superscript are not significantly different ($p < 0.05$)

Groups of fish in 2 replicates	No. of fish	Percentage seropositive	ELISA O.D. of responders		
			Mean ± SE	Max.	Min.
Natural infection (prolonged)	52 (s)	35	$0.354^{A} \pm 0.123$	0.865	0.089
Natural infection (short)	8 (s)	25	$0.192^{B} \pm 0.095$	0.219	0.091
Severe experimental infection (2 wk)	261* (s)	48	$0.297^{A} \pm 0.098$	0.231	0.111
Moderate experimental infection (3 wk)	11** (s) 10 (m)	18 0	$0.207^{A} \pm 0.112$ $0.123^{B} \pm 0.096$	0.231 0.132	0.108 0.106
Control fish (naive)	50 (s) 6 (m)	– –	$0.101^{B} \pm 0.041$ $0.116^{B} \pm 0.036$	0.142 0.127	0.078 0.091

*Collected sera from survivors of experimentally challenged fish (Table 4)
**Collected sera from Trial 3

Bryant et al. (1995) who used another species of amoeba. These workers reported antibody level was not proportional to the dose of amoeba injected and an inoculum of 20 µg protein per fish was sufficient to produce a strong antibody response.

When sheep APA were injected i.p. into Atlantic salmon to passively immunize them, it was shown that these antibodies were persistent (up to 8 wk). Therefore, the persistence of these antibodies could provide fish with passive protection if antibodies were protective to combat the disease.

Crude antigen (wild *Paramoeba* sp.) harvested directly from infected fish was not superior antigenically to vaccines produced from cultured organisms. Fish immunized with this antigen died gradually when they were cohabited with infected fish 2.5 mo p.i. Administration of this crude antigen with FCA into Atlantic salmon did not change the unresponsiveness status of non-responding fish (60% of vaccinated fish were seronegative). Since the most probable reason for lack of immunity when cultured *Paramoeba* sp. was used was thought to be the absence of virulence factors in the cultured organisms it is difficult to explain why a crude vaccine containing antigens from virulent organisms did not work. It was shown from the findings of Lumsden et al. (1994), who injected (i.p. with 0.1 ml) and immersed (in a 1:10 dilution of acetone-killed *Flavobacterium branchiophilum*) rainbow trout, that the level of antibody to the bacterium was highest in the i.p. injected group. However, the percent cumulative mortality was 32.1, 11.7 or 45.3% in i.p. injected, immersed and control fish respectively. The i.p. injected group had the highest serum antibody levels, while bath exposure to the highest concentration of killed bacteria produced the highest gill-associated antibody levels. The lack of a significant *Paramoeba* sp. antibody response in gill mucus at 6 to 8 wk after exposure to fish with AGD is not surprising as Lumsden et al. (1993) only found a significant response to *Flavobacterium branchiophilium* when fish were subjected to a second challenge at 200 d after the initial exposure. Also, it is possible that the monoclonal antibody against rainbow trout serum immunoglobulin (Ig), while suitable for Atlantic salmon serum Ig, may not have been able to detect surface antibodies present in gill mucus. Rombout et al. (1993) showed that not all monoclonal antibodies against carp serum Ig are suitable for detected mucus Ig in this species. Therefore, gill associated antibodies might be the main protective value in immersion vaccinated fish especially against diseases which cause surface infections of gills.

Cohabitation with presumed diseased fish did not produce the disease at the first attempt. However, a second batch of infected fish produced severe AGD. It is possible that the first batch did not carry many *Paramoeba* sp. on their gills possibly due to the development of immunity during the long course of infection.

Regarding the lack of protectiveness of mammalian sera in combating paramoebiasis, it should not be forgotten that in cohabitation, 100% morbidity was not expected. With such a high morbidity it is difficult to assess the protection efficacy. Furthermore, systemic antibody is possibly less important for protection from AGD if the organism infects gill tissues. Therefore, local immune response may play an important role in naturally infected fish. However, in our experiment, local antibody to PA could not be detected using ELISA.

Passively and actively immunized Atlantic salmon that were experimentally infected by cohabitation showed active humoral antibody response (measured by ELISA) to *Paramoeba* sp. in a period of 2 wk after exposure to *Paramoeba* sp. (Table 4). The humoral responses of these fish were significantly different from those of control fish (naive fish). This concludes that fish humoral antibody can be induced by a heavy AGD infestation.

Some groups of the exposed Atlantic salmon which previously were immunized showed significant differ-

ences with each other in antibody levels post-cohabitation (Table 4). This could be due to injection of antigen with adjuvant when they were immunized before cohabitation and the possible effect of adjuvant on their humoral responses. However, this pattern was not consistent between groups of fish, i.e. live PA treated and sheep APA treated fish showed almost the same antibody level as sonicated PA with FCA treated fish.

Not all sera of presumed naturally infected Atlantic salmon appeared to be seropositive (only 35% seropositive) indicating that amoeba in gills evoke a humoral response in some fish (Table 5). Similarly, experimentally infected Atlantic salmon did not show a high response either, even though the percentage of seropositive in this group was higher (48%) than those naturally infected. This is consistent with the results of Grayson et al. (1991) who reported that Atlantic salmon can produce antibodies to artificially injected salmon louse *Lepeophtheirus salmonis* components, but naturally infected Atlantic salmon and rainbow trout did not similarly respond. It is clear that short-term infection of naive fish can stimulate the humoral immune system. Perhaps long-term infestation, particularly with a small number of *Paramoeba* sp. on the gill, may produce immune tolerance. However, the observed unresponsiveness still remains unresolved.

No significant difference was observed between the antibody levels of Atlantic salmon naturally and experimentally infected with paramoebiasis. Thus, long exposure of fish does not seem to stimulate an enhanced immune response.

These initial trials have been disappointing in that protective immunity was not demonstrated. However, this is not surprising as subsquent studies (V. Findlay & B. L. Munday unpubl.) have demonstrated that 'memory' appears to be involved in the expression of immunity in previously infected fish, and there is a delay before protection is apparent on re-exposure. The results obtained in this investigation may have considerable value in relation to future immunization trials, protection experiments and immunological assays. Further work is required to elucidate the gill tissue immune response in naturally and experimentally infected fish in the near future to establish a method of immunization against such a problematic and important fish disease.

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