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Efficacy of Montanide™ ISA 763 A VG as aquatic adjuvant administrated with an inactivated *Vibrio harveyi* vaccine in turbot (*Scophthalmus maximus* L.)

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ABSTRACT

Turbot (*Scophthalmus maximus* L.) is a commercially important fish species in China. Despite of its great economic potential, fish farms often suffer severe economic losses due to certain fish diseases. Vaccination has become a common strategy to prevent diseases caused by pathogens in aquaculture industry. However, no inactivated vaccine against *Vibrio harveyi* of turbot has been reported so far. In this study, we developed an inactivated vaccine using formalin-killed cells of *V. harveyi* and the efficacy of a commercial adjuvant Montanide™ ISA 763 A VG on the inactivated vaccine was evaluated. We found that with an optimum vaccine dosage at 1.0×10^8 CFU/fish, a high relative percent survival (RPS) more than 75% was observed at 4 weeks post vaccination (w.p.v.). Moreover, enhanced antibody titer, lysozyme activity, total serum protein and antibacterial property in sera of vaccinated fish were observed at 4, 8, 12 and 16 w.p.v. In conclusion, we developed an efficient inactivated vaccine against *V. harveyi* in turbot, which not only induced humoral immunity, but also enhanced initial innate immune response for long-term protection.

1. Introduction

Turbot (*Scophthalmus maximus* L.) is a commercially important fish species in China, mainly cultured in Shandong, Liaoning and Hebei province. However, many diseases such as ascites disease, vibriosis and exophthalmic disease often threaten turbot industry, causing serious economic losses [1]. Among these, *Edwardsiella tarda* [2] and *Vibrio harveyi* [3] are the major pathogens, as well as other pathogens such as *Aeromonas salmonicida* subsp. [4], scuticociliate [5], and even viral haemorrhagic septicaemia virus [6].

V. harveyi, a Gram-negative bacterium commonly found in aquatic environment has been reported as a pathogen capable of affecting both vertebrates and invertebrates [7]. For instance, it is the causative agent of luminous vibriosis, affecting shrimp [8], oysters [9] and lobsters [10]. As well, it infects many fish species, such as Japanese flounder (*Paralichthys olivaceus*) [11], sea bream (*Sparus aurata*) [12] and Senegalese sole (*Solea senegalensis*) [13].

Traditionally, antibiotic therapy is a common treatment for pathogenic disease. However, the rise in prevalence of multi-drug resistant pathogens in aquaculture creates an urgent need for more effective solutions. Many vaccines against *V. harveyi* were developed so far. For example, a glutathione peroxidase DNA vaccine of *V. harveyi* ZJ0603 was constructed and showed effective immune protection to orange-spotted grouper (*Epinephelus coioides*) [14]. As well, a formalin-inactivated vaccine provided good protection against *V. harveyi* infection in orange-spotted grouper [15]. Among which inactivated vaccine has been considered as an ideal solution for aquaculture industry due to its effectiveness against extracellular bacteria and relatively low cost [16]. Despite of many efforts in developing different vaccines against *V. harveyi* for turbot [15,17], there is no report on the development of inactivated vaccine against *V. harveyi* in turbot. Since there are still some drawbacks such as short duration [17] and low efficiency of immunoprotection [18], inactivated vaccines are often administrated with adjuvants to enhance the vaccine efficiency. Several adjuvants such as

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oil emulsions, nano/microparticles and even cytokines have been attempted in aquaculture [19]. For example, Soltani et al. used Montanide™ IMS 1312 VG as an adjuvant for *Yersinia ruckeri* vaccine [20]. Freund's complete adjuvant (FCA) has been used for developing vaccines against *Streptococcus iniae* [21], *Aeromonas hydrophila* [22], and *A. salmonicida* [23]. In our previous study, we also found chitosan oligosaccharide was an efficient adjuvant administrated with a formalin-inactivated *Vibrio anguillarum* vaccine [24].

In this study, we developed an inactivated vaccine using formalin-killed cell (FKC) of *V. harveyi* and the efficacy of a commercial adjuvant Montanide™ ISA 763 A VG on this vaccine was evaluated in turbot. Moreover, some immune parameters such as antibody titer, lysozyme activity, total serum protein and antibacterial property in sera from vaccinated turbot were determined and the side effects of the adjuvant for residual *in vivo* were studied as well.

2. Materials and methods

2.1. Fish maintenance

Healthy turbots of 35.0 ± 5.0 g were purchased from a commercial fish farm (Tianyuan, Shandong, China) and maintained in aerated tanks which supplied with a continuous flow of sand-filtered seawater at 15.0 ± 1.0 °C. Turbots were fed twice daily with commercial feed and acclimated at least one week before experiments. Turbots were randomly selected for examination of bacterial recovery from liver, kidney and spleen on thiosulfate citrate bile salts sucrose agar (TCBS, Difco, USA) plates to confirm that the turbot were not infected by *V. harveyi*. Before vaccination, turbots were anaesthetized with MS-222 (100 ng/ml) in seawater. All fish experiments were carried out according to the guidelines and approval of the Animal Research and Ethics Committees of East China University of Science and Technology.

2.2. Preparation of inactivated vaccine

V. harveyi strain used in this study was isolated from diseased turbot suffering from vibriosis occurring in an aquaculture farm in Shandong of China. The strain was cultured on TCBS at 28 °C for 24 h. Colonies from fresh TCBS were sub-cultured into LuriaeBertani (LB) mediums supplemented with 2% sodium chloride (LB20) and were harvested by centrifugation at 5000g for 10 min. Then, cells were washed twice in sterile 2% sodium chloride (2% NaCl). Further, bacterial culture was inactivated with 0.5% formalin at 28 °C for 48 h and the death of bacteria was checked as evidenced by the lack of growth on the TCBS plate after incubating for five days. After confirming the bacteria inactivated thoroughly, appropriate amount of inactivated cells were dissolved in 2% NaCl and mixed with Montanide™ ISA 763 A VG (763 A, Seppic, Paris, France) in a ratio of 3:7 equably. The inactivated vaccine was stored at 4 °C until use.

2.3. Vaccination and challenge

To determine the optimum vaccine dosage of the inactivated vaccine, 480 turbot were randomly divided into 3 groups (160 fish/group). In each group, fish were sub-divided into 8 groups (20 fish/group) randomly. Five groups of fish were intraperitoneally injected with different concentrations of 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/fish. Besides, the other three groups of fish were injected with 10^7 CFU/fish of FKC alone, 763 A alone, and 2% NaCl as controls. After 4 weeks, fish in each group were intramuscularly challenged with 5.0×10^6 CFU/fish of *V. harveyi*. Cumulative survival rate (CSR) was recorded for lasting 21 days and relative percent survival (RPS) was calculated according to the following formula. Both vaccination and challenge were conducted in triplicate.

$$\text{RPS} = (1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{mortality of control fish}}) \times 100\%.$$

Later, immunity duration of the inactivated vaccine was determined. 720 turbot were randomly divided into 3 groups (240 fish/group). In each group, fish were sub-divided into 2 groups (120 fish/group) randomly. One group of fish were vaccinated intraperitoneally with 100 μl of inactivated vaccine containing 1.0×10^8 CFU (V group). The other group of fish were injected with 2% NaCl as a control (C group). At 4, 8, 12, and 16 weeks post vaccination (w.p.v.), 20 fish in each V and C group were intramuscularly challenged with 5.0×10^6 CFU/fish of *V. harveyi*, respectively. Meanwhile, five fish were anaesthetized for blood sampling and dissection. The experiment was conducted in triplicate.

2.4. Specific antibody detection

Blood was extracted from vaccinated fish and control fish at 4, 8, 12, and 16 w.p.v. Sera were collected after centrifugation at 3000 rpm for 10 min and stored at −80 °C until use. Antibody titers in turbot sera against *V. harveyi* were determined using a modified ELISA method. Briefly, microplate was coated with 1.0×10^8 CFU/ml *V. harveyi* in 100 μl/well coating buffer (50 mM carbonate buffer, pH 9.6) at 4 °C overnight. Wells were washed in PBS with 0.05% Tween-20 (PBST) and blocked in PBST with 1% BSA (PBSTB) at 22 °C for 2 h. After blocking, sera from vaccinated fish and control fish were diluted with a 1:512 dilution and added into wells (100 μl/well) in duplicate, respectively. After incubation at room temperature (RT) for 3 h, microplate was washed three times with 300 μl/well PBST, and 100 μl/well of mouse-anti-turbot IgM (Aquatic DiagnosticsLtd, Stirling, UK, 1: 33 dilution in PBSTB) was added to each well. Then, microplate was washed three times with 300 μl/well PBST after incubation at RT for 1 h, followed by incubation with 100 μl/well goat-anti-mouse IgG conjugated to HRP (Abgent, San Diego, CA, USA, 1:200 dilution in PBSTB) for 1 h. Finally, microplate was washed five times with 300 μl/well PBST, and 100 μl/well TMB was added. After incubation for 10 min at RT, 50 μl/well of H_2SO_4 (2 M), which was used as a stop solution, was added to each well. Finally, the absorbance of the solution was determined at OD_{450} using a microplate reader. Each sample was assessed in duplicate.

2.5. Lysozyme activity

Lysozyme activity in sera of turbot was evaluated using a lysozyme assay kit (Cat. No: A050-1, Nanjing Jiancheng Bioengineering Institute, China) according to Zhu et al. with slight changes [25]. Briefly, 200 μl of serum samples, standard solutions, and distilled water were placed on ice, added with 2 ml of bacterial suspension (*Micrococcus lysodeikticus*), respectively, and incubated at 37 °C for 15 min. Double distilled water was used as a blank. Lysozyme activity was evaluated at 530 nm and reported as mg/ml.

2.6. Total serum protein

Total serum protein was determined by Biuret reaction using the total protein quantification kit (Cat. No: A045-3; Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer's instructions. Briefly, 250 μl of biuret reagent was added to serum samples and protein standards or distilled water, respectively. Then the mixture were incubated at 37 °C for 30 min. Total serum protein was calculated at 562 nm following the formula.

$$\text{Total serum protein (mg/ml)} = \frac{\text{ODserum} - \text{ODwater}}{\text{ODstandard} - \text{ODwater}} \times \text{standard concentration of protein.}$$

Table 1
Primers used in RT-qPCR.

Name	Sequence (5′→3′)	EMBL accession number
β-actin	F:ATCGTGGGGCGCCCCAGGCACC R:CTCCTTAATGTCACGCACGATTTC	AY008305
IgM	F:TTGTTCCCGAAGGTGG R:CATTATGTGAGTCGCAGTAG	DQ848956
lysozyme	F:AGCCTGGCCGACTGGGTTTG R:CGCAGCGACACACAGTGAAAGTAAAG	AJ250732

2.7. RNA extraction and real-time quantitative PCR (RT-qPCR)

RNA was extracted and RT-qPCR carried out as previously described [26]. The primers are listed in Table 1.

2.8. Serum bactericidal activity assay

Serum bactericidal activity was analyzed according to our previous report with slight changes [27]. *V. harveyi* was cultured in LB20 at 28 °C for 12 h and then harvested and resuspended in sterile 2% NaCl to 5.0×10^3 CFU/ml. Turbot sera from vaccinated group and control group were then diluted in 2% NaCl and mixed with the same volume (50 μl) of bacterial suspensions. The mixture was incubated at 28 °C. At 0, 2, 4, 6, 8 and 10 h post incubation, the mixture was sampled and plated onto LB20 at 28 °C. The number of colonies that appeared on the plates was calculated after cultivation for 12 h. The assay was conducted in triplicate.

2.9. Statistical analysis

Independent-sample *t*-tests were performed for statistical significance of gene expression using SPSS software as well. Significant differences were considered present at $^*P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. Development of an inactivated V. harveyi vaccine

Firstly, a *V. harveyi* vaccine was developed by formalin inactivation and mixed with adjuvant 763 A according to the specification. Then fish were injected with different concentrations of vaccine containing 10^5, 10^6, 10^7, 10^8 and 10^9 CFU/fish with 763 A as an adjuvant and injected with 10^7 CFU/fish of FKC alone, 763 A alone, and 2% NaCl as controls, and finally challenged with 5.0×10^6 CFU/fish of *V. harveyi* at 4 w.p.v. As shown in Fig. 1, inactivated vaccine exhibited immunoprotection in a dose-dependent manner. Only 10.0% fish died after the challenge which were vaccinated with 1.0×10^9 CFU antigen while the mortality of fish injected with 1.0×10^8 CFU antigen was 15%. However, in contrast, more than 50% fish died which were vaccinated with no more than 1.0×10^7 CFU antigen, FKC alone, 763 A alone and 2% NaCl. Therefore, 1.0×10^8 CFU/fish was determined as the vaccine dose of the inactivated *V. harveyi* vaccine.

3.2. Efficacy of the inactivated V. harveyi vaccine

Later, efficacy of the inactivated *V. harveyi* vaccine was determined. Fish were injected intraperitoneally with 100 μl vaccine containing 1.0×10^8 CFU. At 4, 8, 12 and 16 w.p.v., fish were challenged with 5.0×10^6 CFU/fish of *V. harveyi*. As shown in Fig. 2 and Table 2, significant high survival rates of vaccinated fish than control fish were observed. RPS of vaccinated group at 4 w.p.v. was 75.86%, and reached the highest level of 83.87% at 12 w.p.v. Later, RPS decreased slightly to 70% at 16 w.p.v. These results suggested that the inactivated *V. harveyi* vaccine accompanied with 763A displayed an excellent protective effect in turbot for lasting 16 weeks.

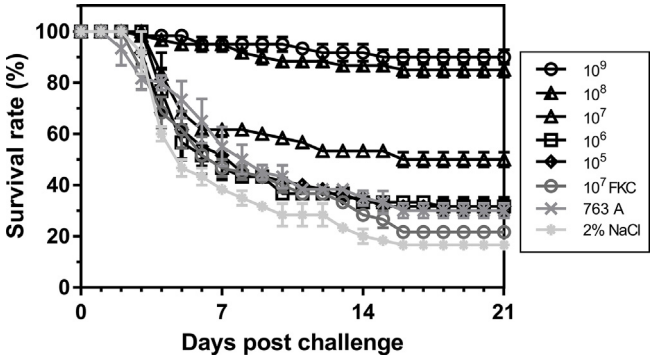

Fig. 1. Survival rate of vaccinated turbot challenged with *V. harveyi* after vaccinated with different dosages of vaccine. Briefly, five groups (20 fish/group) of fish were intraperitoneally vaccinated with different concentrations of vaccine containing 10^5, 10^6, 10^7, 10^8 and 10^9 CFU. Besides, the other three groups (20 fish/group) of fish were injected with FKC alone, 763A alone, and 2% NaCl as a control. Vaccinated fish were intramuscularly challenged with 5.0×10^6 CFU/fish of *V. harveyi* at 4 w p.v. Fish mortality was recorded for 21 days until there were no dead fish. The experiment was conducted in triplicate. Error bars represented standard deviations.

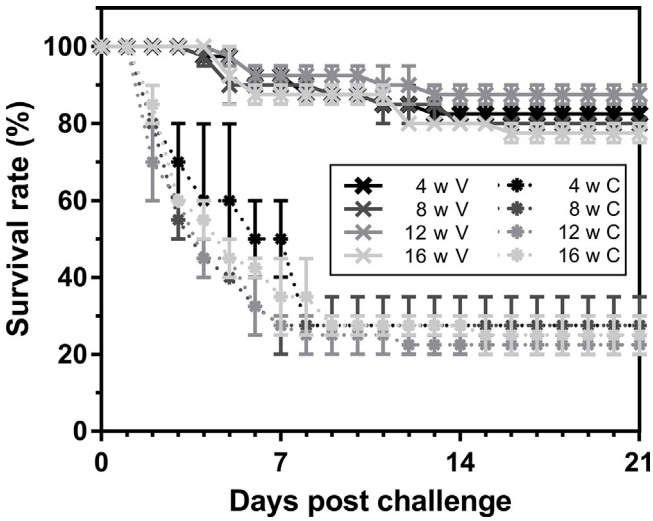

Fig. 2. Survival rate of vaccinated turbot challenged with *V. harveyi* for a long immunity duration. Briefly, 120 fish were vaccinated intraperitoneally with 100 μl of inactivated vaccine containing 1.0×10^8 CFU (V group) and 120 fish were injected with 2% NaCl as controls (C group). At 4, 8, 12, and 16 w post vaccination (p.v.), 20 fish in V and C group were intramuscularly challenged with 5.0×10^6 CFU/fish of *V. harveyi*. Fish mortality was recorded for 21 days until there were no dead fish. The experiment was conducted in triplicate. Error bars represented standard deviations. The rest fish were used for sampling and dissection as mentioned in Figs. 3–5.

Table 2
RPS of turbot vaccinated with inactivated *V. harveyi* vaccine.

w.p.v.	4	8	12	16
RPS (%)	75.86	72.41	83.87	70

3.3. Immune parameters in sera from vaccinated turbot

3.3.1. Antibody titers

Specific antibody production in sera against *V. harveyi* of vaccinated turbot were determined. Blood was extracted from fish of vaccinated group and control group at 4, 8, 12 and 16 w.p.v. As shown in Fig. 3, a first rise then descend trend of antibody titer was observed in vaccinated fish when the sera were diluted to 1:512, and the highest level of

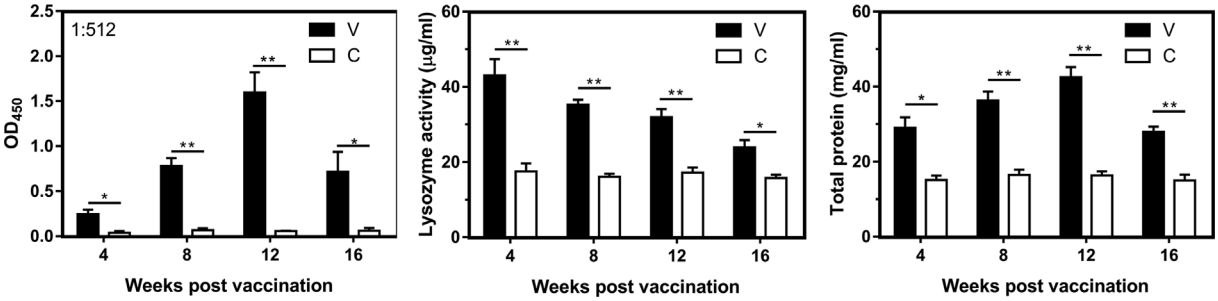

Fig. 3. Specific antibody titers, lysozyme activity and total serum protein in sera of turbot vaccinated with the inactivated vaccine at 4, 8, 12 and 16 w.p.v. Briefly, fish were vaccinated intraperitoneally with 100 μl of inactivated vaccine containing 1.0×10^8 CFU (V group) and injected with 2% NaCl as controls (C group). At 4, 8, 12, and 16 w.p.v., five fish were anaesthetized for blood sampling. Sera were collected from vaccinated fish and control fish. Specific antibody titers were determined by ELISA. Lysozyme activity and total serum protein were determined using lysozyme assay kit and total protein quantification kit. Statistical significance was analyzed between vaccinated and control turbot (*$P < 0.05$, **$P < 0.01$).

antibody titers was exhibited at 12 w.p.v. Generally, notable differences were observed between vaccinated group (V) and control group (C) at each time point. This result suggested that the inactivated *V. harveyi* vaccine accompanied with 763 A induced an intense humoral immunity by producing specific antibody against *V. harveyi* for lasting at least 16 weeks.

3.3.2. Lysozyme activity

Then, lysozyme activities in sera of vaccinated turbot were determined. As shown in Fig. 3, vaccinated fish (V) showed higher level of lysozyme activity than control fish (C). The maximum activity was observed at 4 w.p.v., reaching 43 mg/ml. Even though it gradually decreased later, significant differences were obtained between V and C groups. In contrast, the lysozyme activities of control fish throughout the experiment were ranged from 15 to 18 mg/ml.

3.3.3. Total serum protein

Total serum protein production in sera of vaccinated turbot were also tested. Interestingly, a similar trend to antibody titers was observed as shown in Fig. 3. Briefly, total protein increased from 4 w.p.v. to 12 w.p.v., and then decreased. The concentration of total protein in vaccinated fish (V) was ranged from 27 to 43 mg/ml, while in control fish (C), it remained relatively steady at around 15–17 mg/ml. Notable differences were observed between V and C group at each time point.

3.3.4. Expression of IgM and lysozyme in immune organs

Then, gene expressions of IgM and lysozyme in the spleen and kidney were determined by RT-qPCR. As a result shown in Fig. 4, significant upregulation of IgM was observed in the spleen from 4 to 12 w.p.v., and it increased with 9.2-fold in the kidney at 12 w.p.v. However, no remarkable change of lysozyme expression was obtained in both spleen and kidney with in 16 w.p.v.

3.3.5. Bactericidal properties

Moreover, bactericidal properties of turbot sera were determined by plate count assay. As shown in Fig. 5, all sera from vaccinated turbot exhibited well bactericidal properties. In contrast, sera from fish injected with 2% NaCl didn't showed bactericidal properties at all. These results suggested that the inactivated *V. harveyi* vaccine accompanied with 763 A enhanced antibacterial activity of the vaccinated turbot sera.

3.4. Adjuvant persistence in vaccinated turbot

Finally, fish vaccinated with the inactivated *V. harveyi* vaccine were sampled and dissected at 4, 8, 12 and 16 w.p.v. to determine the persistence of 763 A *in vivo*. Control fish were also analyzed. As shown in Fig. 6, adjuvant residue was observed in peritoneal cavity of vaccinated fish. The gut was covered by 763 A, with a degree of tissue adhesion.

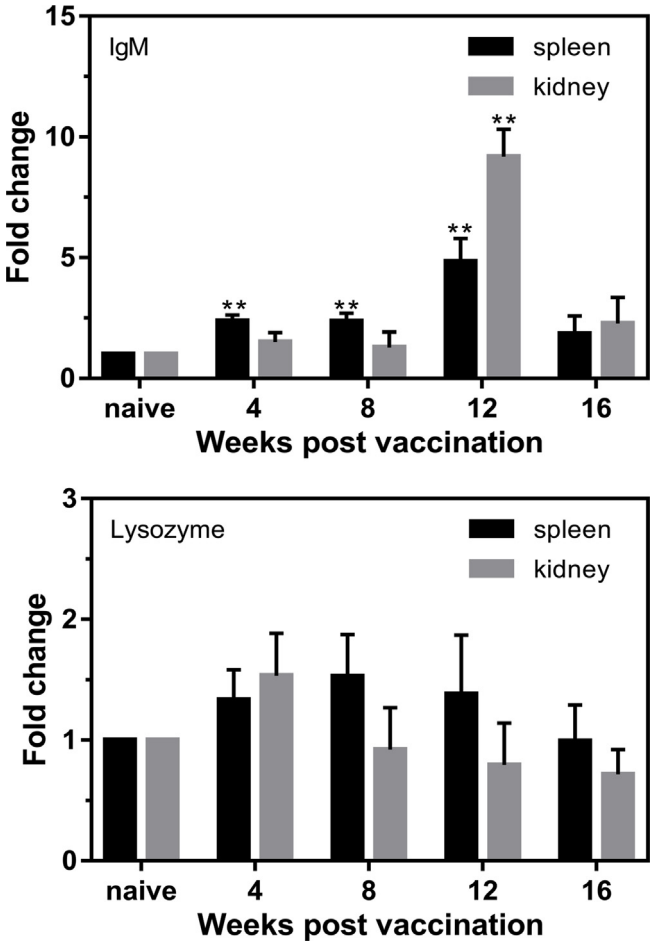

Fig. 4. Gene expressions in vaccinated fish at 4, 8, 12 and 16 w.p.v. RNA from spleen and kidney were extracted. mRNA level of each gene was normalized to that of β-actin and relative expression was calculated by dividing values of the vaccinated tissues by those of the controls. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. Statistical significance was analyzed between vaccinated fish and normal fish (*$P < 0.05$, **$P < 0.01$).

However, the residue reduced gradually as time passed. Comparatively, fish injected with 2% NaCl showed normal appearance of internal organs.

4. Disscusion

Vaccination becomes a common strategy to prevent disease caused

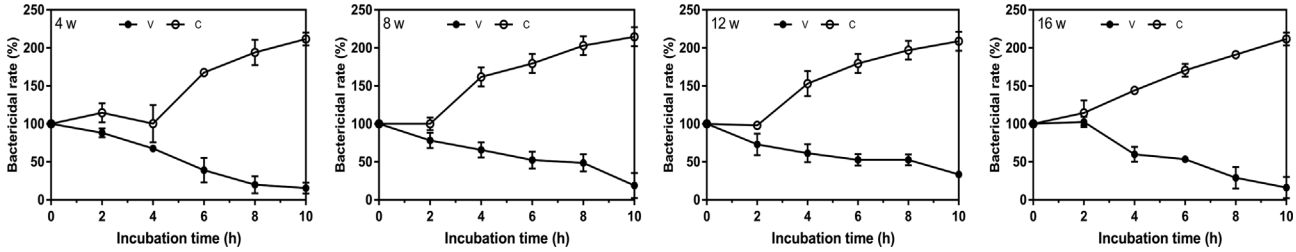

Fig. 5. Bactericidal activities in sera of turbot vaccinated with the inactivated vaccine at 4, 8, 12 and 16 w.p.v. The experimental procedure was as described in the legend for Fig. 3. 50 μl of isolated serum was incubated with the same volume of bacterial suspensions of *V. harveyi*. The mixture was incubated at 28 °C. At 0, 2, 4, 6, 8 and 10 h post incubation, the mixture was sampled and plated onto LB20 at 28 °C. The number of colonies that appeared on the plates was calculated after cultivation for 12 h. The assay was repeated three times. Statistical significance was analyzed between the vaccinated and control turbot (*$P < 0.05$, **$P < 0.01$).

by pathogens in aquaculture industry [28]. *V. harveyi* is a common pathogen that found ubiquitously in aquatic environment. In this study, we prepared a formalin-killed *V. harveyi* cell that isolated from diseased turbot suffering from vibriosis occurring in China, mixed the inactivated cells with a commercial adjuvant Montanide™ ISA 763 A VG, and evaluated the immunoprotection of the vaccine in turbot. The vaccine exhibited efficient immunoprotection with enhanced antibody production, lysozyme activity, total serum protein and antibacterial property in sera of vaccinated fish. Herein, we developed an efficient inactivated vaccine against *V. harveyi* in turbot, which can not only induce humoral immunity, but also enhance strong innate immune response for long-term protection.

Before we developed the inactivated *V. harvey* vaccine, a *V. harvey* infection model in turbot was established in our preliminary experiment. The median lethal dose (LD_{50}) of intramuscular injection and intraperitoneally injection in turbot were 1.58×10^7 and 8.39×10^8 CFU/ml, respectively. Fish with intramuscular injection developed symptoms such as lingering near the surface of the water, blackened body, visceral hemorrhage and swelling spleen, which were similar with the symptoms of *V. harvey* infection. Therefore, intramuscular injection was utilized for the following challenge test. Moreover, in our lab, to determine the immune efficacy of a vaccine, we often use a challenge dose once the cumulative mortality rate of the control infected group is over than 70%. Therefore, a dose of 5×10^6 CFU/fish was selected as the challenge dose according to LD_{50} value.

Antigen concentration of the inactivated vaccine was firstly determined. Low dosage of antigen may not induce an efficient protection. Dash et al. found that the dose of 2.0×10^4 CFU/fish produced weak immunity against the infection in comparison to dose of 2.0×10^6 CFU/fish and 2.0×10^8 CFU/fish, and a dose dependent specific and non-specific immune response was induced in major carp after injected with formalin-killed *A. hydrophila* whole cells [29]. Moreover, earlier reports indicated that the carp need a highest dose of antigen to produce increased agglutination and antibody titer [30]. Herein, RPS of fish vaccinated with 1.0×10^8 and 1.0×10^9 CFU were all greater than 80%. Considering that high dosage may increase the product-cost and workload, 1.0×10^8 CFU per fish was finally used as the optimum vaccine dosage.

Immunity duration is an important parameter for vaccine. In this study, we measured the RPS of the vaccine within 16 weeks. The maximum value was 83.87% at 12 w.p.v. Interestingly, instead of increasing gradually, the RPS at 8 w.p.v. (72.41%) was lower than that at 4 w.p.v. (75.86%). Since both innate and adaptive immune responses were induced after the vaccination, and the former sustained a shorter time than the latter, we believed that innate immune response induced by the vaccine reached the maximum at 4 w.p.v. and then decreased, while adaptive immune response reached the maximum until 12 w.p.v. Therefore, a first decrease and then increase and then decrease again trend of RPS was observed after the vaccination. This observation was

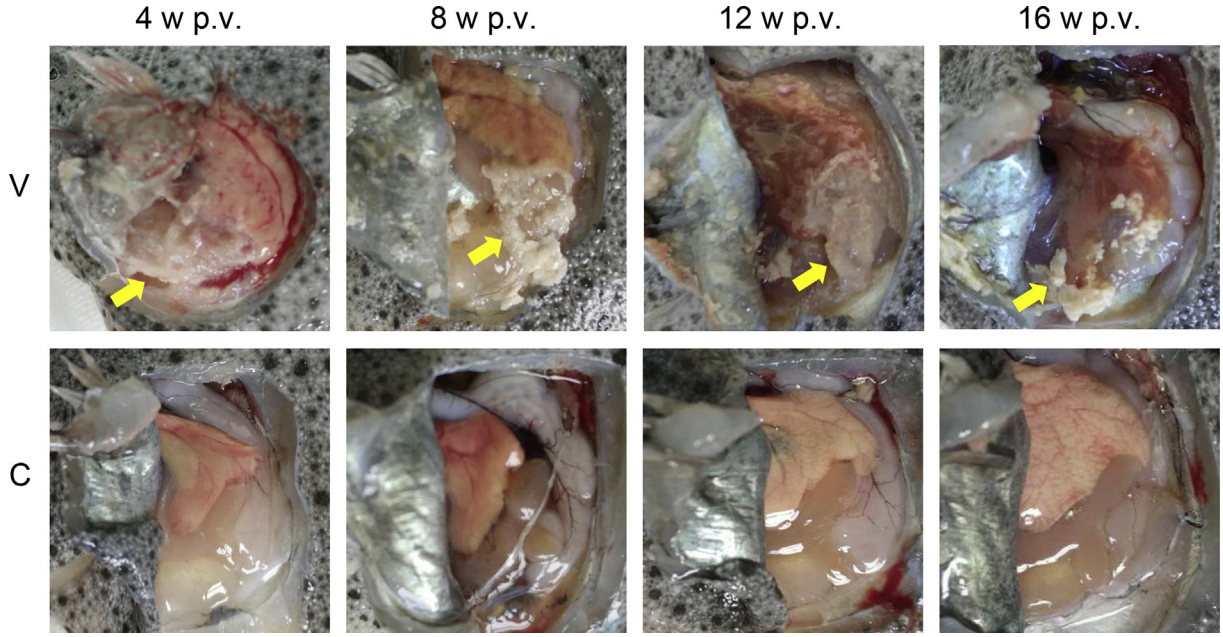

Fig. 6. Adjuvant persistence in vaccinated turbot. The experimental procedure was as described in the legend for Fig. 2. Finally, fish were anaesthetized for and dissection.

consistent with Vinay et al.'s work in which an inactivated vaccine against viral hemorrhagic septicemia emulsified with squalene and aluminum hydroxide adjuvant was also reported to provide long-term protection in olive flounder (*P. olivaceus*), and the highest RPS was observed at 12 w.p.v [31]. This result suggested that the inactivated *V. harveyi* vaccine we developed could induce long-term protection. Similarly, a formalin-inactivated vaccine against *V. harveyi* in orange spotted grouper also exhibited good protection during the 12 w.p.v [15]. The long-term protection may be attributed to oil adjuvant for slow release. Usually, side effects are inevitable once oil adjuvant is used. It was found that the residue of adjuvant in peritoneal cavity of vaccinated fish was reduced gradually, suggesting that the adjuvant could be absorbed and metabolized in fish slowly. However, tissue adhesion was observed within 16 w.p.v.

Antibody is another vital factor in humoral immunity. Significant higher specific antibody level was obtained of vaccinated group compared with that in control group. Highest level was showed at 12 w.p.v. In a previous study, a notable increase of antibody was observed earlier from 2 w.p.v [15]. This might due to the lower temperature used in this study and different species for immune system evocation. Lysozyme is recognized as an important protein in defense to bacteria by activating complement system and phagocytes [32]. Total lysozyme level is a measurable humoral component of the non-specific defense mechanism [33]. Soltani et al. found that a higher level of lysozyme activity in immunized fish with *Y. ruckeri* containing Montanide was observed during 8 w.p.v. compared with fish immunized with *Y. ruckeri* antigen alone [20]. In the present study, lysozyme activity was notably increased as well, and the peak level was observed at 4 w.p.v. Enhanced antibacterial property of sera in vaccinated fish was also observed. Another parameter we tested was serum total protein. It is reported that serum total protein plays an important role in immune response, which is thought to be associated with a strong innate immune response in fish [33]. We found that the change of serum total protein was consistent with the antibody titers. These results suggested that the inactivated vaccine not only induced a strong humoral immunity, but also enhanced innate immune response.

In conclusion, the formalin-killed cell of *V. harveyi* adjuvanted with Montanide™ ISA 763 A VG inducd efficient immunoprotection in turbot. The optimum vaccine dosage was 1.0×10^8 CFU/fish, and it promoted the antibody production. Moreover, enhanced lysozyme activity, total serum protein and antibacterial property in sera of vaccinated fish were observed. Therefore, we developed an efficient inactivated vaccine against *V. harveyi* in turbot.

Acknowledgments

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