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Efficacy of chitosan oligosaccharide as aquatic adjuvant administrated with a formalin-inactivated *Vibrio anguillarum* vaccine

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ABSTRACT

Vaccine is one of the efficient candidates to prevent fish disease through activating host immune response in aquaculture. Actually, several vaccines are often administered with adjuvants to increase immunostimulation, especially for some water-based formalin-killed vaccines. However, side effects are inevitable after vaccination of some adjuvants. Therefore, exploration for effective and harmless aquatic adjuvants is urgently needed. In this study, immunoprotection of a formalin-inactivated *Vibrio anguillarum* vaccine applied with chitosan oligosaccharide (COS) was analyzed. High levels of protection were achieved in zebrafish and turbot vaccinated with inactivated vaccine and COS (RPS of 89.0 ± 4.5% and 80.0 ± 6.9%) compared with fish vaccinated with inactivated vaccine alone (RPS of 47.8 ± 6.6% and 64.7 ± 5.8%) at 4 week post vaccination. Moreover, high antibody reaction and cross-protection against *Vibrio alginolyticus* and *Vibrio harveyi* were observed of turbot vaccinated with inactivated vaccine and COS. In conclusion, COS can enhance immunoprotection of a formalin-inactivated *V. anguillarum* vaccine, significantly activate humoral immune response of host, and be benefit for inhibition against pathogens. Therefore, COS would be a potential adjuvant for aquatic vaccine design in the future.

1. Introduction

Vaccination is one of the strategies for preventing fish disease and is considered to be essential for reducing antibiotic use [1]. Over the past few decades, people have been committed to research and develop vaccines for aquatic animals [2]. Inactivated vaccine, live attenuated vaccine and subunit vaccine are common in aquaculture, and DNA vaccine becomes a novel form. Thereinto, inactivated vaccine was considered to be one of the efficient vaccines against extracellular bacteria and the fact that inactivated vaccines can be produced at low cost makes them ideal for use in aquaculture industry [3]. For instance, the first commercial available bacterial vaccines against enteric redmouth disease and vibriosis were based on inactivated whole-cell formulations [4].

Protective efficacy of vaccines correlates well with their ability to activate immune response. Although inactivated vaccines treated with formalin can induce both innate and adaptive immune response to some extent, there are still some cases of vaccines that could not reach an acceptable level of protection [3]. For example, a formalin-killed cells of *Edwardsiella tarda* promoted humoral immunity in ginbuna crucian carp, but it was ineffective in preventing *E. tarda* infection [5]. Moreover, water-based formalin-killed vaccine might provide a short duration of protection [6–8]. Thus, inactivated vaccines are often administered with adjuvants to increase immunostimulation [9,10]. Adjuvants have been defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen [11]. Several adjuvants such as oil emulsion, microparticle, and even danger molecule as well as inflammatory cytokine have been attempted in aquaculture [12]. However, some adjuvants have also been found to cause side effects such as injection site lesions, peritoneal adhesions with the internal organs, and decreased growth rates [13–16]. Therefore, exploration for effective and harmless aquatic adjuvants is extremely essential [17].

Chitosan, possessing several biological activities such as antioxidant [18], antibacterial and antifungal properties [19–21], has been considered as a potential adjuvant for vaccination [22]. In

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mammals, chitosan has been proved to be equipotent to incomplete Freund's adjuvant (IFA) and superior to aluminum hydroxide [22]. The effect of chitosan as a novel adjuvant to an inactivated influenza vaccine was studied by Chang et al. They found that antibodies in mice serum increased remarkably using chitosan as an adjuvant, enhancing the immune reaction to the vaccine [23]. However, low solubility under physiological condition limits its application in some fields since an aqueous solution is essectial for its use as an immunostimulant in clinical applications [22], in aquaculture for instance [24]. Chitosan oligosaccharides (COS), derived from degradation and deacetylation of chitosan, has been demonstrated having versatile biological functions such as antioxidation, antitumor and against bacterial infection [25]. Due to lower molecular weight and ready solubility in water, COS becomes a popular bioactive molecule in many aspects. Recently, COS has been shown to have immune-enhancing characteristics, anti-inflammatory activities and abilities to reduce the establishment of pathogens in *vitro*. For instance, oligomers of COS were found to significantly enhance peritoneal macrophages migratory activity in mouse [26]. Besides, COS with a high molecular weight (90-HMWCOS) may have anti-inflammatory effect via down-regulation of transcriptional and translational expression levels of TNF-a, IL-6 and iNOS and COX-2 [27]. Moreover, COS was proved effective at reducing the adhesion of enteropathogenic *Escherichia coli* to human cells [28]. However, until now, no direct evidence has been provided on the protective efficacy of COS as an adjuvant on aquatic vaccines.

Vibrio anguillarum is the causative agent of hemorrhagic septicemia in a variety of commercial farmed fish species, resulting in heavy losses in aquaculture [29]. The rise in prevalence of multidrug resistant pathogens makes an urgent need for efficient vaccines to combat *V. anguillarum*. In the current study, immunoprotection of a formalin-inactivated *V. anguillarum* vaccine applied with COS was firstly determined using zebrafish. Moreover, MONTANIDE™ ISA763A, an adjuvant producing stable vaccine emulsions with low viscosity, was analyzed as well. MONTANIDE™ ISA763A has been proved to improve immune responses and provide safe and long-lasting protection in teleost [30]. In our previous study, we also found MONTANIDE™ ISA763A can enhance protective efficacy of vaccines. But the adjuvant was difficult to be absorbed by fish. Therefore, MONTANIDE™ ISA763A was used as a comparative adjuvant here. Subsequently, immunoprotection of the inactivated vaccine applied with COS and/or MONTANIDE™ ISA763A was evaluated in turbot. Moreover, antibody titers, cross-protection and antibacterial properties of serum of vaccinated turbot were analyzed.

2. Materials and methods

2.1. Fish maintenance

Zebrafish (*Danio rerio*) weighing 0.20 ± 0.05 g were obtained from a local fish farm (Jiading, Shanghai, China) and acclimatized in running dechlorinated and aerated water at 24 ± 2 °C. Fish were reared under a 12-h light/12-h dark rhythm and fed with commercial blood worm twice per day. Turbot (*Scophthalmus maximus*) weighing 35.0 ± 5.0 g were purchased from a commercial farm (Tianyuan, Shandong, China) and maintained at 15 ± 1 °C in aerated tanks that were supplied with a continuous flow of sand-filtered seawater. The salinity of the seawater was 3.0–3.1% and the dissolved oxygen content was at least 6.0 mg/L. Fish were fed twice daily with commercial turbot feed. In the experiment, fish were immersed in 100 ng/ml tricaine methanesulphonate (MS-222, Sigma, USA) for anesthetization and in 300 ng/ml MS-222 at least 10 min for euthanasia. All animal experiments were carried out according to the guidelines and approval of respective Animal Research and Ethics Committees of East China University of Science and Technology.

2.2. Preparation of inactivated vaccine

V. anguillarum MVM425 strain was cultured on thiosulfate citrate bile salts sucrose agar (TCBS) at 30 °C for 36 h. Colonies from fresh TCBS were subcultured into Luria–Bertani (LB) mediums supplemented with 2% NaCl (LB_{20}) and were harvested by centrifugation at 5000 × g for 10 min. Then, cells were washed twice in sterile physiological seawater (PSW). Further the bacterial culture was inacticated with 0.5% formalin at 30 °C for 48 h and the death of bacteria was checked as evidenced by the lack of growth on the TCBS plates after incubating for five days.

2.3. Vaccination and challenge

150 zebrafish were randomly divided into vaccinated and control groups (30 fish/group): v, fish vaccinated with inactivated vaccine; v_{763}, fish vaccinated with a mixture of equal volume of inactivated vaccine and MONTANIDE™ ISA763A (ISA763A, Seppic, France); $v_{COS\ (1)}$ and $v_{COS\ (0.1)}$, fish vaccinated with a mixture of equal volume of inactivated vaccine and COS (Bozhihuili, Shandong, China), and the final concentrations of COS were 1 and 0.1 mg/ml, respectively; c, fish mock-vaccinated with sterile PSW. All fish received an intramuscular injection (i.m.) with 5 μl vaccine or PSW, and the final vaccination dose was 2×10^5 CFU per fish. At 4 week post vaccination (p.v.), zebrafish were intramuscularly challenged with 1×10^3 CFU/ml of *V. anguillarum* MVM425. Cumulative survival rate was recorded for lasting 14 days and relative percent survival (RPS) was calculated according to the following formula. Both vaccination and challenge were conducted in triplicate.

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

320 turbot were randomly divided into vaccinated and control groups (40 fish/group): V, fish vaccinated with inactivated vaccine; V_{763}, fish vaccinated with a mixture of inactivated vaccine and ISA763A in a ratio of 3:7; V_{COS}, fish vaccinated with a mixture of inactivated vaccine and COS, and the final concentration of COS was 1 mg/ml; $V_{763+COS}$, fish vaccinated with a mixture of inactivated vaccine, ISA763A and COS; C, fish mock-vaccinated with sterile PSW; $C_{763,}$ fish mock-vaccinated with ISA763A; $C_{COS,}$ fish mock-vaccinated with COS, and the final concentration of COS was 1 mg/ml; $C_{COS+763}$, fish mock-vaccinated with a mixture of ISA763A and COS. All fish received an intraperitoneal injection (i.p.) with 100 μl vaccine or/and adjuvant, and the final vaccination dose was 2×10^7 CFU per fish. At 4 and 12 w p.v., 30 fish in each group were intramuscularly challenged with 5×10^6 CFU/ml of *V. anguillarum* MVM425. Manipulation was operated as described above.

2.4. Specific antibody detection

Blood was extracted from three vaccinated and mock-vaccined fish in each group at 4 and 12 w p.v. Sera were collected after centrifugation at 3000 r/m for 10 min and stored at −80 °C until use. Antibody titers in turbot sera against *V. anguillarum* were determined using a modified ELISA method. Briefly, microplate was coated with 1×10^8 CFU/ml *V. anguillarum* MVM425 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, 2-fold serial dilution series of serum ranging from 2 to 2^{10} were