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Immunogenicity and protective efficacy of *Vibrio harveyi* pc*Fla*A DNA vaccine in *Epinephelus awoara**

QIN Yingxue (覃映雪)[†], SU Yongquan (苏永全)[††], WANG Shifeng (王世峰)[†††], YAN Qingpi (鄢庆枇)[†,**]

[†] *Fisheries College, Jimei University, Xiamen 361021, China*

[††] *Department of Oceanography, Xiamen University, Xiamen 361005, China*

[†††] *Ocean College of Hainan University, Haikou 570228, China*

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Abstract The *Fla*A gene from *Vibrio harveyi,* with a short nucleotide sequence encoding the Flag marker, was cloned into the eukaryotic expression vector pcDNA3.1(+) (designated as pc*Fla*A). Ninety grouper (*Epinephelus awoara*) were separated into three equal size groups. An experimental group was immunized with pc*Fla*A, Control I group was immunized with the vector pcDNA3.1(+), and Control II group was immunized with PBS. The expression of pc*Fla*A mRNA and protein was examined using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. We also evaluated the immunogenicity and protective efficacy of pc*Fla*A against *V. harveyi* by measuring the lymphocyte proliferation response and serum levels of specific antibody and conducting a bacterial challenge test. We successfully transfected the fish muscle with pc*Fla*A. The pc*Fla*A mRNA and protein was expressed in the muscle cells for up to one month following injection. The proliferation response of lymphocytes in fish immunized with pc*Fla*A was significantly higher than in control group II. Furthermore, the immunized fish generated specific antibody. The vaccination also resulted in significantly higher survival during the bacterial challenge test.

Keyword: *Vibrio harveyi*; DNA vaccination; immunogenicity; protective efficacy

1 INTRODUCTION

Vibrio, the major causal agent of vibriosis, affects a wide range of marine cultured organisms throughout the world. Despite this, prophylactic or therapeutic measures have been unavailable to date. Although vaccines have long proven their efficacy in herd protection, there has been little research into vaccines against *Vibrio*.

An early report by Hansen (1991) documented the expression of a reporter gene in carp muscle after injection of plasmid DNA. Since then, the potential for DNA vaccines has captured the widespread attention of scientists working in the field of vaccine development, primarily because of the advantages over classical antigen vaccines (Nie et al., 2007). DNA vaccines have been developed for use in aquaculture, but of the majority of fish DNA vaccines are effective against viruses alone, relatively few have been developed against bacteria (Garver et al., 2006; Rout et al., 2007; Kumar et al., 2007, 2008).

The flagellin core (FC) protein in *Vibrio* consists of a single protein subunit and is conserved among different groups of bacteria (McCarter, 1995; McGee et al., 1996; Das et al., 1998). Furthermore, the FC protein is a major immunogenic antigen (Yang et al., 1977; Das et al., 1998). Because it is a relatively simple protein, FC is easily studied at the gene level. In addition, its role in the pathogenicity and immunoprotection of the bacteria mean that it is an ideal candidate for the development of a vaccine. Because it is conserved among different groups of bacteria it is also possible that FC could be used as a universal antigen against bacterial infection.

As part of an effort to develop practical technologies for immunizing fish against bacteria, we evaluated the expression of the *V. harveyi* FC protein gene

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** Corresponding author: Yanqp@jmu.edu.cn

following direct injection of DNA into a fish. The FC protein is encoded by the *Fla*A gene in *V. harveyi*. We used the *Fla*A gene as the antigen gene and constructed a recombinant plasmid (pc*Fla*A) which was used to immunize grouper (*Epinephelus awoara*). We then measured the duration and level of *Fla*A expression in the grouper. Our results may be used to direct future research into the development of DNA vaccines for teleosts.

2 MATERIALS AND METHODS

2.1 Construction and preparation of DNA vaccines pc*Fla*A

Recombinant pc*Fla*A were constructed from the eukaryote expression vector pcDNA3.1(+) (donated by Fujian Academy of Agriculture Sciences). Briefly, the *Fla*A gene of *V. harveyi* was amplified by polymerase chain reaction (PCR) using the forward primer 5' CAG AAG CTT ACC ATG ACC ATT AAC GTT AAT ACT AAC G 3' (containing restriction sites *HindIII*) and the reverse primer 5'CAG TCT AGA TTA GAT TAC AAG GTA GAC GAC GAT AAG CTG CAA TAA GGA CAT TGC AGAA3'(containing restriction sites *Xba*I and FLAG octapeptide). The PCR products were digested with *Hind*III/*Xba*I and cloned into the pcDNA3.1(+) vector, also digested with *Hind*III/*Xba*I. The recombinant plasmid was transformed into *Escherichia coli* JM109 competent (Takara) cells and identified by restriction enzymes and sequencing, then designated as pc*Fla*A.

The pc*Fla*A constructs were amplified in *E. coli* JM109 cells grown in LB media. Plasmid DNA was prepared using the alkaline-lysis procedure, purified with Polyethylene Glycol 8000, and resuspended in PBS (0.5 mg/ml DNA) for use.

2.2 DNA vaccination

Three groups of 30 adult *E. awoara* (150–200 g) were placed in separate tanks. The fish were immunized by multipoint intramuscular injection with one of the following treatments (in 100 μl of sterilized PBS): (1) 50 μg of pc*Fla*A (experimental group), (2) 50 μg of pcDNA3.1(+) vector (control group I), or (3) no plasmid DNA (control group II). The fish were injected intramuscularly to a depth of 2 mm, on the right side below the dorsal fin. On days 7, 14, 21, and 28, we collected muscle, blood, and head kidney tissue samples from three fish in each group (selected at random). The muscle tissue was collected at the site of injection and divided into two samples. One sample was frozen for immuno-histochemistry and the other was immediately frozen in liquid nitrogen for RNA extraction.

2.3 Expression of the DNA vaccine in musculature of immunized fish

Total RNA was prepared from muscle samples using Trizol reagent (Life Technologies, Carlsbad, USA). First-strand cDNA was synthesized using a random primer Oligo$(dT)_{18}$ (Fermentas, Glen Burnie, USA). Aliquots of first-strand cDNA were subjected to PCR amplification (95°C for 5 min followed by 30 cycles of 94°C for 45 s, 58°C for 50 s, 72°C for 90 s) using the sense primer and anti-sense primers described above. To eliminate template contamination with plasmid DNA, we used a reverse-transcription reaction, in which reverse-transcriptase was omitted, as a negative control. A positive control was performed using the RNA template and primers provided in the First Strand cDNA Synthesis Kit (Fermentas).

The fusion protein FC-FLAG was detected following the procedure described by Heppell (1998) with minor modifications. Briefly, the muscle tissue was immediately frozen and sectioned (7 μm) using a Leica CM1900 rapid sectioning cryostat (Leica, Wetzlar, Germany). The tissue sections were collected on clean slides and fixed in cold acetone for 10 min. The slides were air-dried at room temperature and washed with PBS before being dipped in 0.3% H_2O_2-methanol solution for 30 min. Following this, the slides were re-washed in PBS, dipped in 0.2% Triton-X-PBS at 37°C for 30 min, treated with 10 mg/mL BSA for 20 min, and incubated with mouse anti-FLAG M2 McAb (Sigma, St. Louis, USA) over-night. Last, the tissue sections were washed with PBS, incubated with a secondary antibody (rabbit anti-mouse IgG HRP conjugate: BaiAo, Zhuhai, China) for 1 h and stained with DAB/H_2O_2 for ~5 min.

2.4 Lymphocyte proliferation assay

Lymphocyte proliferation was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5–diphenyltetrazolium bromide (MTT) assay (Daly et al., 1995). On days 7, 14, 21, and 28 after immunization, three fish from each group were killed and the head kidney was removed and sieved through a sterile metal mesh into RPMI 1640 medium. We used Ficoll-paque PLUS lymphocyte solution to isolate the lymphocytes. The isolated cells were suspended at a density of 5×10^6 cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum. We then added 200 μl of the cell suspension and 50 μl flagellin (25 mg/ml) from

V. harveyi (Qin et al., 2006) into the wells of a 96-well tissue culture plate. The mixture was cultured at 27°C in 5% CO_2. Wells without flagellin served as the control and wells without the lymphocyte suspension served as the blank. The cells were cultured for 68 h then incubated with 20 μl of MTT per well for 4 h. Following incubation, we added 150 μl of dimethyl sulfoxide (DMSO) to each well. The plates were shaken slowly for 10 min. We then measured the absorption of each well at 570 nm using a microtiter plate reader (BioRad, Hercules, USA). Lymphocyte proliferation was expressed using a stimulation index (SI) where SI= $OD_{test}/OD_{control}$.

2.5 Enzyme-linked immunosorbent assay

We collected blood samples from three fish in each group by caudal venipuncture on days 7, 14, 21, and 28 after immunization. Pooled serum samples were prepared by mixing an equal volume of serum from each group for ELISA. We used flagellin from *V. harveyi* (Qin et al., 2006) as the antigen. The secondary antibody consisted of mouse anti-grouper IgM (ADL, Stirling, UK), the third antibody consisted of horseradish peroxidase-conjugated rabbit anti-mouse IgG (BaiAo, Zhuhai, China). The color reaction was initiated by the addition of 3,3′,5,5′-tetramethyl-benzidine (TMB). The absorbance was read at 450 nm using an ELISA reader (BioRad).

2.6 Challenge test

The remaining fish were challenged by intra-abdominal injection with a 0.2 ml bacteria suspension (10^7cfu ml^{-1}) per fish, 28 d after immunization. We monitored the fish twice daily during the following week. Any dead fish were removed and standard husbandry procedures were followed throughout the experiment.

2.7 Statistical analysis

All data from this study were analyzed using SPSS 16.0. $P<0.05$ were considered to be significantly different.

3 RESULTS

3.1 Construction and identification of the DNA vaccine

The *Fla*A gene (~1.1 kb) was cloned into the expression vector pcDNA3.1 to construct the recombinant plasmid pc*Fla*A. The recombinants, picked out using a selective plate, were digested with *Hind*III/*Xba*I to excise the insert (Fig.1). The positive eukaryotic recombinants were confirmed by sequencing.

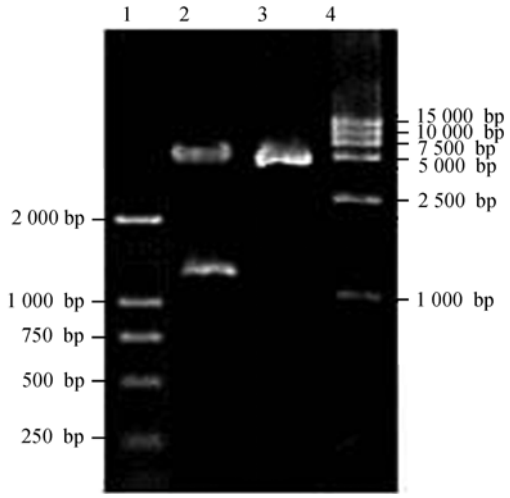

Fig.1 Restrictive enzymes digestion identification of recombinant plasmid pc*Fla*A

1) DNA Marker DL2 000; 2) Plasmid digested by *Hind*III/*Xba* I; 3) Plasmid pc*Fla*A; 4) Marker DL15 000

3.2 *In vivo* transcription of recombinant *Fla*A gene

We used RT-PCR of the DNase-treated RNA from immunized fish to determine transcription of the recombinant *Fla*A gene in the muscle tissue following injection. The RT-PCR yielded a 1.1 kb fragment in the muscle samples obtained from the site of injection 7, 14, 21, and 28 d after immunization. However, the efficiency of amplification was low (Fig.2).

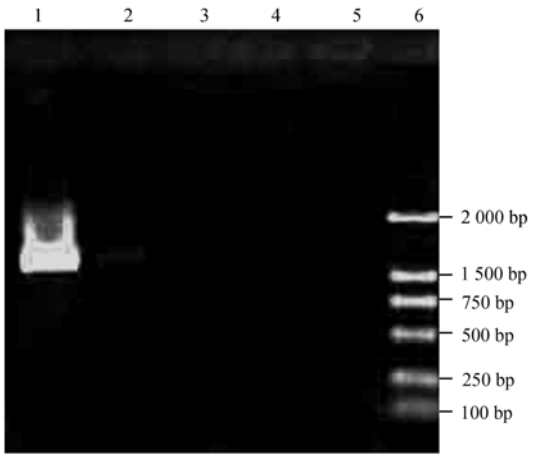

Fig.2 *In vivo* transcription of the recombinant *Fla*A gene 28 d after immunization

1. Positive control; 2. Experimental group; 3. Control group I; 4. Control group II; 5. Negative control; 6. DNA marker DL 2 000

3.3 Expression of FC-FLAG Proteins

We evaluated expression of the fusion protein (FC-FLAG) in muscle tissue sections from fish at the site of injection using immunohistochemistry. FC-FLAG was observed in the tissue of fish injected with pc*Fla*A 7, 14, 21, and 28 d post-injection. The FC-FLAG protein was primarily expressed near the periphery of the muscle fibrils (Fig.3). Tissue sections from fish in the control group were negatively stained.

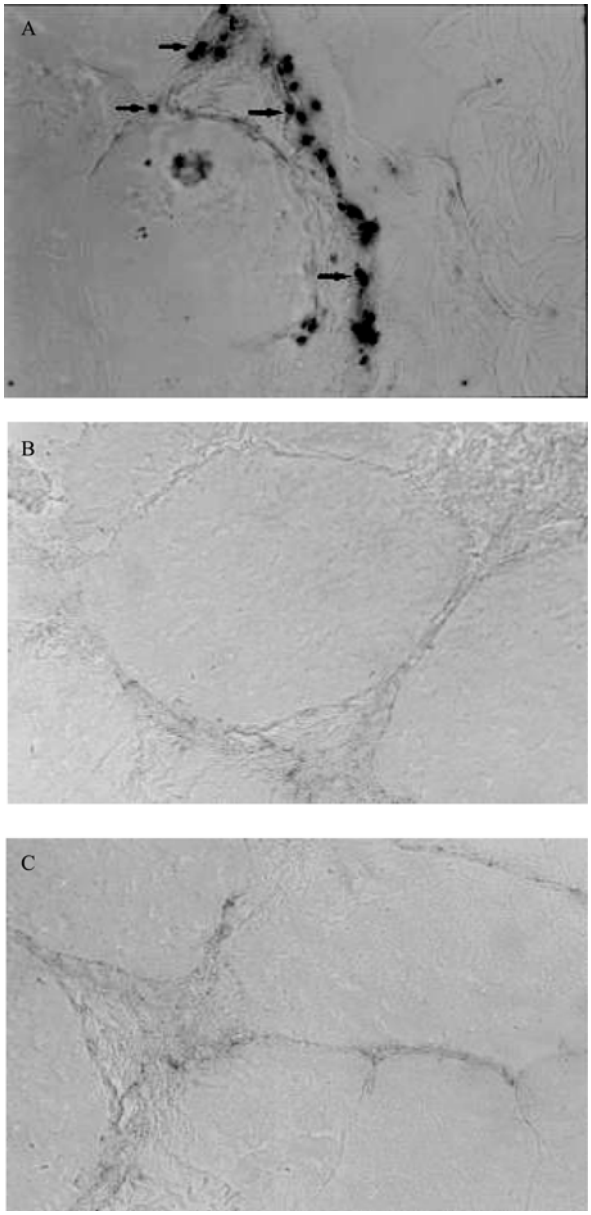

Fig.3 Expression of the fusion protein FC-FLAG in fish muscle injected with pc*Fla*A 28 d after immunization

A. Experimental group; B. Control group I; C. Control group II. Note: Dark granulations are the positive blots (arrow).

3.4 Lymphocyte proliferation response

The proliferation response of lymphocytes from fish in the three groups was similar during the first two weeks (Fig.4). However, four weeks after immunization the proliferation response of lymphocytes from fish immunized with pc*Fla*A was significantly higher than in control group II (P<0.05).

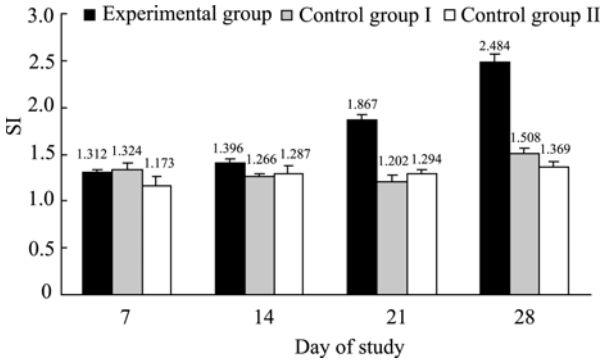

Fig.4 Proliferation of grouper head kidney lymphocytes

3.5 Antibody response to pc*Fla*A vaccination

We did not measure any serum antibody response during the first three weeks after immunization (Fig.5). In contrast, the serum antibody levels in fish immunized with pc*Fla*A were significantly higher than those in fish from control group I (P<0.05) and control group II (P<0.01) 28 d post-immunization (Fig.5).

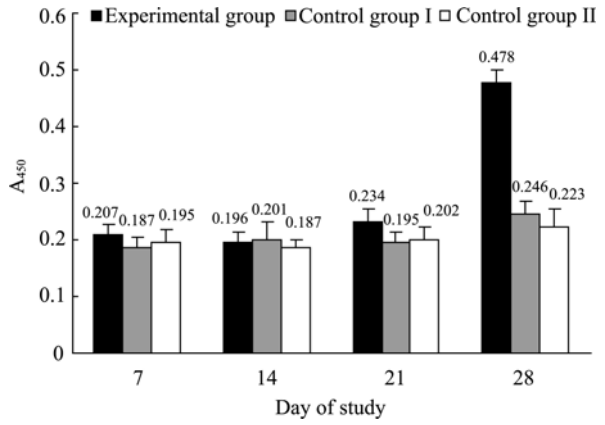

Fig.5 Antibody responses of *E. awoara* immunized with DNA vaccine

3.6 Challenge test

We measured the effectiveness of immunization by challenging all remaining fish with an injection of bacteria 4 weeks after immunization. The fish in control group II were all dead within 3 d after the challenge. In contrast, four fish in control group I and

11 fish in the experimental group remained alive 7 d after the challenge (Table 1). The survival of the experimental group was significantly higher than that of control group Ⅰ ($P<0.05$) and control group Ⅱ ($P<0.01$).

Table 1 Survival after bacterial challenge

	Total	Number of survival	Survival rate (%)
Experimental group (pc*Fla*A)	17	11	64.7
Control I (pcDNA3.1)	15	4	26.67
Control II (PBS)	15	0	0
Total	47	15	31.91

4 DISCUSSION

DNA immunization is an effective vaccination technology that delivers DNA constructs encoding specific immunogens into host cells, thus inducing an immune response. Despite the potential, there has been limited research into the use of DNA vaccines in fish. Our results demonstrate that the muscle cells of *E. awoara* can incorporate recombinant plasmid pc*Fla*A and express the *Fla*A gene for at least 28 d following intramuscular injection of naked plasmid DNA. Furthermore, the recombinant pc*Fla*A elicited a cellular and humoral immune response which contributed to the protective immunity against *V. harveyi* infection. Based on our results, we are optimistic that recombinant plasmid pc*Fla*A may be used in a DNA vaccine against *V. harveyi*.

However, we were only able to generate a weak signal from the mRNA and fusion protein FC-FLAG, suggesting that transfection of plasmid pc*Fla*A and transcription of the *Fla*A gene *in vivo* was inefficient. The fusion protein FC-FLAG was primarily expressed in the periphery of muscle fibrils at the injection site, and not at muscle cells of other sites. This suggests that plasmid diffusion and distribution was limited, which may be related to the method of vaccination. Previous studies have shown that an excessively high dose of DNA can result in a lower expression level of the foreign gene (Schulte et al., 1998; Gómez-Chiarri et al., 1996). Heppell (1998) pointed out that typical doses for fish fall in the range of 1–50 μg of DNA, in a volume of 10–50 μl. Heppell et al. (2000) believed the optimal dose of DNA probably varies according to the species and size of the animal, but it does not increase proportionally with the weight of the fish. In addition, Hansen et al. (1991) noted that young, fast growing carp had much higher levels of chloramphenicol acetyl transferase (CAT) reporter gene activity than older fish. In general, rainbow trout (*Oncorhynchus mykiss*, Walbaum) fingerlings (2–4 g) are used as model fish in most studies of DNA vaccines in teleosts. In contrast, we used grouper (wt 100–150 g) and administered a dose of 50 μg of DNA in a volume of 100 μl. It is unclear whether this was the most effective concentration for this species. Experimental fish are also typically treated with bupivacaine or a high-concentration sucrose solution before DNA immunization. This results in muscle relaxation or myofibril necrosis, which may improve plasmid uptake by muscle cells (Rosas et al., 1998; Caselli et al., 1999). To avoid the confounding effects of muscle relaxation or myofibril necrosis we did not treat the sites of DNA inoculation before immunization.

The fish immunized with pc*Fla*A did not display an obvious antibody response or proliferation of lymphocytes until the 4th week post-injection. This may be due to differences in metabolic rate as teleosts are cold-blooded vertebrates so may have a slower cellular and humoral immune response to antigens when compared with warm-blooded animals. Development of pc*Fla*A into a viable DNA vaccine requires additional research into of the method of vaccination and the appropriate doses for each species.

5 CONCLUSION

DNA vaccination using recombinant pc*Fla*A resulted in transfection in fish muscle and expression in the muscle cells for up to a month. DNA vaccination induced both a cellular and humoral immune response which contributed to immunity against *V. harveyi* infection in *E. awoara*.

6 ACKNOWLEDGMENTS

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