マダイの非定型Edwardsiella tarda感染症に対する不活化ワクチンの効果
Protective Efficacy of a Formalin-Killed Vaccine against Atypical *Edwardsiella tarda* Infection in Red Sea Bream *Pagrus major*

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ABSTRACT—*Edwardsiella tarda* causes high mortality infections in fish. To determine the efficacy of a vaccine against *E. tarda*, a formalin-killed preparation of atypical *E. tarda* FPC503 was intraperitoneally injected into red sea bream *Pagrus major* with or without the oil adjuvant, Montanide ISA 763 A VG. Both vaccine regimens showed protective efficacy against experimental infection with *E. tarda* FPC503 at 28 and 56 days post-vaccination. An enzyme-linked immunosorbent assay (ELISA) revealed the specific antibody production in vaccinated fish but the relationship between protective efficacy and antibody titer was not clear. The present results indicate that the formalin-killed vaccine is useful for the prevention of atypical *E. tarda* infection in red sea bream.

Key words: *Edwardsiella tarda*, formalin-killed vaccine, oil adjuvant, *Pagrus major*, red sea bream

In Japan, *Edwardsiella tarda*, a member of the family Enterobacteriaceae, causes significant mortality in the cultured red sea bream *Pagrus major* (1), the Japanese flounder *Paralichthys olivaceus* (2) and the Japanese eel *Anguilla japonica* (3,4). Phenotypically distinct *E. tarda* strains have been reported in diseased fish. Typical *E. tarda* strains that show higher virulence in Japanese flounder and freshwater fish are motile and do not produce acid from arabinose, galactose, mannitol, and mannose (1,5,6). Atypical *E. tarda* strains isolated from diseased red sea bream and yellowtail *Seriola quinqueradiata* are non-motile and produce acid from the aforementioned carbohydrates (5,6).

Studies on a vaccine to protect against *edwardsielosis*, caused by typical *E. tarda*, have been conducted mostly in flat fish, including Japanese flounder (7-12) and turbot *Scophthalmus maximus* (13-15). However, no vaccine studies have investigated protection from atypical *E. tarda* infections. In this study, we evaluated the efficacy of a formalin-killed vaccine against atypical *E. tarda* in red sea bream.

Materials and Methods

Bacterial strain and vaccine preparation

An atypical *E. tarda* FPC503 strain isolated from a diseased red sea bream (9) was used in this study. FPC503 was cultured at 27°C in 500 mL of heart infusion (HI) broth (BD Biosciences, USA) supplemented with NaCl to a final concentration of 2.0%. Formalin (final concentration 0.5%) was added when the bacterial culture reached 1.2 x 10⁹ CFU/mL and the culture was incubated for 48 h at 4°C. After centrifugation at 3,000 x g for 10 min, the supernatant (475 mL) was discarded and the bacterial pellet was resuspended in 25 mL of medium. This bacterial suspension was emulsified in 75 mL of adjuvant (Montanide ISA 763 A VG) to create an adjuvanted vaccine preparation. A second vaccine preparation was prepared by mixing 75 mL of phosphate-buffered saline (PBS) with 25 mL of the bacterial suspension. The final concentration of bacterial cells in these concentrated vaccines was estimated to be 6.0 x 10⁹ CFU/mL.

Vaccination

Red sea bream (average body weight, 21 g) were acclimated at 25°C in flow-through 60-L tanks. A fixed volume (100 µL) of adjuvanted or non-adjuvanted vaccine was intraperitoneally injected into individual fish using a 27-gauge needle and syringe. Additionally, fish were intraperitoneally injected with 100 µL of emulsified adjuvant or PBS alone as controls.

Challenge test

*Edwardsiella tarda* FPC503 was inoculated on HI agar (BD Biosciences) and incubated at 25°C for 48 h. Bacterial colonies (1 mg) were suspended in 1 mL of sterile PBS and the bacterial suspension was adjusted to the appropriate bacterial titer. This live bacterial suspension (100 µL) was injected into the peritoneal cavity of a vaccinated fish and a non-vaccine control fish. The challenge test was started at 28 days post-vaccination (dpv) or 56 dpv. The fish were then held in a 60-L tank at 25°C and mortality was recorded up to 28 days post-challenge. The relative percentage survival (RPS) was determined following the formula: RPS = (1 - [% mortality of vaccinated fish / % mortality of control (PBS-injected fish)]) x 100.

Sampling of antiserum from vaccinated fish

Antisera collected from red sea bream immunized with the non-concentrated vaccines were analyzed to determine time-dependent changes of antibody titer. Non-concentrated vaccines were prepared by either...
emulsifying 25 mL of the formalin-treated culture with 75 mL of Montanide ISA 763 A VG or mixing it with 75 mL of PBS. The concentration of bacterial cells contained in these non-concentrated vaccines was estimated to be 3.0 x 10^8 CFU/mL. The bream were immunized with either of the non-concentrated vaccines by the procedure outlined above. Peripheral blood was collected from the vaccinated fish at 10, 20, and 56 dpv using a 21-gauge needle and syringe. Peripheral blood of non-vaccine control fish was collected at 0 dpi. The serum was separated by centrifugation at 3,000 x g for 10 min and diluted 64-fold with PBS before being analyzed by enzyme-linked immunosorbent assay (ELISA).

ELISA

An overnight culture of *E. tarda* FPC503 grown at 27°C in 15 mL of Hi broth with 2% NaCl was collected by centrifugation at 3,000 x g and the pellet was washed twice with PBS. Bacterial cells were resuspended in 15 mL PBS and sonicated on ice for 5 min using a Tomy ultrasonic vibrator UR-200P (Tomy Seiko, Japan). After centrifuging at 3,000 x g for 10 min, the supernatant served as solubilized bacterial antigens. The concentration of proteins in this supernatant was determined to be 32 μg/mL.

For analysis of sera, 50 μL of the supernatant was loaded into each well of a microtiter plate and incubated at 4°C for 12 h. After washing three times with Tris-buffered saline (pH 7.6) containing 0.5% Tween20 (TBST), wells were incubated with blocking buffer (3% skim milk, 0.02% sodium azide in TBST) at 4°C for 12 h, and then washed another three times with TBST. Diluted serum (50 μL) was loaded into independent wells of the plate and then incubated at 4°C for 12 h. Specific binding of serum IgM reactive to *E. tarda* antigens in vaccinated fish was detected by a murine monoclonal antibody (mAb) that was raised against red sea bream IgM in our laboratory. A goat anti-mouse IgG horseradish peroxidase-conjugate and TMB microwell peroxidase substrate system (KPL, USA) were used for color development. The reaction was stopped by the addition of 2 N H₂SO₄ and the absorbance was measured at 450 nm. Each assay was carried out with three replicate fish and significant differences in the data were determined by Student's t-test.

### Results and Discussion

Both the adjuvanted and non-adjuvanted vaccines containing concentrated formalin-killed *E. tarda* FPC503 cells showed effective protective efficacy (*p* < 0.05, Fisher’s exact test) in the first experimental run at 28 dpv (Table 1). The cumulative mortality of both vaccinated groups upon challenge was 12.5%, whilst those for the adjuvant-injected and PBS-injected control groups were 100% and 87.5%, respectively. Upon challenge, the RPS of vaccinated groups in this experiment was 85.7%. In the second experiment at 56 dpv, no mortality was observed in the vaccinated group, and the RPS of both vaccinated groups was 100%. However, the efficacy of the vaccine was not statistically significant (*p* > 0.05, Fisher’s exact test) due to the low cumulative mortality in the control groups (< 33.3%).

A significant increase (*p* < 0.05, Student’s *t*-test) in the level of antibody reactive to *E. tarda* was only observed at 10 and 20 dpv in the non-adjuvanted vaccine group when compared with the non-vaccinated control group (Fig. 1). On the other hand, no significant increase in antibody titer was detected in the adjuvanted vaccine group. This result suggests that the significant production of specific antibody against FPC503 was only induced in the non-adjuvanted vaccine group. However, we observed similar vaccine efficacy in both vaccine groups at 28 dpv (Table 1). In addition, protective efficacy was still observed in both vaccine groups even after antibody titers returned to basal levels at 56 dpv. Hence, the relationship of antibody titer and vaccine efficacy was not clear in this study.

### Table 1. Bacterial challenge of red sea bream immunized with formalin-killed cells of atypical *E. tarda*

<table>
<thead>
<tr>
<th>Experimental run</th>
<th>Challenge test at</th>
<th>Bacterial dosage of challenge test (CFU/fish)</th>
<th>Treatment</th>
<th>No. of dead fish</th>
<th>Percent mortality (%)</th>
<th>RPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>28 days post-</td>
<td>3.1 x 10^6</td>
<td>Adjuvanted vaccine</td>
<td>1/8</td>
<td>12.5</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>vaccination</td>
<td></td>
<td>Non-adjuvanted vaccine</td>
<td>1/8</td>
<td>12.5</td>
<td>85.7</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Adjuvant</td>
<td>8/8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PBS</td>
<td>7/8</td>
<td>87.5</td>
<td></td>
</tr>
<tr>
<td>Run 2</td>
<td>56 days post-</td>
<td>1.1 x 10^7</td>
<td>Adjuvanted vaccine</td>
<td>0/12</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>vaccination</td>
<td></td>
<td>Non-adjuvanted vaccine</td>
<td>0/12</td>
<td>0</td>
<td>100</td>
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<td></td>
<td>Adjuvant</td>
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<td>33.3</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td>PBS</td>
<td>2/12</td>
<td>16.6</td>
<td></td>
</tr>
</tbody>
</table>

* The relative percentage survival (RPS) was determined following the formula RPS = (1− [% loss of immunized fish/% loss of PBS-injected fish]) x 100.
An equivocal protective effect of formalin-killed vaccine against *E. ictaluri* in channel catfish *Ictalurus punctatus* and typical *E. tarda* in Japanese flounder has been suggested in previous studies. Since these bacteria are facultative intracellular pathogens, specific antibody production in the host would not be expected to protect from infection. Furthermore, atypical strains of *E. tarda* also seem to resist phagocyte killing. Components other than antibody are likely crucial in the protective mechanisms of fish immunized with the formalin-killed vaccine against a typical *E. tarda* infection.

Negative effects were observed in fish treated with oil adjuvant in this study, where the highest mortality in both experimental run 1 and 2 was observed in adjuvant-injected control group. In addition, antibody titers in the adjuvanted vaccine group were lower than those in the non-adjuvanted vaccine group. Additional analyses conducted using samples without red sea bream serum to confirm background due to non-specific binding of anti-red sea bream IgM mAb and goat anti-mouse IgG horseradish peroxidase-conjugate (Background). *Significant increase of absorbance was observed in non-adjuvanted group at 10 and 20 dpv when compared with the non-vaccinated control group (*p* < 0.05, Student's *t*-test).

In conclusion, we demonstrated the protective effects of a formalin-killed vaccine against experimental atypical *E. tarda* infection in red sea bream. Thus, this treatment could become a useful prophylactic treatment for protection from Edwardsiellosis in cultured red sea bream. However, we did not observe an improved vaccine efficacy when the oil adjuvant was co-administered with the formalin-killed vaccine. Additional analyses are required to find an appropriate adjuvant to improve vaccine efficacy. It takes 2–3 years for red sea bream to reach marketable size in Japan. Hence, further assessment of the long-term effect is needed for the practical use of this formalin-killed vaccine in the field setting.

**References**

マダイの非定型 Edwardsiella tarda 感染症に対する不活化ワクチンの効果

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マダイの非定型 Edwardsiella tarda 感染症に対する不活化ワクチンの感染防御効果を調べた。非定型 E. tarda FPC503株のホルマリン不活化菌体をワクチンとしてマダイの腹腔内に接種し、その28日および56日後矢ワクチン株で攻撃した。その結果、オイルアジュベント（Montanide ISA 763 A VG）の添加の有無にかかわらず、ワクチンの感染防御効果が認められた。ELISA法によりワクチン株に対する抗体産生が確認されたが、抗体価と感染防御効果との関係は不明瞭であった。

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