

Immune responses of flounder *Paralichthys olivaceus* vaccinated by immersion of formalin-inactivated *Edwardsiella tarda* following hyperosmotic treatment

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ABSTRACT: The aim of the present study was to evaluate the effects of hyperosmotic immersion (HI) vaccination and determine the optimum hyperosmotic salinity for flounder *Paralichthys olivaceus* by investigating its immune responses following vaccination. Flounder were immersed in 1 of 3 hyperosmotic solutions at 50, 60 and 70‰ salinity, then transferred into 30‰ salinity normal seawater containing formalin-inactivated *Edwardsiella tarda* for vaccination (3 HI groups), or were immersed in normal seawater as direct immersion (DI group). The results showed that the percentages of surface membrane immunoglobulin-positive (sIg+) cells in peripheral blood leukocytes and spleen leukocytes induced by HI were significantly higher than that with DI ($p < 0.05$), and the 50‰ salinity group showed the strongest response among the HI groups, which reached peaks at Week 4. ELISA assay showed that the specific serum antibodies gradually increased after vaccination and reached peak at Day 32, and the fish treated with HI showed stronger antibody responses; among the HI groups, a significantly higher specific antibody level was detected in the 50‰ salinity group at Day 32 ($p < 0.05$). Similarly, the fish treated with HI showed higher specific mucosal antibody levels compared to the DI group, and the mucosal antibody showed a faster response, with peak time arriving 1 wk earlier than for the serum antibody. The relative percent survival (RPS) of flounder treated with HI at 50, 60 and 70‰ salinities were 79, 71 and 57% respectively, while this was 43% in the DI group. These results demonstrated that HI, especially the 50‰ salinity, could efficiently enhance the immune response of flounder and show higher RPS. This has significant value for immunological prevention of edwardsiellosis in flounder.

KEY WORDS: Flounder · *Paralichthys olivaceus* · *Edwardsiella tarda* · Hyperosmotic immersion · Immune response

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INTRODUCTION

Edwardsiella tarda, as the causative agent of edwardsiellosis, was one of the most serious bacterial pathogens in fish culture (Bang et al. 1992, Leung et al. 2012). The disease resulted in extensive economic losses and became a concern to the aquaculture industry. Immunoprophylaxis has become an impor-

tant strategy for fish disease prevention and control, benefiting the economic and environmental sustainability of the fish-farming industry. Therefore, vaccines are considered the most effective and secure strategy to resolve infectious diseases, and are widely used for disease control in the fish industry (Plant & Lapatra 2011). A great many effective vaccine candidates against edwardsiellosis have been

developed, such as whole-cell bacterins, live attenuated *E. tarda*, cell ghosts, cell contents, recombinant vaccines and DNA vaccines (Sommerset et al. 2005, Park et al. 2012, Brudeseth et al. 2013). Among these vaccine candidates, inactivated whole-cell bacterins were the most widely adopted prophylactic strategy, as they offer an economical, safe, effective and convenient way of preventing edwardsiellosis (Shutou et al. 2007, Dumrongphol et al. 2009).

The bacterins were generally delivered by injection, immersion or oral administration. Immersion vaccination was first reported by Amend & Fender (1976) and became a common practice in aquaculture, with advantages in terms of reduced stress on the fish, low labor costs, easy administration for large numbers of small fish and operator safety. An additional valuable advantage is that immersion vaccination could evoke the specific mucosal immunity of fish (Lumsden et al. 1993, Gudding et al. 1999, Dos Santos et al. 2001, Feng et al. 2009). Immersion vaccination can be effectively performed by direct immersion (DI), spray and flush (Amend & Fender 1976, Gould 1978, Anderson et al. 1979). However, immune responses following immersion vaccination are generally less robust and of shorter duration than those obtained through injection (Crosbie & Nowak 2004, Xu et al. 2009, Valdenegro-Vega et al. 2013). So, the most important issue for researchers was how to increase the uptake of vaccines by fish mucosal tissues to maximize the efficacy of immersion vaccination. For this purpose, several specific methods were employed to improve immersion vaccine efficacy, such as hyperosmotic immersion (HI), ultrasound-mediated immersion and adjuvant for immersion of fish (Thune & Plumb 1984, Zhou et al. 2002, Huising et al. 2003, Navot et al. 2004, Cobo et al. 2013, 2014, Soltani et al. 2014). Among these strategies, HI could increase the antigen uptake and enhance the antibody response and protection rate, and is considered an effective and promising method. However, to our knowledge, little information concerning the effects of HI vaccination on flounder has been reported, and the optimal hyperosmotic salinity still needs to be researched.

The aim of the present study was to evaluate the effects of HI vaccination and determine the optimum hyperosmotic salinity for flounder by investigating the immune responses following vaccination. Flounder were vaccinated by immersion of formalin-inactivated *E. tarda* following hyperosmotic treatments, then the percentages of surface membrane immunoglobulin-positive (sIg+) cells in peripheral blood leukocytes (PBL) and spleen leukocytes (SL) were

monitored by flow cytometry, and the specific antibody levels in serum and mucus were detected by indirect ELISA. Additionally, relative percent survival (RPS) was determined by challenge tests.

MATERIALS AND METHODS

Experimental fish

Apparently healthy flounder *Paralichthys olivaceus* (weight: 15–25 g, body length: 10–15 cm) were obtained from a fish farm in Rizhao, Shandong Province, PR China. The experimental fishes were then randomly selected and subjected to standard microscopic and bacteriological examination to ensure that they were uninfected with *Edwardsiella tarda*. A total of 1000 fish were kept in the laboratory, supplied with running seawater at 20–22°C, and fed daily with dry food pellets. The salinity of the running seawater was 27–29‰, and the dissolved oxygen concentration was $6.5 \pm 0.5 \text{ mg l}^{-1}$. After acclimation to laboratory conditions for 1 wk, fish were randomly divided into 5 groups for the immersion vaccination experiments (3 different HI groups, a DI group, and a blank control group).

Preparation of inactivated *E. tarda* bacterin

The strain of *E. tarda* HC01090721 was previously isolated from the ascites of Japanese flounder *P. olivaceus* and stored in saline with 15% glycerol at –80°C in our laboratory (Tang et al. 2010). The *E. tarda* bacterin was prepared according to procedures described previously (Feng et al. 2009). Briefly, frozen stocks were directly inoculated onto brain heart infusion (BHI) agar plate and cultured at 37°C for 48 h, and then single clones were selected and transferred into BHI broth for shaking incubation at 37°C. After reaching the stationary phase, bacteria were harvested and washed with 0.01 M phosphate-buffered saline (PBS; pH 7.2) by centrifuging. The concentration of bacteria was calculated directly using an Accuri C6 flow cytometer (BD Biosciences). The bacteria suspension with the concentration $2.5 \times 10^9 \text{ cfu ml}^{-1}$ was treated with 0.5% formalin (v/v) for 72 h at 4°C, and inactivation of the bacteria was confirmed by incubating the solution on BHI agar at 37°C. The inactivated cells were washed 3 times with sterilized PBS by centrifuging at $8000 \times g$ for 15 min. After the last wash, the concentration of bacterin suspension was adjusted to $1.0 \times 10^{10} \text{ cfu ml}^{-1}$ and stored at 4°C until

use. The safety of the bacterin was checked again by intraperitoneally injecting 0.5 ml of the bacterin into healthy flounder at a density of 1.0×10^8 cfu ml⁻¹.

Immersion vaccination

The vaccine solution was prepared by diluting the stored inactivated *E. tarda* to a concentration of 1.0×10^8 cfu ml⁻¹ with normal seawater at a salinity of 30‰ (Feng et al. 2009). HI operations were done as described by Huising et al. (2003). Different hyperosmotic solutions at salinities of 50, 60 and 70‰ were prepared and aerated 1 d in advance. For HI, fish were immersed in one of the 3 hyperosmotic solutions for 20 min and then immediately transferred to the vaccine solution for 30 min. The fish without hyperosmotic treatment were directly immersed in the vaccine solution for 30 min and served as negative controls (DI group). The fish that were exposed to neither hyperosmotic solution nor vaccine solution served as blank controls.

Sampling

The skin mucus was sampled on Days 3, 5, 7, 11, 18, 25, 32 and 39 after vaccination. Six fish from each group were anesthetized and the skin mucus was collected by gently scraping one side of the fish from head to tail using a slide and then mixed together, with enough care not to damage the skin to avoid contamination with blood. Subsequently, the skin mucus was homogenized with an equal volume of PBS and vigorously vortexed for 2 min, then centrifuged at $15\,000 \times g$ for 30 min at 4°C. The supernatant was collected and frozen at -20°C until use. After the mucus sampling, the blood of the 6 fish was drawn by caudal venepuncture with a sterile needle and clotted at 4°C overnight. The serum was obtained by centrifugation at 4°C, pooled together and stored at -20°C for analysis.

Six fish from each experimental group were sampled at Weeks 1, 2, 3, 4, 5 and 6 after vaccination for leukocyte isolation according to procedures described previously (Tang et al. 2010). Briefly, blood was drawn from the caudal vein, diluted 1:1 in solution (65% RPMI-1640 containing 20 IU ml⁻¹ heparin, 0.1% w/v NaN₃ and 1% w/v BSA), and stored at 4°C for 1 h, then centrifuged at $100 \times g$ for 10 min to get rid of partial red blood cells. After the blood draw, the spleen of sample fish was excised. The cell suspensions of spleen were prepared by squeezing the tis-

sue through a nylon gauze filter. Subsequently, the cell suspensions of blood and spleen were laid over a discontinuous Percoll gradient and centrifuged at $840 \times g$ for 30 min. The leukocytes at the Percoll interface were collected and washed 3 times with PBS containing 5% (v/v) newborn calf serum by centrifugation at $640 \times g$, and then suspended in PBS for fluorescence staining and flow cytometric analysis.

Flow cytometric analysis

The cell density of leukocytes was adjusted to 1×10^6 cells ml⁻¹ in PBS, and this was then incubated with monoclonal antibody (MAb) 2D8, which was previously produced by our laboratory (Li et al. 2007). After 1 h incubation at 37°C with gentle shaking, the leukocytes were washed 3 times with PBS containing 5% (v/v) newborn calf serum by centrifugation at $640 \times g$ for 5 min, then incubated with goat anti-mouse Ig-fluorescein isothiocyanate (FITC) (1:256; Sigma) for 1 h at 37°C. After washing as above, PBL and SL were suspended in 1 ml PBS for flow cytometric analysis using the MXP flow cytometer (Beckman Coulter). Further processing of the data was performed using WinMDI 2.9 software. Myeloma culture supernatant was substituted for MAb 2D8 as the negative control.

ELISA

All steps were carried out in 96-well microplates (Costar) for specific antibody detection in serum and mucus using an indirect ELISA according to previous studies (Tang et al. 2010, Xu et al. 2011). Between each successive step, the wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T). Briefly, formalin-inactivated *E. tarda* (1.0×10^8 cfu ml⁻¹) were coated on the flat bottom microplates in carbonate buffer (CB buffer, 35 mM NaHCO₃, 15 mM Na₂CO₃, pH 9.6) in triplicate and this was incubated overnight at 4°C. The following day, wells were blocked with 200 µl of 3% BSA for 1 h at 37°C. Then 100 µl per well of serum (1:50 diluted in PBS) or mucus (1:1 diluted in PBS) collected after vaccination was added and this was incubated for 2 h at 37°C. MAb 2D8 (100 µl) diluted 1:1000 in PBS was added and this was incubated for 1 h at 37°C. Then 100 µl per well of goat anti-mouse Ig-alkaline phosphatase conjugate (Sigma) diluted 1:5000 in PBS was added followed by incubation for 1 h at 37°C. Finally, 100 µl of 0.1% (w/v) *p*-nitrophenyl phosphate (*p*NPP; Sigma) in CB buffer containing 0.5 mM MgCl₂ was added to each well and

this was incubated for 30 min at room temperature in the dark. The reaction was stopped with 50 μ l per well of 2 M NaOH, and absorbance was measured with an automatic ELISA reader at 405 nm (Molecular Devices). As a control, myeloma culture supernatant instead of MAb 2D8 was used.

Challenge

Thirty fish were randomly selected from each experimental group for a challenge test at Day 42 after immunization. Fish were challenged by intraperitoneal injection with live *E. tarda* HC01090721, which was cultured in BHI broth at 37°C for 24 h. The challenge dose was 100 μ l (containing 5.0×10^6 cfu

per fish. The concentration of bacteria was determined using the Accuri C6 flow cytometer. Mortality was recorded for 21 d post-challenge. The fish without vaccination were challenged as control. RPS was calculated as previously described (Croy & Amend 1977).

Statistics

Data were given as arithmetic mean values. Statistical analysis was performed using the software SPSS 19. One-way ANOVA and Duncan's multiple comparisons of the means were done to compare the data obtained. Differences were considered statistically significant when $p < 0.05$.

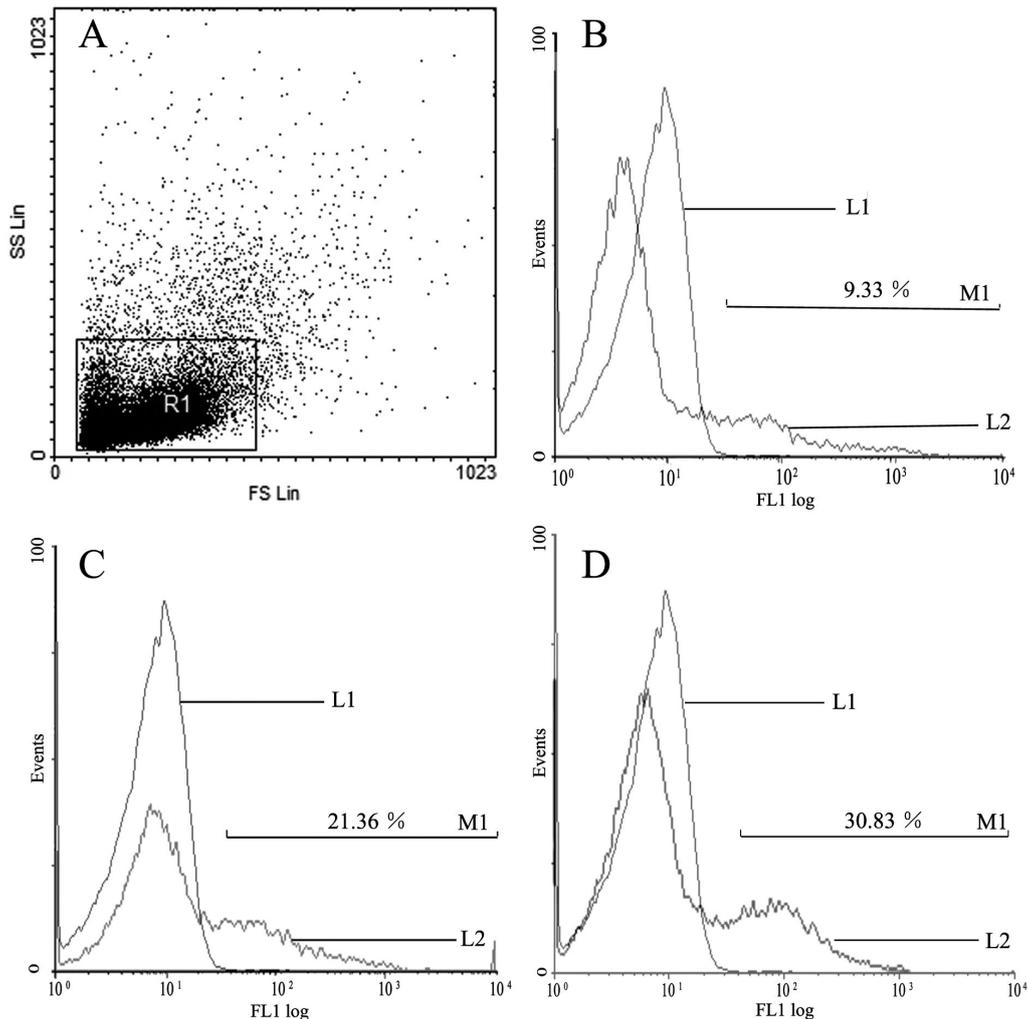


Fig. 1. Flow cytometric analysis of leukocytes in spleen of *Paralichthys olivaceus*. (A) Leukocytes were gated (R1) on forward and side scatter (FS-SS) dot plot; (B–D) combined fluorescein isothiocyanate (FITC) fluorescence histogram of gated leukocytes showing the percentage of surface membrane immunoglobulin-positive (sIg+) lymphocytes in total gated leukocytes (scale of M1) at (B) Week 4 in the blank control, (C) Week 3 in the 50‰ salinity group and (D) Week 4 in the 50‰ salinity group

RESULTS

Responses of sIg+ cells following immersion vaccination

The forward and side scatter (FS-SS) dot plots gated on leukocytes and 3 representative combined fluorescence histograms are shown for SL (Fig. 1) and PBL (Fig. 2) at Weeks 3 and 4 after vaccination in the 50‰ salinity group, together with the blank control group at Week 4. All the FS-SS dot plots of PBL and SL displayed very similar profiles, and the fluorescence histograms show that the leukocytes stained with myeloma culture supernatant only had a single peak (L1) with very weak self-fluorescence, which was used as a negative control. The leukocytes stained with MAb

2D8 exhibited 2 peaks on the fluorescence histogram, and the second peak (L2) with strong fluorescence indicates the subpopulation of sIg+ lymphocytes. Based on the histograms, the percentages of sIg+ cells in blood and spleen have an obvious rise with different levels after vaccination. The sIg+ lymphocytes in spleen showed a much higher level than in blood, which was also effectively triggered and increased to a high level (30.83% in spleen) at Week 4 after HI vaccination in the 50‰ salinity group.

Changes in the percentages of sIg+ lymphocytes in SL and PBL in different vaccination groups during 6 wk after immunization are summarized in Figs. 3 & 4, respectively. In the blank control, the percentages of sIg+ cells in SL and PBL remained stable during the experimental period, whereas in the vaccinated

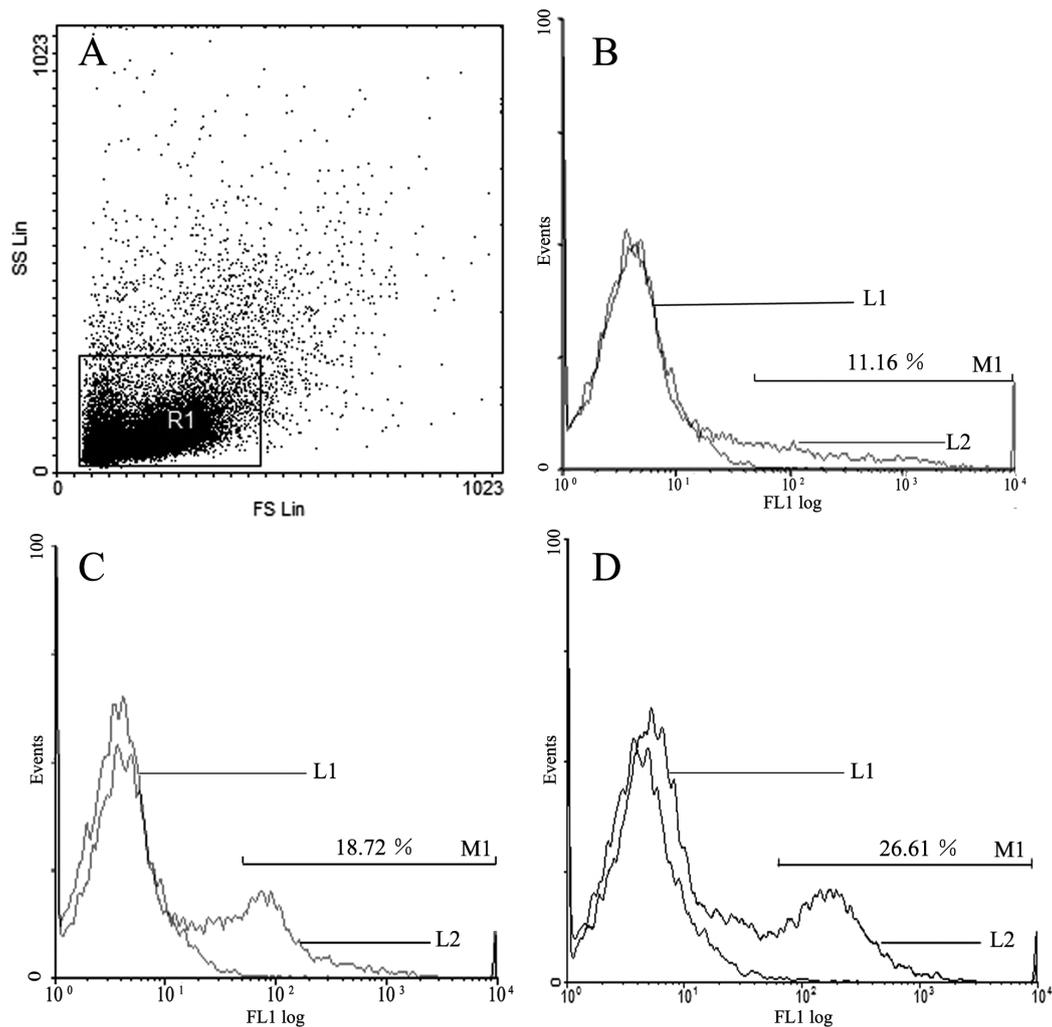


Fig. 2. Flow cytometric analysis of leukocytes in peripheral blood of *Paralichthys olivaceus*. (A) Leukocytes were gated (R1) on forward and side scatter (FS-SS) dot plot; (B–D) combined fluorescein isothiocyanate (FITC) fluorescence histogram of gated leukocytes showing the percentage of surface membrane immunoglobulin-positive (sIg+) lymphocytes in total gated leukocytes (scale of M1) at (B) Week 4 in the blank control, (C) Week 3 in the 50‰ salinity group and (D) Week 4 in the 50‰ salinity group

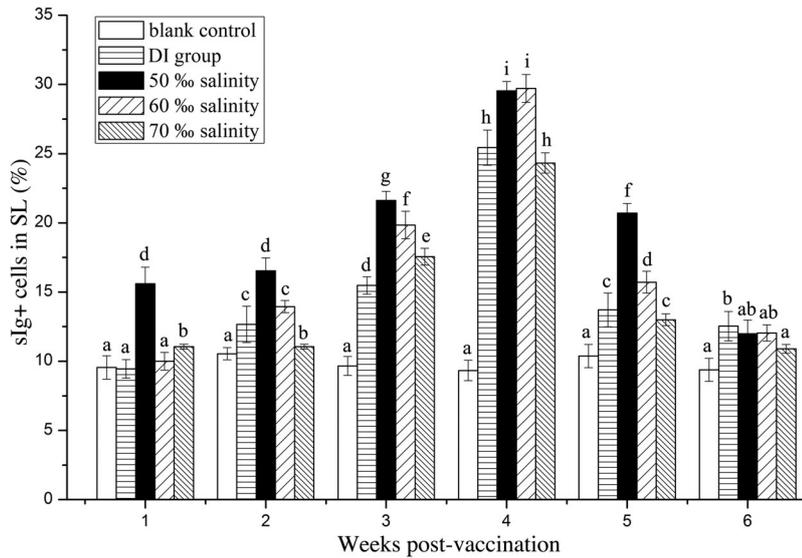


Fig. 3. Percentage of surface membrane immunoglobulin-positive (sIg+) cells in spleen leukocytes (SL) of *Paralichthys olivaceus* following immersion vaccination with inactivated *Edwardsiella tarda*. Values are mean \pm SE of 3 replicates, and different letters denote significant differences among groups ($p < 0.05$). DI: direct immersion

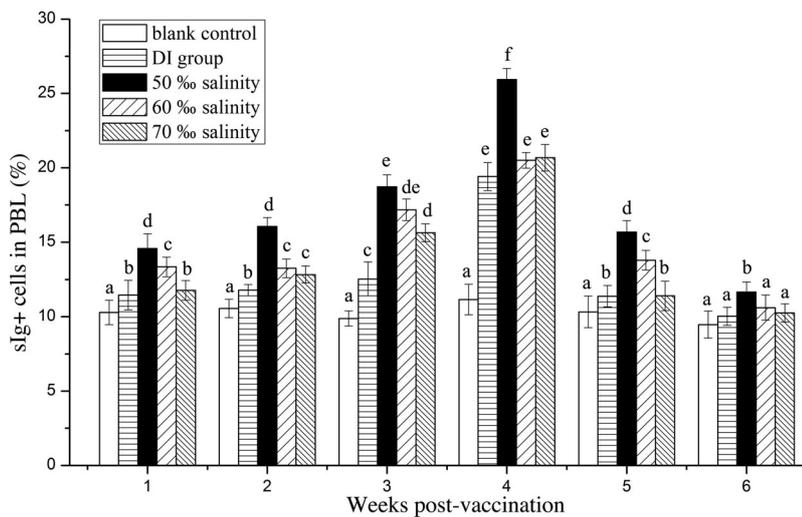


Fig. 4. Percentage of surface membrane immunoglobulin-positive (sIg+) cells in peripheral blood leukocytes (PBL) of *Paralichthys olivaceus* following immersion vaccination with inactivated *Edwardsiella tarda*. Values are mean \pm SE of 3 replicates, and different letters denote significant differences among groups ($p < 0.05$). DI: direct immersion

groups, the number of sIg+ cells in the 2 lymphoid organs showed a marked rise, and the percentages of sIg+ cells in PBL were significantly higher ($p < 0.05$) than the blank control group 1 wk after immunization. From Week 2 after vaccination, the percentages of sIg+ cells in SL and PBL were significantly higher than the blank control group and reached their peak levels at Week 4 after immunization, then gradually decreased.

In SL and PBL, the percentages of sIg+ cells in the 50 and 60‰ salinity HI groups were significantly higher than that of the DI group ($p < 0.05$), and the highest level was present in the 50‰ salinity group at Week 4, whereas there was no significant difference between the 50‰ and 60‰ salinity groups in SL. The percentages of sIg+ cells in the 50‰ salinity group ascended quickly after vaccination and reached peak level (30.83% in SL, 26.61% in PBL) at Week 4, and these levels were significantly higher than the other HI groups during the experimental period ($p < 0.05$). Compared to the DI group, the percentages of sIg+ cells in the 60‰ salinity group showed higher level from Week 3 to Week 5 ($p < 0.05$) and peaked (30.21% in SL, 21.05% in PBL) at Week 4. However, there was no significant difference between the 70‰ salinity group and the DI group ($p > 0.05$).

Serum and mucosal antibody responses

The change in specific serum antibodies in different experimental groups after vaccination is shown in Fig. 5. Compared to the blank control, the specific antibody levels in the serum of all vaccinated groups shared the same dynamics trend. The levels increased steadily and peaked at Day 32, then descended slowly. There was no significant difference in the specific antibodies between the HI and DI groups within the first 11 d ($p > 0.05$), whereas the specific antibody levels of the HI groups were significantly higher than the DI group from Day 18 onwards ($p < 0.05$). Among the 3 HI groups, the specific antibody levels in the 50‰ salinity group were significantly higher than the other 2 HI groups. In the 70‰ salinity group, antibody levels were not significantly different from the DI group at Days 32 and 39 ($p > 0.05$), but were significantly lower at Day 25 ($p < 0.05$).

The change in specific mucosal antibodies is shown in Fig. 6. Compared to the blank control, the specific mucosal antibody levels in the mucus of the vac-

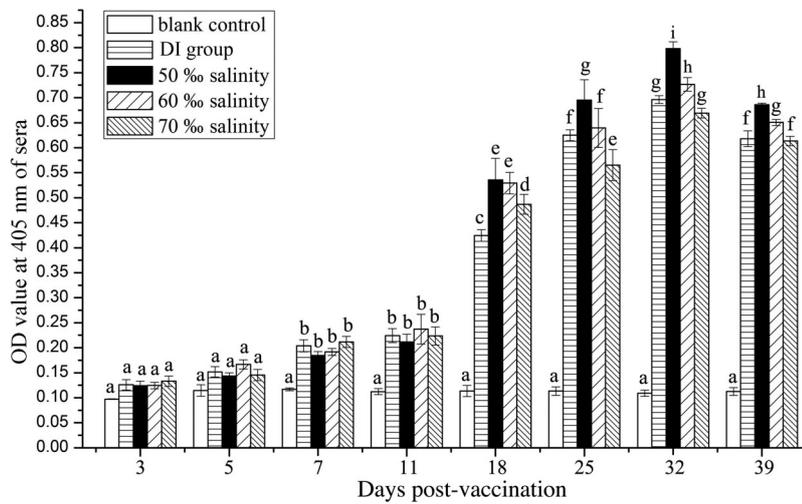


Fig. 5. Absorbance of specific antibodies from the sera of *Paralichthys olivaceus* following immersion vaccination with inactivated *Edwardsiella tarda*. Values are mean \pm SE of 3 replicates, and different letters denote significant differences among groups ($p < 0.05$). OD: optical density; DI: direct immersion

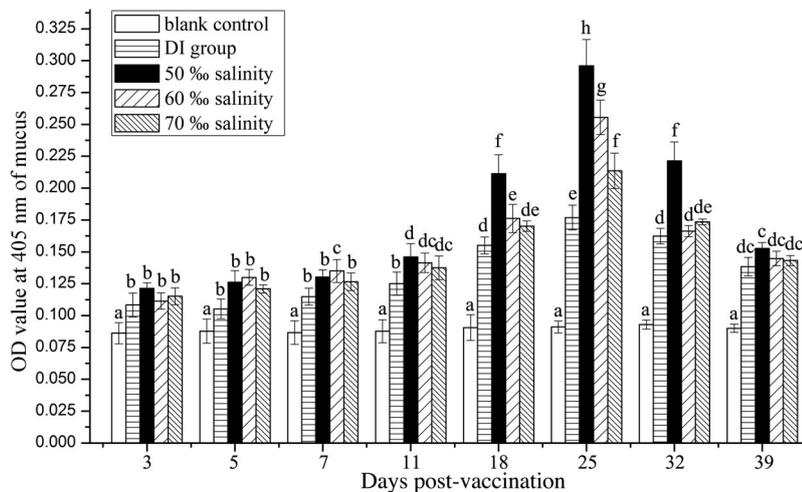


Fig. 6. Changes of specific antibodies in the mucus of *Paralichthys olivaceus* following immersion vaccination with inactivated *Edwardsiella tarda*. Values are mean \pm SE of 3 replicates, and different letters denote significant differences among groups ($p < 0.05$). DI: direct immersion

nated groups ascended steadily after vaccination and reached peak levels at Day 25, then descended slowly. After 3 d post-vaccination, the values in all the vaccinated groups were significantly higher than the blank control group ($p < 0.05$). Compared to the DI group, the HI groups showed significantly higher levels at Days 11 and 25 post-vaccination ($p < 0.05$). The 50‰ salinity group had the highest level among the 3 HI groups, and was significantly higher than the other 2 HI groups from Day 18 post-vaccination onwards ($p < 0.05$), until Day 39, when no significant difference was seen among the 4 vaccinated groups.

Protection against *Edwardsiella tarda* infection

Fish cumulative mortality rates in all the experimental groups after being challenged with live *E. tarda* are shown in Fig. 7. The fish began to die 3 d after challenge, and the cumulative mortality rate of the blank control group increased rapidly at 6–14 d and reached 93.33% at Day 15. Infected flounder exhibited typical clinical signs of edwardsiellosis, including abdominal distension and rectal hernia, exophthalmia and opacity of the eye, and peripheral hyperemia in mandible lesion; and pure cultures of *E. tarda* were re-isolated from liver, kidney and ascites in the moribund flounder. However, the fish treated with DI and HI had significantly lower cumulative mortality rates than the blank control group ($p < 0.05$). RPS varied among the different vaccination groups. The RPS of flounder treated with HI at 50, 60 and 70‰ salinities were 79, 71 and 57% respectively, and the RPS was 43% in the DI group.

DISCUSSION

Though cellular and humoral responses following intraperitoneal injection with immunogen have been investigated in flounder (Tang et al. 2010, Xu et al. 2011), little information concerning the effect of HI on the immune responses has been reported.

The present study monitored the changes in sIg+ cells in SL and PBL, the specific antibody levels in serum and mucus of flounder after HI and DI vaccination, and the protection efficacies of different vaccination groups. Based on the immune responses and protection efficacies, the immune response enhancement by HI was analyzed and the optimum salinity for HI in flounder was determined.

Previous studies have found that stronger immune responses and higher immune protection against pathogens could be elicited using HI in rainbow trout (Fender & Amend 1978), sockeye salmon (Antipa et al. 1980), channel catfish (Thune & Plumb 1984) and

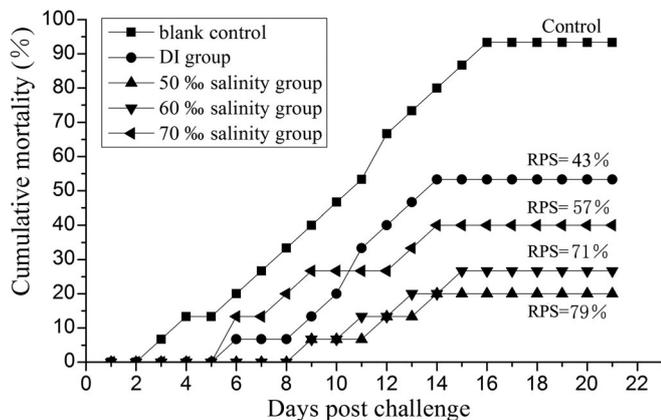


Fig. 7. Cumulative mortality rates of *Paralichthys olivaceus* in different experimental groups after challenge with live *Edwardsiella tarda* on Day 42 post-vaccination. DI: direct immersion; RPS: relative percent survival

common carp (Huising et al. 2003). The present study showed that the sIg+ cell levels and the specific serum and mucosal antibodies of flounder in HI groups were significantly higher than in the DI group, and higher immune protection rates were also gained in the HI groups, which possibly suggests that there was a close positive correlation between the immune levels and the protection efficacies against *Edwardsiella tarda*.

In the present study, the strongest immune response and the highest protection efficacy in flounder were observed in the 50‰ salinity group, while the weakest was in the 70‰ salinity group. This phenomenon might be because the salinity tolerance of the fish is within certain limits; a suitable salinity treatment for a certain amount of time could result in a temporary disruption of the integrity of the mucosal epithelia, with this mild damage recovering quickly yet having the adjuvant functions of facilitating antigen entry and stimulating mucosal immunity. When salinity is set too high, unrecoverable damage to the mucosal epithelia would be caused, resulting in loss of capacity to respond to vaccination (Huising et al. 2003). In the present study, we found that flounders in the 70‰ salinity solution exhibited a little stress and had abnormal behaviors, including being anxious and active, breathing fast and swimming to the water surface, whereas no obvious stress response was observed in the 50‰ salinity group. Conversely, if salinity was too low and the immersion period too short, there was likely insufficient time for the cellular structures response on the mucosal surface, and the ideal vaccination effect also cannot be obtained (Fishelson 1980, Jonassen et al. 1997, Morgan & Iwama 1998). Therefore, only immersion under the proper

hyperosmotic salinity for a suitable time period would maximize the vaccination effect; further research is needed to determine the optimal immersion parameters of time, salinity, temperature and vaccine concentration.

In teleosts, the skin, gills and gut are the main mucosal surfaces and physical barriers to infection, which play important roles in immune defense (Linden et al. 2008, Gomez et al. 2013). In the present study, we interestingly found that the mucosal antibody exhibited a faster response and the peak time arrived a week earlier than the serum antibody, which indicated that immersion vaccination could quickly activate the mucosal immune system of flounder, and then mucus antibody was produced rapidly by local mucosal antibody secretion cells. This speculation could be supported by several studies, which found differential binding of monoclonal antibodies to mucosal- and serum-derived IgM in common carp and flounder (Rombout et al. 1993, Sheng et al. 2012), suggesting that structural differences exist between mucosal- and serum-derived antibodies. On the other hand, it takes longer for an antigen to be transferred from the mucosal system to stimulate the systemic immune system for serum antibody production (Hatten et al. 2001, Luo et al. 2007).

This study evaluated the immune effect by measuring fish survival upon infective challenge, and also by examining cellular and humoral immune response levels; this provides a tentative approach to evaluating the effect of various vaccination strategies. Based on the cellular and humoral immune responses of flounder treated with DI and HI, the HI can effectively enhance the immune response and the protection against *E. tarda*, and the 50‰ salinity HI could elicit the highest immune response, suggesting its use as the reference treatment concentration for HI vaccination against *E. tarda* for flounder in practical applications.

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