Efficacy of Oil-adjuvanted Vaccine for Coldwater Disease in Ayu Plecoglossus altivelis

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\textbf{ABSTRACT} — We investigated the potency of two oil adjuvants, Montanidae ISA 763A (763A) and a squalene emulsion (Squalene), to enhance the response of ayu Plecoglossus altivelis to a formalin-killed bacterin (FKB) made from the coldwater disease etiologic agent, Flavobacterium psychrophilum. Ayu were challenged 4 wk after vaccination by an intramuscular injection with live pathogenic F. psychrophilum. Mortalities of fish injected with FKB of F. psychrophilum combined with either adjuvant showed that the adjuvanted vaccines had significantly higher (P<0.05) potencies than the FKB vaccine alone or sterile distilled water. Moreover, both adjuvanted vaccines produced significantly higher antibody titers than the FKB vaccine without adjuvant. From these investigations it is concluded that the use of Squalene and 763A adjuvants provides enhanced protection against coldwater disease in ayu compared to the use of FKB alone.

\textbf{Key words}: oil adjuvant, vaccine, coldwater disease, Flavobacterium psychrophilum, ayu, Plecoglossus altivelis, squalene, bacterin

\textit{Flavobacterium psychrophilum} was originally isolated in 1948 from coho salmon Oncorhynchus kisutch in the USA, and is well known as the causative agent of bacterial coldwater disease (Holt et al., 1993) and rainbow trout fry syndrome (RTFS, Lorenzen et al., 1997). In Japan this pathogen was first isolated from ayu Plecoglossus altivelis in 1987, and has spread throughout most prefectures (Wakabayashi et al., 1994). Recently, bacterial coldwater disease has caused high mortality of ayu in farms and natural waters and is considered to be one of the most serious fish diseases in Japan. Therapeutic treatments are not applicable in natural waters and therefore development of vaccines is needed. Although an effective vaccine using an inactivated strain of \textit{F. psychrophilum} has been reported for the prevention of bacterial coldwater disease in trout (Obach and Baudin Laurencin, 1991), no vaccine for ayu is currently available.

Commercial vaccines to protect fish against bacterial pathogens typically consist of killed cells, extracellular fractions, or cell culture supernatants (Ellis, 1997). In a preliminary experiment, we attempted to develop a vaccine using a formalin killed bacterin (FKB) of \textit{F. psychrophilum}, but encountered low efficacy. It is well recognized that development of a successful vaccination strategy depends on the presence of protective antigens, stimulation of the immune system and appropriate delivery to the host. Adjuvants have become important tools for increasing vaccine potency (Anderson, 1997), and the use of oil adjuvants combined with antigens has resulted in a substantial reduction in the use of antibacterial drugs in Norway (Markestad and Grave, 1997). Recently, squalene has been reported to be an effective
adjuvant for vaccines in humans and other mammals (Souza and Playfair, 1995; Allison, 1999), although there is no information available for the use of squalene in fish vaccines.

In the present study we attempted to develop an effective vaccine against coldwater disease in ayu using oil adjuvants combined with FKB of *F. psychrophilum*.

**Materials and Methods**

**Fish**

Juvenile ayu *Plecoglossus altivelis* were obtained from a hatchery (Nissin Marine Tech) in Aichi Prefecture, Japan, where coldwater disease has not been observed. The fish were confirmed to be free of *F. psychrophilum* before starting the experiments. The fish were maintained in 200 L tanks with running freshwater at 15°C, fed with commercial dry pellets, and acclimatized to the laboratory for at least 4 wk before starting each experiment.

**Bacteria**

*F. psychrophilum* strain PH-9304, originally isolated in 1993 from an infected ayu in Hiroshima Prefecture, Japan, was used in this study. Bacteria were cultured on modified cytophaga agar (MCY agar, Wakabayashi and Egusa, 1974) for 2 days and then on casitone gelatin yeast agar (CGY agar, pH 7.2, Ototake and Wakabayashi, 1985) for 1 day.

**Vaccines**

FKB vaccine: FKB of *F. psychrophilum* was prepared by suspending the harvested bacteria in sterile distilled water at a concentration of 10⁸ CFU/mL, then adding formalin to a final concentration of 0.3% and incubating at 4°C for 48 h.

763A vaccine: The adjuvant Montanidae ISA 763A (763A) (Seppic, France) was combined with FKB vaccine at a concentration of 10⁸ CFU/mL in a 7:3 (v/v) ratio. The adjuvant was mixed with the FKB by passing the mixture through a double-ended needle from one syringe to another repeatedly, until it became a thick homogenous emulsion.

Squalene Vaccine (Squalene): The squalene adjuvant was composed of a homogenous mixture of 7.5 mL canola oil (Sigma), 1 mL squalene (Wako, Japan) and 1.3 mL arlacel-A® (Nacalai tesque, Japan). We prepared one mixture consisting of the canola oil, squalene and arlacel-A® and another mixture consisting of 10 mL FKB vaccine (10⁸ CFU/mL) with 0.2 mL Tween 40 (Nacalai tesque, Japan). Subsequently, the two mixtures were mixed very well by using a double-ended needle.

**Vaccination**

The potency of each of the vaccines was assessed by intraperitoneal injection of 1.5 ± 0.5 g (average body weight ± standard deviation) juvenile ayu. One hundred ayu of each group were anaesthetized with 0.015% FA-100 (4-allyl-2-methoxyphenol, Tanabe Seiyaku Co, Japan), and then vaccinated with 10 μL per fish of the Squalene-, the 763A-, or the FKB vaccine, and another group was injected with distilled water as a control. Injections were carried out using a repeating micro-dispenser (Eppendorf 4780) with a 28 gauge, 4 mm long needle. A polypropylene tube was attached to the needle so that it would not enter the fish deeper than 1.5 mm (Fig. 1). With these improvements, only 3 ayu out of 400 (1.5 g) died within several days after the injection. After vaccination, fish were held at 15°C and fed daily with commercial dry pellets.

**Challenge test**

To determine the virulence of the *F. psychrophilum* strain PH-9304, a series of 10-fold dilutions of live bacteria cultures were prepared and injected intramuscularly into 3 groups of 20 ayu. Injected fish were reared for 15 days at 15°C and mortality was recorded daily. Infection with *F. psychrophilum* was confirmed by a slide agglutination test with anti-*F. psychrophilum* rabbit serum and bacteria isolated from the kidney of each dead fish. The median lethal dose (LD₅₀) was calculated from mortalities according to Reed and Muench (1938).

To determine the efficacy of vaccines, challenge tests were performed 4 wk after vaccination. Fish were...
anaesthetized and then challenged by intramuscular injection with 25 μL of a live F. psychrophilum suspension at concentrations of 1.5 × 10⁴ CFU and 1.5 × 10³ CFU per fish. Challenged fish were kept in tanks with running freshwater at 15°C for 15 days. Dead fish were collected daily and infection with F. psychrophilum was confirmed by morphological characterization of the colonies on MCY agar containing 5% horse serum. Relative percentage survival (RPS) (Croy and Amend, 1977) was calculated from the cumulative mortalities as

\[ RPS = \left(1 - \frac{\text{mortality of vaccinated group}}{\text{mortality of unvaccinated control group}}\right) \times 100 \]

Agglutination antibody titer

Sera were collected by caudal puncture from 15 fish in each group 4 wk after immunization. Sera were incubated at 44°C for 20 min to inactivate complement activity (Sakai, 1981). Agglutinating antibody titers against F. psychrophilum were determined using the micro-titer method.

Statistical analysis

Significant differences between treatments in levels of mortality after challenge were detected by Fisher's exact probability test. The relative percentage survival was calculated when a significant difference was detected between mortalities in control and vaccinated groups. Data for the agglutination antibody titer were first subjected to the F test in order to detect significant differences between variances. When the difference in variances was significant, the data were subjected to the Cochran-Cox test in order to detect significant differences between means. The rest of the data were subjected to the Student's unpaired t test in order to detect significant differences between means. Significance level for all statistical analyses was P<0.05.

Results

The lethal dose (LD₅₀) of the F. psychrophilum strain PH-9304 was 10⁴.³ CFU/fish (Table 1). Fish began to die on the 3rd day post injection in the group inoculated with the highest dose. Disease signs and mortalities continued for 10 days in all injected groups.

In the challenge with a high dose of the pathogen, fish began to die on the 5th day post challenge and continued to die until the 10th day post challenge in the vaccinated groups. Cumulative mortality was 24% in the Squalene-vaccinated group, 30% in the 763A-vaccinated group and 45% in the FKB-vaccinated group. In contrast, fish in control group continued to die until the 11th day post challenge and the cumulative mortality was 75%. In the group challenged with a low dose, mortalities began on the 5th day post challenge and continued until the 9th day post challenge. The mortalities of groups administered the adjuvanted vaccines were

### Table 1. Mortality of ayu injected intramuscularly with F. psychrophilum strain PH-9304

<table>
<thead>
<tr>
<th>Inoculum dose (CFU/fish)</th>
<th>Dead fish / injected fish</th>
<th>Mortality (%)</th>
<th>LD₅₀ (CFU/fish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7 × 10⁴</td>
<td>15/20</td>
<td>75</td>
<td>10⁴.³</td>
</tr>
<tr>
<td>3.7 × 10³</td>
<td>3/20</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>3.7 × 10²</td>
<td>4/20</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Relative percentage survival (RPS) of vaccinated ayu after challenge with F. psychrophilum

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Challenge dose (CFU/fish)</th>
<th>Total (fish)</th>
<th>Specific loss (fish)</th>
<th>Mortality (%)</th>
<th>RPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>1.5 × 10⁴</td>
<td>25</td>
<td>6</td>
<td>24</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>1.5 × 10³</td>
<td>25</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>763A</td>
<td>1.5 × 10⁴</td>
<td>20</td>
<td>6</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1.5 × 10³</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>FKB</td>
<td>1.5 × 10⁴</td>
<td>20</td>
<td>9</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1.5 × 10³</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.5 × 10⁴</td>
<td>20</td>
<td>15</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 × 10³</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Agglutinating antibody titers in sera of vaccinated and non-vaccinated ayu

<table>
<thead>
<tr>
<th>Vaccinated with</th>
<th>Individual serum titers</th>
<th>Av.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>32, 32, 16, 8, 16, 16, 32, 32, 16, 32</td>
<td>26**</td>
</tr>
<tr>
<td>763A</td>
<td>64, 32, 16, 32, 16, 32, 16, 16, 64, 32</td>
<td>26*</td>
</tr>
<tr>
<td>FKB</td>
<td>2, 8, 2, 2, 2, 2, 4, 2, 4, 4, 2, 4</td>
<td>4⁴</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2</td>
<td>&lt;2⁴</td>
</tr>
<tr>
<td>Pre-immunized</td>
<td>&lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2, &lt;2</td>
<td>&lt;2⁴</td>
</tr>
</tbody>
</table>

*: significant (P<0.05) differences among a, b and c. Av.: geometric average.
significantly lower than those administered the FKB vaccine alone, while the FKB vaccine showed significantly higher potency than the control (sterile distilled water). There was no significant difference between the two adjuvanted vaccines (Table 2).

The agglutinating antibody titers of sera from fish in the vaccinated, non-vaccinated and pre-immunized groups are shown in Table 3. The highest antibody titers were found in the Squalene vaccinated group and the 763A vaccinated group. The FKB vaccinated fish showed higher antibody titers than control fish which showed antibody titers similar to those of pre-immune fish. The antibody titers of fish vaccinated with the adjuvanted vaccines were significantly higher than those of fish in the FKB vaccinated group or control group. However, there was no significant difference in titer between the groups vaccinated with the 763A- and the Squalene vaccine.

Discussion

Ayu vaccinated with the 763A vaccine showed significantly (P<0.05) greater protection against coldwater disease than ayu vaccinated with the FKB vaccine or distilled water. This result agrees with previous studies on the use of adjuvants in vaccines for other fish species. Krantz et al. (1963) first reported that formalin-killed Aeromonas salmonicida with an adjuvant (one part arlacel to nine parts klearol mineral oil) increased antibody titer in brown trout. Adams et al. (1988) reported that an adjuvant combined with formalin-killed cells of A. salmonicida gave better protection in rainbow trout against furunculosis compared to the formalin-killed cells alone. Similarly, Holt (1987) reported the high potency of an adjuvanted vaccine for coldwater disease in coho salmon. We have conducted several challenge tests to further investigate the efficacy of the 763A vaccine and found similar results to those shown in Table 2. However, 763A adjuvant was retained in ayu for more than 4 wk but less than 8 wk (data not shown). For the development of commercial ayu vaccines, it will be important to find adjuvants with shorter retention times. Screening of adjuvants with shorter retention periods is under investigation in our laboratory.

Souza and Playfair (1995) reported the effectiveness of an oil-in-water emulsion squalene vaccine for humans against blood-stage malaria, and Estuningsih et al.  (1997) reported that squalene Montanidae 80, MF59-100 with antigen induced moderate to high antibody titer in cattle. Allison (1999) reported that squalene emulsions are efficient adjuvants, eliciting both humoral and cellular immune responses in primates. In the present study, the vaccine containing Squalene as an adjuvant was very effective in ayu. Fish vaccinated with the Squalene-adjuvanted FKB showed significantly (P<0.05) greater protection against coldwater disease in ayu than those vaccinated with the FKB vaccine or distilled water. This result suggests that squalene is a good candidate for inclusion in adjuvanted vaccines for fish as well as those for mammals. Additional studies have confirmed the efficacy of the Squalene vaccine as shown in Table 2, although, as the 763A vaccine, the retention period of Squalene in ayu was between 4 and 8 wk (data not shown).

In the present study two adjuvanted vaccines produced significantly higher antibody titers in ayu than the FKB vaccine without adjuvant. These findings are consistent with previous reports in coho salmon (Paterson and Fryer, 1974) and rainbow trout (Cossarini-Dunier, 1985). It is generally accepted that the adjuvant forms a depot that releases the antigen slowly into the tissue or blood, enhancing and prolonging the humoral immune response (Anderson, 1992). We also found good correlation between protection and antibody titer, as previously reported for carp vaccinated with A. hydrophila (Karunasagar et al., 1997). Midtlyng et al. (1996) also observed that antibody could play a very important role in protection of Atlantic salmon against furunculosis. In the present study, similar phenomena may have occurred in adjuvant-vaccinated ayu, which showed higher antibody titers than those of FKB-vaccinated ayu.

Brown et al. (1997) showed that F. psychrophilum was vertically transmitted in coho salmon, and Kumagai et al. (1998) reported that povidone-iodine treatment was ineffective to eliminate F. psychrophilum from salmonid eggs. These reports suggest that the vertical transmission of the bacterium is possible in ayu. Ayu broodstock are sometimes seriously affected by coldwater disease. Therefore, it is essential to keep broodstock pathogen-free to prevent the vertical transmission. In the present study, we found that many more ayu were free from pathogen in the groups treated with adjuvanted vaccine than the group treated with non-adjuvanted vaccine on the 5th day post challenge with live pathogen (data not shown). This result indicates that injection vaccination with the Squalene- or the 763A vaccine can be applied to protect ayu broodstock from coldwater disease.

Coldwater disease also occurs in juvenile ayu, with smaller fish showing higher sensitivity (Iida and Mizokami, 1996). Therefore, it is necessary to immunize ayu at the earliest stage possible. In a preliminary experiment, fish weighing approximately 0.5 g were injected with either of the adjuvanted vaccines or FKB alone, but most of the fish died the following day. However, most fish weighing 1.5 g survived after injection. In order to avoid side effects, the dose given to fish was limited to 10 μL, which was less than 1% of the body weight. In another preliminary experiment, the FKB vaccine was administered to fish weighing about 1.5 g by bath. However, no efficacy was observed (data not shown). In order to immunize fish weighing
less than 1 g, a new method of administration would be necessary.

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References


