Aquaculture 454 (2016) 125–129

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

Effects of a subunit vaccine (FlaA) and immunostimulant (CpG-ODN 1668) against *Vibrio anguillarum* in tilapia (*Oreochromis niloticus*)

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ARTICLE INFO

Article history:
Received 6 September 2015
Received in revised form 3 December 2015
Accepted 9 December 2015
Available online 12 December 2015

Keywords:
Subunit vaccine
Vibrio anguillarum
Flagellum
Flagellin-A

ABSTRACT

Vibriosis, caused by *Vibrio anguillarum*, is a common bacterial disease in cultured fish. Treatment of vibriosis can lead to the development of antibiotic resistance or tolerance. Therefore, alternative methods for combatting vibriosis are needed. In this study, we investigated the effects of a subunit vaccine (recombinant flagellin-A [rFlaA]) and adjuvant (CpG-ODN 1668 [CpG]) candidate against *V. anguillarum* in tilapia (*Oreochromis niloticus*). In an in vivo experiment, fish in the rFlaA + CpG and rFlaA-only groups were first treated with CpG or PBS, followed by vaccination with rFlaA the next day. In the control group, both vaccine and adjuvant were replaced by PBS. The highest survival rate (60%) was observed in fish treated with rFlaA + CpG, compared with 0% in PBS-treated fish or 35% in fish immunized with rFlaA-only. Moreover, sera purified from tilapia treated with rFlaA + CpG exhibited significantly stronger agglutination activity (approximately 3-fold higher) than sera obtained from control fish, whereas sera from fish treated with rFlaA-only showed values 2.5-fold higher than those in control fish. The bacterial survival rates were also lower in sera from tilapia treated with rFlaA + CpG (41%) than in sera from fish treated with rFlaA alone (61%) or control fish (100%). Thus, fish treated with both rFlaA and CpG exhibited the highest immune activation in terms of agglutination and bactericidal activity. In summary, FlaA could be a promising subunit vaccine, and CpG-ODN 1668 could be applied as an adjuvant for immunization against *V. anguillarum*.

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1. Introduction

Vibriosis, a common bacterial disease found in cultured fish, causes hemorrhagic septicemia in brackish water and freshwater fish, thereby leading to enormous economic losses in the aquaculture industry (Actis et al., 1999; Austin and Austin, 1993). Although chemotherapeutics are often used for treating these bacterial and infectious diseases, such drugs can promote antibiotic resistance in bacteria and can negatively affect the environment and human health. Therefore, most countries have attempted to reduce the use of chemotherapeutics, and rather have developed effective vaccines to treat vibriosis. Although several commercial vaccines produced in formalin-inactivated cultures containing mixtures of whole *Vibrio anguillarum* cells and extracellular products can protect against *V. anguillarum* infections in farmed fish, the existence of many O-serotypes in *V. anguillarum* (Rasmussen, 1987; Rasmussen and Larsen, 1987; Sørensen and Larsen, 1986) has limited the extensive use of these vaccines in various fish species and countries. Therefore, new vaccine strategies are needed to overcome these technical limitations.

To date, vaccines used for *Vibriosis* prevention have generally been inactive vaccines containing formalin-killed cells (FKCs), and the prophylactic effects of these vaccines vary widely (Joosten et al., 1997; Toranzo et al., 1997; Vervarcke et al., 2004; Vervarckel et al., 2004; Wong et al., 1992). In addition, some formalin-inactivated vaccines have been widely used in fish farms of South Korea. However, inactivation of pathogenic bacteria by formalin or heating inevitably impairs immune responses in the host because of modification of the epitopes of surface antigens, which alters the physico-biochemical characteristics of the antigens, particularly T cell-dependent antigens (Tola et al., 1999; Kwon et al., 2006, 2007). To induce an effective immune response, the outer membranes of target bacteria must not be changed after physical or chemical treatment of live infectious bacteria. Consequently, subunit vaccines may be used to overcome these limitations.

In *V. anguillarum*, the motility of the bacterium is controlled by the flagellum and is related to the pathogenesis of vibriosis. The importance of flagellin-A (FlaA), the outer protein in the flagellum, as a potential virulence factor has been demonstrated in other bacteria (Milton et al., 1996). Indeed, nonmotile mutant bacteria propagate but do not cause characteristic systemic infection, indicating that motility contributes to the invasive capabilities of this organism (Grant et al., 1993; Ormonde et al., 2000).

To date, several studies have been conducted to examine the potential of CpG-oligodeoxynucleotides (CpG-ODNs) as immunotherapeutic

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http://dx.doi.org/10.1016/j.aquaculture.2015.12.005
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agents and adjuvants. CpG-ODNs 2007, 1826, and 2006 have been shown to have immunostimulatory effects or function as adjuvants in mice, female calves, hens, and mammals (Kwant and Rosenthal, 2004; McCluskie et al., 2000; Wedlock et al., 2005). However, CpG-ODN 1668 has not been shown to have potential as an adjuvant for vaccines in fish thus far.

In addition, CpG-ODN 1668 exhibits therapeutic and prophylactic effects in olive flounder infected with scuticociliatosis caused by *M. avidus* (*Miamiensis avidus*) (Kang and Kim, 2012, 2015) and with *Edwardsiella tarda* (Lee et al., 1999). At 3 days after injection of CpG-ODNs 1668 and 2216, olive flounder showed enhanced resistance against *M. avidus* and VHSV infections. Additionally, fish injected with CpG-ODN 1668 showed increased survival in an in vivo study, and their serum had higher scuticocidal activity compared with fish injected with PBS. These findings suggest that immunostimulation should be performed continuously for three days. Therefore, in this study, we administered the adjuvant (CpG-ODN 1668) 1 day before the injection of the antigen as an intermediate method between the typical method of mixing the antigen and CpG-ODN 1668 and the administration of the antigen at the third day of treatment with CpG-ODN 1668 as described in a previous study (Kang and Kim, 2012).

Additionally, we aimed to determine whether rFlaA and CpG-ODN 1668 could be used as a vaccine and adjuvant, respectively, against *V. anguillarum* infection in tilapia (*Oreochromis niloticus*).

2. Materials and methods

2.1. Bacterial strains and plasmid

The pET-28a vector (Novagen, USA) and BL21 (DE3) competent cells were used as the expression system. Expression of 6× histidine (His)-tagged fusion proteins in *Escherichia coli* was achieved by culturing at 37 °C in Luria–Bertani (LB) broth (BD Difco, USA) containing 30 μg/mL kanamycin and ampicillin. The *V. anguillarum* strain used in this study was isolated from olive flounder (*Paralichthys olivaceus*) in a natural outbreak of vibriosis on a commercial farm in Korea.

2.2. Cloning and sequence analysis of FlaA

The *FlaA* gene derived from *V. anguillarum* was cloned and sequenced; the sequence has been registered in the GenBank database of the National Center for Biotechnology Information (NCBI, USA). The sequences of the primers used to amplify the *FlaA* gene were as follows (*BamH*I and *Not*I sites are underlined): forward primer, 5′-GGATCCATGACCATTACAGTAAATACTAACG-3′; and reverse primer, 5′-GCGGCCGCTTACTGCAATAGTGACATTGC-3′. For gene amplification, genomic DNA from *V. anguillarum* was isolated using the *AccuPrep* Genomic DNA Extraction Kit (Bioneer, Korea). Using this template, the *FlaA* gene was amplified by the polymerase chain reaction (PCR) using a primer pair in an iCycler (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: one cycle of 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C; and a final extension step of 7 min at 72 °C. The sequence of the cloned product was confirmed using the GenBank database in the NCBI BLAST program. The amplified PCR product was purified with a Gel Extraction Kit (Nucleogen, Si Hung, Korea), visualized on a 0.7% agarose gel stained with ethidium bromide, and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) for sequencing.

2.3. Construction of a vector expressing recombinant FlaA (rFlaA)

The *FlaA* gene and pET-28a (Novagen) vector were digested by restriction enzymes (*BamH*I and *Not*I) and ligated together to construct the pET28a-FlaA plasmid. This recombinant plasmid was used to transform *E. coli* BL21 (DE3) competent cells.

2.4. Expression and purification of recombinant protein

BL21 (DE3) cells harboring the pET28a-FlaA plasmid were grown in LB broth containing 30 μg/mL kanamycin (Sigma) at 37 °C. For the expression of FlaA, isopropyl 1-thio-β-D-galactoside was added to a final concentration of 1 mM when the optical density at 600 nm (OD_{600}) reached approximately 1.0. Four hours later, rFlaA-expressing *E. coli* BL21 (DE3) cells were harvested by centrifugation at 2000 × *g* at 4 °C for 10 min and then incubated with His-tag binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9) and lysozyme. The *E. coli* cells were then sonicated using a Sonic Dismembrator (Fisher Scientific) for 5 min at intervals of 30 s (three times at each point) and centrifuged at 10,000 × *g* at 4 °C for 30 min, and the pellet was collected. The collected pellet was resuspended in binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) containing urea (USB, UK), adjusted to a concentration of 8 M. The His-tagged rFlaA protein was dialyzed and purified by chromatography under native conditions on Ni-nitrilotriacetic acid resin (histidine [His]-affinity column; Novagen), according to the manufacturer's protocols. The purified protein was quantified using a bicinchoninic acid assay (Sigma, St. Louis, MO, USA).

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

To confirm the purity of rFlaA, the protein was analyzed by SDS-PAGE using a 4% stacking gel (upper gel) containing 0.5 M Tris–HCl and 10% SDS, and a separating gel (lower gel) containing 1.5 M Tris–HCl, 10% SDS, and 30% acrylamide. The purified rFlaA protein was diluted 1:4 to 40 μg/mL with sample buffer (0.35 M Tris–HCl [pH 6.8], 20% SDS, 36% glycerol, 0.5% 2-mercaptoethanol, and 0.012% bromophenol blue), and 20 μL per lane was then loaded. Protein markers (5 μL; Thermo Scientific PageRuler Prestained Protein Ladder, 10–80 kDa) were loaded onto the gel, and samples were separated by electrophoresis. Protein bands were detected by Coomassie blue staining. For Western blotting, a second gel was transferred to a nitrocellulose membrane. The membranes were treated with blocking solution (3% bovine serum albumin in Tris-buffered saline [TBS] containing 150 mM NaCl and 10 mM Tris–HCl [pH 7.5]) for 2 h at room temperature (RT) and then washed with TBS containing 0.05% Tween 20 (TTBS; pH 7.5). After blocking, the membrane was incubated with a rabbit anti-6× His-tag antibody (1:500 dilution; Santa Cruz Biotechnology, Inc., CA, USA) for 2 h at RT, washed again with TTBS, and incubated for 2 h at RT with a secondary antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase; 1:200 dilution; Santa Cruz Biotechnology, Inc.). The membrane was then washed three times with TTBS and developed after the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate with substrate buffer (Sigma, USA).

2.6. In vivo experiments

2.6.1. CpG-ODN 1668

CpG-ODN 1668 was synthesized by Bioneer (South Korea). The sequence of CpG-ODN 1668 was: 5′-TCCATGACGTTCCTGATGCT-3′, where the underlined nucleotides indicate the presence of phosphorothioate bonds.

2.6.2. Fish

Tilapia (*O. niloticus*; average size of 10 cm, weighing 3.5 g each) were received from the Inland Research Center of the National Fisheries Research and Development Institute. Fish were acclimated for 1 week before use in experiments. For in vivo experiments, *V. anguillarum* was cultured on TSB containing 1.5% NaCl and incubated for 24 h at 27 °C. The LD_{50} of *V. anguillarum* was determined by intraperitoneal (i.p.) injection in tilapia fry.

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