The Effect of Levamisole as an Adjuvant on the Humoral Immune Response of Atlantic Salmon (Salmo salar L.)

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Abstract
Pre and post-smolt Atlantic salmon (Salmo salar L.) were administered a levamisole adjuvanted Vibrio anguillarum vaccine by bath or intraperitoneal (IP) injection. A significant serum anti-V. anguillarum antibody response was elicited in groups of fish administered an IP injection of bacterium only. Fish (pre and post-smolt) treated with the levamisole adjuvanted vaccine (IP and bath) showed a suppressed response relative to the respective positive (vaccinated) control groups. No detectable antibody response was elicited in fish not treated or treated with a placebo. Results from this trial suggest that levamisole as an adjuvant has a narrow range of efficacy.

Introduction
Vaccination of fish is an elegant means of enhancing the protective capabilities of fish to specific pathogens. While many vaccines are effective when used insularly, the efficacy of others may benefit from the inclusion of adjuvants. Recent studies have demonstrated that levamisole (used as an adjuvant) increased resistance to pathogenic challenge through the elevation of antibody response and non-specific immune parameters (Siwicki et al., 1990; Anderson and Jeney, 1992; Jeney and Anderson, 1993; Midtlyng et al., 1996). Evidence has suggested that the efficacy of levamisole as an adjuvant is dependent on the antigen with which it is administered. The aim of this study was to determine the effect of levamisole as an adjuvant with a commercial Vibrio anguillarum vaccine on the humoral immune response of Atlantic salmon (Salmo salar L.).

Materials and Methods
Fish
Atlantic salmon (S. salar L.) pre-smolt (10 g) or post-smolt (585 g) were held in fresh water (0 ‰) or sea water (35 ‰) respectively. Pre-smolts (n = 378) were held in eighteen 50 L tanks in a flow through system, while post-smolts (n = 32) were individually tagged and held in a single 4000 L rathburn tank in a recirculation system. Fish were fed commercial salmon diets (Gibson’s). Both groups of fish were kept at a constant temperature using temperature control apparatus. The pre-smolts were held at 14 ± 0.7°C, whilst the post-smolts were held at 11 ± 0.5°C.

Vaccination
Fish were divided into six (pre-smolt) or four (post-smolt) treatment groups (Table 1). Bath vaccine was prepared by diluting a Vibrio anguillarum (serovar O1) whole cell vaccine...
Levamisole (Levamisole gold, Virbac, Peakhurst, NSW) in the form of levamisole hydrochloride (5 µg mL⁻¹) was added to the bath. Injectable vaccine was prepared by centrifuging 10 mL of Anguillivac-C for 3 minutes at 2130 g. After removal of the supernatant, cells were washed twice with sterile 0.1 M PBS (pH = 7.2) then resuspended in the same volume of PBS. Injectable levamisole in the form of levamisole phosphate (Nilverm®, Coopers, Sydney, NSW) was diluted in PBS and vaccine to create the required volume and concentration (table 1).

Fish were acclimated to the tanks over fourteen days prior to treatment. Fish in all groups were anaesthetised with benzocaine (ethyl-p-aminobenzoate; 50 mg L⁻¹) and weighed prior to administration of treatments. Bathing of fish occurred over a one hour period in an adjuvanted or non-adjuvanted vaccine bath. For the injected treatments, fish were intraperitoneally (IP) injected

### Table 1. Vaccination treatments (levamisole concentration µg/g fish⁻¹)

<table>
<thead>
<tr>
<th>No</th>
<th>Treatment</th>
<th>Pre-smolt treatment</th>
<th>Post-smolt treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No treatment</td>
<td>naᵇ</td>
<td>naᵇ</td>
</tr>
<tr>
<td>2</td>
<td>PBS (IP)</td>
<td>0.2 ml (0 µg/g fish)</td>
<td>0.5 ml (0 µg/g fish)</td>
</tr>
<tr>
<td>3</td>
<td>Vaccine (IP)</td>
<td>0.2 ml (0 µg/g fish)</td>
<td>0.5 ml (0 µg/g fish)</td>
</tr>
<tr>
<td>4</td>
<td>Vaccine + Levamisole (IP)</td>
<td>0.2 ml (0.5 µg/g fish)</td>
<td>0.5 ml (0.5 µg/g fish)</td>
</tr>
<tr>
<td>5</td>
<td>Vaccine (bath)</td>
<td>2 ml/l (0 µg/g fish)</td>
<td>niᶜ</td>
</tr>
<tr>
<td>6</td>
<td>Vaccine + Levamisole (bath)</td>
<td>2 ml/l (5 µg/g fish)</td>
<td>niᶜ</td>
</tr>
</tbody>
</table>

(Anguillivac-C; DPIWE, Launceston, Tasmania) containing a minimum of 1x10¹⁰ cells mL⁻¹ of formalin killed whole cells.

**Sampling**

When sampling, fish were euthanased with an overdose of benzocaine (100 mg L⁻¹). At 4 weeks post-treatment approximately half the pre-smolts (n = 10 from each triplicate tank) and all the post-smolts (n = 8) were euthanased. Blood was removed from the caudal vein and allowed to clot on ice. Blood was centrifuged at 1890 g for 3 minutes and serum removed and frozen (-80°C) until required. At 8 weeks post-treatment, the remaining pre-smolts were sampled as described.

**Enzyme linked immunosorbent assay (ELISA)**

The serum anti-V anguillarum antibody responses were monitored after an indirect ELISA had been developed (Crowther, 1995). Briefly, plates were coated with sonicated V. anguillarum diluted in carbonate buffer and incubated overnight at 4°C. Wells were blocked with 3% casein sodium in PBS (pH = 7.2) for 30 minutes at 37°C. Serum samples were added to each well and incubated for 90 minutes at room temperature. Mouse anti-Atlantic salmon IgM monoclonal antibody (MAb; 4610) or rabbit anti-Atlantic salmon IgM were used to probe the pre-smolt and
post smolt antibody titres respectively (60 minutes at 37°C). Rabbit anti-mouse HRP (DAKO P161) or goat anti-rabbit HRP (DAKO P0448) was added to each well for 60 minutes at 37°C and colour developed using o-phenylene diamine in sodium citrate phosphate buffer. Plates were washed 4 times with PBS in between each step. Plates were read using a Titertek Plus MS212 plate reader at 492 nm. Positive (vaccinated) control as well as negative (non-vaccinated) control serum were included on each plate to enable data comparison. Statistical analysis of treatment groups was by one way ANOVA after the assumptions of the test had been satisfied. Differences between individual treatment groups were detected using Tukey’s posteriori test.

Results

Pre-smolt administered an IP injected vaccine alone exhibited a significant elevation of antibody level (Figure 1). In addition, the fish bathed in vaccine displayed a detectable antibody response at week 4. Antibody levels of fish treated with the vaccine and levamisole (bath and IP) were above the negative control groups. However, when compared to the respective positive control groups the antibody levels of fish treated with the levamisole adjuvanted vaccine were suppressed. Statistically, the means of treatment groups were significantly different (P = 0.003) however, at week 8 the response had tapered and means were not significantly different (P = 0.107). A similar trend in antibody response amongst treatment groups was observed in post-smolt salmon. Little or no antibody was observed in post-smolt from the control (null and PBS treatment) groups (Figure 2). Fish in groups administered the vaccine showed a significant elevation of antibody titre (P = 0.000) while the antibody level of fish administered the levamisole adjuvanted vaccine was suppressed relative to the positive control group. However the difference between the positive control and treatment groups was not significant.
Discussion

ELISA optical density (O.D.) data from the current study indicate that levamisole suppresses the humoral antibody response when administered with the *V. anguillarum* vaccine, regardless of the route of administration and life stage. Suppression of the antibody response (post-treatment) by levamisole adjuvanted vaccines has not been reported in the literature. However, the number of antibody producing cells (PFC) was suppressed when rainbow trout (*Oncorhynchus mykiss*) spleens were stimulated *in vitro* using either DNP-Ficoll or *Yersinia ruckeri* and levamisole (Siwicki et al., 1990). The numbers of PFCs produced was critically dependant on both antigen and levamisole concentration. DNP-Ficoll produced significantly higher numbers of PFCs than *Y. ruckeri*, while levamisole (25 µg.mL⁻¹), significantly suppressed the abundance of PFCs and at a concentration of 50 µg.mL⁻¹, levamisole reduced the number of PFCs to zero.

Atlantic salmon (*S. salar*) vaccinated with a levamisole adjuvanted *Aeromonas salmonicida* vaccine had significantly higher ELISA O.D. readings than negative and positive controls, 6, 12 and 24 weeks post vaccination (Midtlyng et al., 1996). Unfortunately, the concentration of levamisole was not specified. A difference in levamisole concentration or type of antigen may have accounted for the discrepancy in ELISA results, when compared to the current trial. In a similar experiment, rainbow trout (*O. mykiss*) vaccinated against *A. salmonicida*, using levamisole (5 µg.fish⁻¹) as an adjuvant, produced significantly higher levels of circulating antibody titres, 14 days post vaccination (Anderson and Jeney, 1992). Rainbow trout (*O. mykiss*) immersed in levamisole (5 µg.mL⁻¹) prior to bathing in an *A. salmonicida* vaccine exhibited heightened circulating antibody levels after 2 weeks, however, at 4 weeks post-vaccination, antibody titres of fish bathed in the vaccine alone exceeded those treated with the adjuvanted vaccine (Jeney and Anderson, 1993). This is in agreement with the findings of Ackerman (1995), after rainbow trout (*O. mykiss*) were treated with a levamisole adjuvanted *A. salmonicida* vaccine. These data suggest that levamisole may hasten the antibody response in the ensuing period after vaccination however, the response of fish vaccinated with bacterium alone “catches up”, then surpasses that of the levamisole treated fish. This raises two important questions; what cellular/biochemical mechanism(s) is/are involved in the initial antibody response and is it possible to prolong that response over a longer time period? The mechanisms by which levamisole functions in fish are yet to be identified, so the answers to these questions will remain speculative until further work is undertaken.

Intraperitoneal injection with the vaccine (with or without levamisole) elicited a higher serum antibody response compared with bath vaccinated fish (week 4 and week 8 post vaccination). These results are consistent with those of Whittington et al. (1994), who demonstrated a slight elevation of serum antibody titres in rainbow trout (*O. mykiss*), 3 weeks post-bath vaccination, followed by a decline to basal levels after 8 weeks. An elevated serum antibody titre has been correlated with resistance of rainbow trout (*O. mykiss*) to *V. anguillarum* in a previous trial (Farrell et al., 1975). However, bath vaccinated fish remain
protected for up a year without significant elevation of the serum antibody titre (Thuvander et al., 1987). Hence, bath vaccination remains efficacious by either cell mediated immunity, immune memory or local mucosal immunity. Further work is required to determine if levamisole affects these components of the immune system.

In conclusion, this trial has demonstrated the suppressive effects of levamisole as an adjuvant on the antibody response after bath and IP vaccination against vibriosis. Further work should determine if the suppression of the antibody response demonstrated in the current trial exists after repeat vaccination. In addition, the effect of levamisole as an adjuvant on the ability of the V. anguillarum vaccine to offer protection requires attention.

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