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Protection of Japanese flounder (*Paralichthys olivaceus*) against *Vibrio anguillarum* with a DNA vaccine containing the mutated zinc-metalloprotease gene

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ABSTRACT

Vibrio anguillarum is one of the causative agents of vibriosis, a systemic disease of fish characterized by acute hemorrhagic septicemia. The extracellular zinc metalloprotease (EmpA) is a putative virulence factor involved in pathogenicity of *V. anguillarum*. Here we described the results of immunization against *V. anguillarum* with the plasmid expressing the mutated EmpA (m-EmpA7), which had no protelytic activity or cytotoxicity. In vitro protein expression of m-*empA*7 gene was determined by fluorescent microscopy and Western-blot after transfection of Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cell lines. All three groups of fish immunized with a single intramuscular (i.m.) injection of different doses of the m-EmpA7 DNA vaccine showed significant serum antibody levels after vaccination, compared with the fish injected with the control eukaryotic expression vector pEGFP-N1 and PBS. In addition, fish receiving the DNA vaccine developed a protective response to a live *V. anguillarum* challenge 4 weeks post-inoculation, as demonstrated by increased survival of vaccinated fish over the control and by decreased histological alterations in vaccinated fish. Furthermore, humoral immune responses and protective effects were significantly increased at higher vaccine doses using a single intramuscularly injection route.

1. Introduction

The marine bacterium *Vibrio anguillarum* is the causative agent of vibriosis, a systemic disease of fish characterized by acute hemorrhagic septicemia [1]. Outbreaks of vibriosis result in high mortality rates of infected fish. The disease is a major obstacle to the spread of commercial aquaculture [1]. Japanese flounder (*Paralichthys olivaceus*) is one of the important fish species for marine aquaculture in China. Bacterial diseases mainly caused by *V. anguillarum* have evolved into a major problem in Japanese flounder farming industries [2]. Although several studies have shown that different vaccine formulations including formalin killed bacteria, heat-inactivated *V. anguillarum* cells and *V. anguillarum* bacterin may provide protection against *V. anguillarum* infections [3–5], there are currently no effective vaccines available against this important pathogen, and the disease control is still one of the serious problems in marine aquaculture [6].

Starting with the report by Anderson et al. [7], who showed that rainbow trout fry injected with the glycoprotein gene (G) of infectious hematopoietic necrosis virus (IHNV) were more resistant to subsequent challenge with the virus. Several other reports have demonstrated the effectiveness of DNA vaccination in fish against viral infections, including infectious hematopoietic necrosis virus, viral hemorrhagic septicaemia virus (VHSV), red seabream iridovirus (RSIV) and hirame rhabdovirus (HIRRV) [8–14]. Immunization with antigen-encoding plasmid DNA can elicit strong and long-lasting humoral and cellular immune responses. This approach also offers economic, environmental and safety advantages, which are particularly attractive for the aquaculture industry [15,16]. To date, there have been many studies of the protective effect of viral DNA vaccines in fish [17–19], but only a few studies have been reported on the expression and protective efficiency of bacterial DNA vaccines in fish except *Aeromonas veroni* [20] in sandbass, *Mycobacterium marinum* in hybrid-striped bass [21] and *V. anguillarum* in seabass [6].

V. anguillarum W-1 was originally isolated from diseased sea perch (*Lateolabrax japonicus*) in China [22]. The extracellular zinc metalloprotease (EmpA) of the bacterium possessed high proteolytic activity, and could cause death to turbot (*Scophthalmus maximus*) and Japanese flounder when injected intraperitoneally, resulting in similar symptoms with diseased fish infected by *V. anguillarum* in nature [23,24]. EmpA encoding gene, *empA*, has already been cloned, sequenced and expressed in *Escherichia coli* strain BL21(DE3) [25,26]. Several EmpA mutants (m-EmpA1–m-EmpA15) have been formed by single-point mutagenesis at active sites of the enzyme, and the proteolytic activities and cytotoxicities

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to flounder gill cell line (FG) of them have also been investigated. The mutant m-*empA*7 (with the $Glu^{347} \rightarrow Lys$ mutation) has been found to lose the proteolytic activity and cytotoxicity to flounder gill cell line (FG) completely but retained its antigenicity to the antibody of EmpA of *V. anguillarum* [27].

In this study, we used the gene of a mutant of EmpA, m-*empA*7 (with the $Glu^{347} \rightarrow Lys$ mutation), which expressed the enzyme with no protelytic activity or cytotoxicity, to construct DNA vaccine, and investigated its efficiency in eliciting immune response and protection in Japanese flounder against *V. anguillarum* in experimental infections.

2. Materials and methods

2.1. Cells and bacterial strains

Transfections using the constructed eukaryotic expression plasmid were performed in Chinese hamster ovary (CHO) and human embryonic kidney (HEK293T) cell lines (Type Culture Collection of Chinese Academy of Sciences, Shanghai, China). Both cell lines were cultivated at 37 °C with 5% CO_2 in minimum essential cell culture medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (also Gibco BRL).

V. anguillarum W-1 was originally isolated from diseased seabass (*Lateolabrax japonicus*) in China [22]. TCBS (Trisodium Citrate Bile salts sucrose) agar and nutrient broth with 2% NaCl medium were used for growing *V. anguillarum* at 28 °C. *E. coli* JM109 (New England Biolaboratories, Beverly, MA, USA) grown at 37 °C on Luria–Bertani (LB) medium was used as a host for DNA manipulations.

2.2. Plasmid construction

The gene encoding EmpA of *V. anguillarum* W-1 was cloned, sequenced and mutated in our previous work [25,27]. We chose the mutant pET24d(+)/m-*empA*7 (with the $Glu^{347} \rightarrow Lys$ mutation), which expressed the enzyme with no protelytic activity or cytotoxicity [27] as the template to amplify the m-*empA*7 gene by polymerase chain reaction (PCR) with specific primer set (Table 1). The DNA vaccine was designed on the pEGFP-N1 plasmid backbone (Clontech Laboratories, Palo Alto, CA, USA) with cytomegalovirus (CMV) immediate early promoter driven the expression of the cloned gene. The ORF of the m-*empA*7 gene was inserted in frame with the EGFP reporter gene and the resulting plasmid named pEGFP-N1/m-*empA*7. The construction of the recombinant plasmid was done by conventional cloning procedures. The plasmid was then purified using Qiagen plasmid kits (Qiagen, Valencia, CA, USA), and verified by sequencing prior to use. The concentration of the purified plasmid was determined by spectrophotometry at 260 nm.

2.3. In vitro transfection of pEGFP-N1/m-empA7 in cell lines

The ability of the recombinant plasmid to express mutated EmpA of *V. anguillarum* in eukaryotic cell lines was tested by transfection of CHO and HEK293T cells. The cells were seeded in 6-well plates at ~75–80% confluence and transfected with purified pEGFP-N1/m-*empA*7 according to the calcium phosphate-mediated transfection protocol (Beyotime Inst. Biotech., Haimen, China). In short, 4 μg of the plasmid DNA was added to 100 μl of the transfection reagent and the mixture was then added to each well of the 6-well plate of CHO or HEK293T cells seeded 1 day earlier. The pEGFP-N1 plasmid was used as the negative control.

Table 1
Oligonucleotide primers used in the studies.

Name	Sequence[a]	References
P11	5′-CGCGGATCCATGAAAAAAGTACAACGTC-3′	Chen et al. [25]
P12	5′ -CCGCTCGAGATCCAGTCTTAACGTTACAC-3′	Chen et al. [25]
P51	5′-CCTTTAACCAAGTGGGCGTA-3′	Chen et al. [25]
P52	5′-CGATTTGTAAGGGCGACAAT-3′	Chen et al. [25]

[a] Underlined bases, respectively, indicate BamHI and XhoI restriction sites.

2.4. Detection of m-EmpA7 expressed in transfected eukaryotic cells

The expressed m-EmpA7 in transfected eukaryotic cells was detected for specificity by Western-blot with anti-EmpA serum. Cells expressing m-EmpA7 were washed once with PBS and then detached by the addition of trypsin-EDTA solution. The cell suspensions were transferred into 1.5 ml tubes. After centrifugation (10 min at 1500 × *g*), the supernatant was discarded, and the pellets were resuspended in 1 ml of sample loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue). Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel [28] and then transferred onto a nitrocellulose membrane (Millipore Co., Billerica, MA, USA). The membrane was treated with the rabbit anti-EmpA serum (1:4000), generated by immunizing rabbits with the purified metalloprotease EmpA of *V. anguillarum* W-1 [23], followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) (Wuhan BoShiDe Biotech., Wuhan, China). Development was performed with H_2O_2 and dianilinobenzene as the substrates. The expression of m-EmpA7 in transfected eukaryotic cells was further confirmed by observation of the green fluorescence from the EGFP fusion protein under a fluorescent microscopy.

2.5. Prechallenge

A prechallenge trial was used to determine *V. anguillarum* W-1 infective dose most suitable for the experimental challenge. Eighteen Japanese flounder (*P. olivaceus*) of 9 g were randomly divided into three groups. The fish in each group were injected intraperitoneally (i.p.) with the bacterial suspension of approximately 3.5×10^7, 3.5×10^6 or 3.5×10^5 CFU $fish^{-1}$. The disease progress was followed for 14 days. The randomly sampled dead fish were recorded and the median lethal dose (LD_{50}) was determined according to the method described by Wardlaw [29].

2.6. Immunization and experimental challenge

Seventy Japanese flounder with a mean weight of approximately 9.38 g were used for immune experiment. The fish were randomly divided into five groups and kept in aerated seawater at ~20 °C (half of the water was changed daily throughout the study). The groups were injected intramuscularly (i.m.) with 5, 20, 50 μg of pEGFP-N1/m-*empA*7, 50 μg of pEGFP-N1 or 0.1 ml of PBS, respectively. Four weeks later, each of the immunized fish was challenged by i.m. injection with 0.1 ml of *V. anguillarum* W-1 cell suspension (3.5×10^7 CFU ml^{-1}). Cumulative mortality and clinical signs were recorded daily until the mortality progress decreased in all groups at 10 days post-challenge (dpc). The relative percent survival (RPS) was calculated according to Amend's method [30].

At the end of the experiment, the survived fish were collected and examined. Tissues from kidney, intestine, liver and spleen were dissected for histopathological analyses. The protective efficiency was determined by comparing the RPS and the histopathological damage among experimental treatments.

2.7. Detection of injected DNA in fish tissues

Muscle tissues were taken from the Japanese flounder at 3, 10 and 24 days after vaccination. Tissue samples (0.5–1.0 cm^3) were

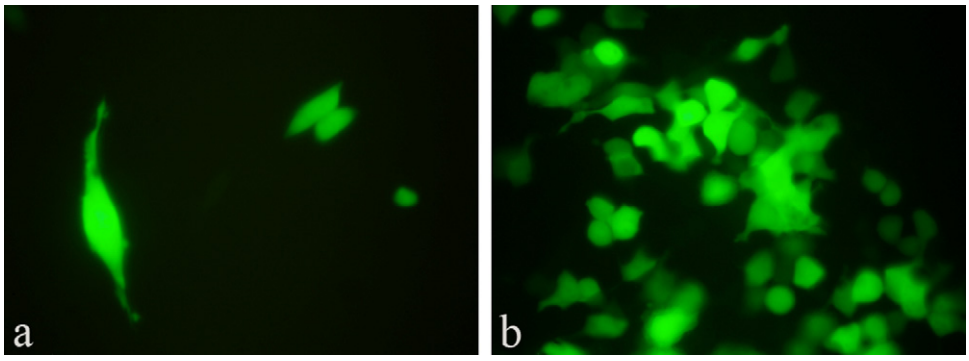

Fig. 1. Expression of m-EmpA7 in cell lines. m-EmpA7 expressed as fusion proteins with the EGFP fusion partner in CHO and HEK293T cells under fluorescence microscopy. (a) m-EmpA7 expressed in CHO and (b) m-EmpA7 expressed in HEK293T.

taken from two fish per group to cover the area of injection, pulverized together to powder in liquid nitrogen, then dissolved in 3 ml genomic DNA isolation buffer (1.0% SDS, 100 mM NaCl, 50 mM Tris–HCl, 100 mM EDTA, pH 8.0, 20 $\mu g\,ml^{-1}$ RNase) and incubated for 1 h at 37 °C. Proteinase K was added to a concentration of 150 $\mu g\,ml^{-1}$ and the samples were incubated at 60 °C overnight. DNA was extracted by conventional phenol-chloroform procedures, and then subjected to 30 PCR cycles consisting of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C, with specific primers (Table 1) targeting the 1484–1731 bp segment of *V. anguillarum* W-1 genomic DNA. PCR products were electrophoresed on a 1.5% agarose gel [25].

2.8. Detection of in vivo transcription of m-empA7 gene by RT-PCR

Total RNA was isolated from muscle tissues (0.5–1.0 cm^3) of two fish in each vaccinated group at 10 days post-vaccination using TRIZOL Reagent (Invitrogen). cDNA was reverse-transcribed from 10 μg of the total RNA in a 25-μl reaction system containing the following: 5 μl of 5× reaction buffer, 1 μl of M-MLV reverse transcriptase (Promega), 1 μl of RNAsin (Toyobo), 2 μl of 0.1 M DTT, 1 μl of oligo-dT primers (50 $\mu g\,ml^{-1}$) (Promega), 6 μl of dNTP mix (10 mM) (Promega) and DEPC-treated water. The samples were heated to 70 °C for 5 min, followed by incubation at 42 °C for 1 h. Reactions were terminated by heating at 95 °C for 5 min. Normalized cDNA samples (concentration of 100 $\mu g\,ml^{-1}$) were used in subsequent PCR reactions.

A 248-bp product of m-*empA*7 gene segment of *V. anguillarum* was amplified using primers P51 and P52 (Table 1) from the cDNA samples. The quality of the RNA samples and the linearity of the RT-step were verified by the simultaneous amplification of endogenous flounder β-actin mRNA in each sample.

2.9. Detection of in vivo expression of m-EmpA7 by Western-blot

The ability of the DNA vaccine plasmid to express m-EmpA7 in Japanese flounder was assayed by Western-blot of muscle tissues sampled from two fish per group, 10 days after immunization. The muscle samples (0.5–1.0 cm^3) were biopsied at the site of injection, homogenized in 1 ml of buffer (100 mM Tris–HCl, pH 6.8, 1.0 mM PMSF, 6% SDS, 2% β-mercaptoethanol) on ice for 20 min and subsequently mixed with equal volume of sample loading buffer. After heat denaturation, the samples were centrifuged at 12,000 × *g* for 20 min and the pellets were discarded. The supernatant was electrophoresed on a 12.5% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane (Millipore). The membrane was then blotted with anti-EmpA serum as described above.

2.10. Analysis of antibody response

At different days post-vaccination, 1–3 fish from each group were assayed for antibody response against m-EmpA7 by enzyme-linked immunosorbent assay (ELISA). A 96-well plate (Costar, Cambridge, MA, USA) was coated overnight at 4 °C with *V. anguillarum* m-EmpA7 (10 $\mu g\,ml^{-1}$) in 100 μl of carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The plate was washed with PBS containing 0.05% Tween 20 (PBST) and then blocked with 3% bovine serum albumin (BSA) in PBS at 37 °C for 2 h. After washing with PBST, the diluted sera (1:50) obtained from individually vaccinated fish were added to triplicate wells of the plate and incubated at 37 °C for 2 h. The plate was then washed twice and rabbit anti-flounder immunoglobulin M serum (1:1000) was added to the plate, and incubated at 37 °C for 2 h. After washing thrice, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) (Wuhan BoShiDe Biotech.) was added to the wells and incubated at 37 °C for 2 h. The reaction was developed by addition of TMB (Wuhan BoShiDe Biotech.) and stopped by 2 N H_2SO_4. The absorbance was then measured with a microtiter plate reader (Labsystems Multistank, MS, Finland) at the wavelength of 450 nm.

2.11. Statistical analysis

A statistical analysis was performed using one-way ANOVA (SPSS, Chicago, IL) for antibody response. If significant, the least significant difference test was further used. The chi-square test was used to assess the protective efficacy of the different vaccine preparations in the comparison with the control groups. *p*-Values were set at the 0.05 level of significance.

3. Results

3.1. Expression of m-EmpA7 in cell lines

The expression of m-EmpA7 as a fusion protein with EGFP was seen as fluorescence in both CHO and HEK293T cells transfected with pEGFP-N1/m-*empA*7 (Fig. 1). The expression of m-EmpA7 was further confirmed by Western-blot analysis. The result revealed specific bands of ~36 kDa corresponding well to the molecular weight of the stable derivative of m-EmpA7 in both cells (Fig. 2). The bands observed were much smaller than the expected for the m-EmpA7–EGFP fusion protein (m-EmpA7: ~36 kDa + EGFP: ~27 kDa). It could be the result of a general incomplete proteolysis at C-terminus of m-EmpA7 depending on the protein itself during preparation of samples by heat treatment, as previously mentioned [26,27]. No specific bands were observed in cells transfected with the empty vector.

Fig. 2. Western-blot analysis of m-EmpA7 expressed in transfected cells. The membrane was probed with a polyclonal antibody raised against EmpA. Lane 1, CHO cells; lane 2, CHO cells transfected with pEGFP-N1; lane 3, CHO cells transfected with pEGFP-N1-m-*empA7*; lane 4, HEK293T cells; lane 5, HEK293T cells transfected with pEGFP-N1; lane 6, HEK293T cells transfected with pEGFP-N1-m-*empA7*.

3.2. Persistence of pEGFP-N1/m-empA7 in muscle tissues

To confirm the presence of the pEGFP-N1/m-*empA7* plasmid in muscle tissues sampled at different time intervals, PCR was performed with *V. anguillarum* m-EmpA7 specific primers. A PCR product of 248 bp was obtained from DNA extracted from muscle tissues sampled at 3, 10, and 24 days post-injection. No amplification was detected in DNA extracted from tissues of the control group (Fig. 3).

3.3. In vivo transcription of m-empA7 gene

Transcription analysis of the m-*empA7* gene in injected muscle tissues was performed by a RT-PCR reaction on DNaseI-treated RNA from fish injected with pEGFP-N1/m-*empA7*, pEGFP-N1 and PBS. The amplification product of 248 bp was obtained from muscle samples taken at the site of injection 10 days after immunization with pEGFP-N1/m-*empA7* (Fig. 4). No amplification was observed in fish either injected with control pEGFP-N1 or PBS.

3.4. In vivo expression of m-EmpA7

Specific bands of ~36 kDa were observed from the muscle biopsies of fish injected with pEGFP-N1/m-*empA7* by Western-blot analysis 10 days after immunization, corresponding well to the molecular weight of the degraded m-EmpA7 (Fig. 5). These findings were consistent with the results obtained from the cells transfected with pEGFP-N1/m-*empA7*, supporting the hypothesis that the ~36 kDa polypeptide is derived from thermo-induced proteolysis of C-terminus of the m-EmpA7 precursor.

3.5. Antibody response to DNA immunization

The humoral immune responses of Japanese flounder to immunization with different doses of pEGFP-N1/m-*empA7* DNA vaccines were assessed by ELISA at different days post-vaccination. No specific antibody against m-EmpA7 was detectable by ELISA in all groups before vaccination (Fig. 6).

Significant specific antibody ($p < 0.05$) was observed in the vaccinated groups at 2 weeks post-vaccination (Fig. 6). Thereafter, the levels of the specific antibody in the vaccinated groups kept on increasing, with the maximum plateau of anti-m-EmpA7 antibody

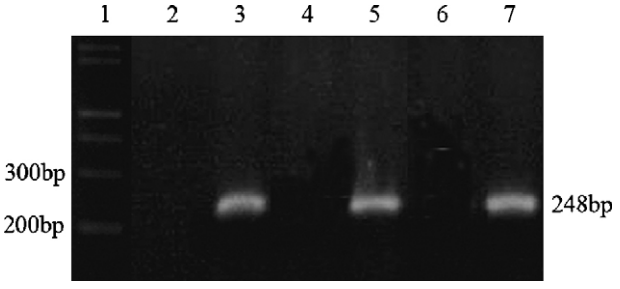

Fig. 3. Detection of vaccine DNA in fish muscle tissue extracts by PCR. Total DNA was extracted from Japanese flounder muscle at 3, 10 and 24 days p.i., and PCR was conducted with the m-EmpA-specific primers described in Table 1. 1, DNA marker; lanes 2, 4, and 6, non-injected groups respectively at 3, 10, and 24 days p.i.; lanes 3, 5, and 7, vaccine DNA injected groups respectively at 3, 10, and 24 days p.i.

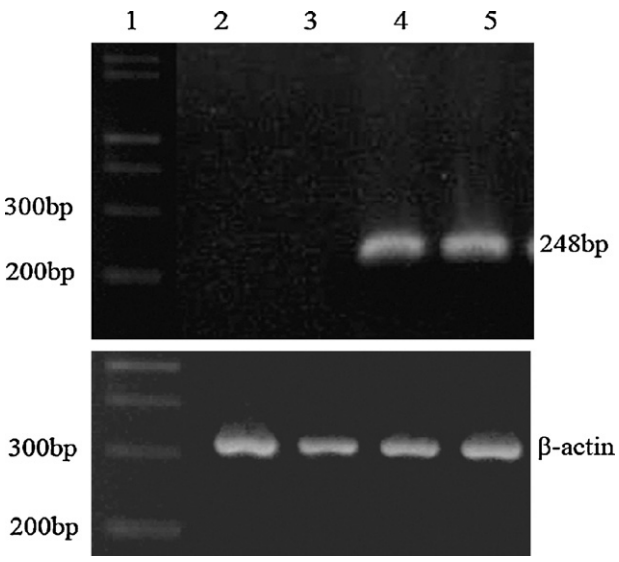

Fig. 4. RT-PCR analysis of transcription of the m-*empA7* gene in fish muscle tissues. 1, DNA marker; 2, muscle injected with negative control pEGFP-N1; 3, muscle injected with PBS; 4–5, muscle injected with vaccine DNA at 10 days p.i. (β-actin put underside).

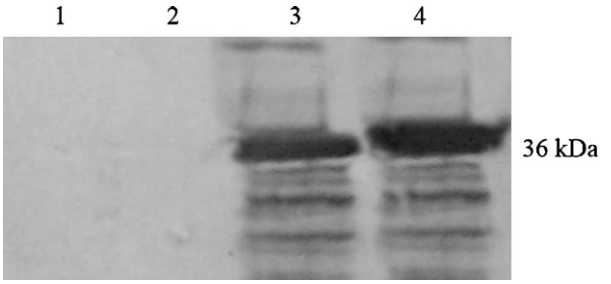

Fig. 5. Western-blot analysis of m-EmpA7 expressed in fish muscle tissues. Lane 1, muscle injected with negative control pEGFP-N1; 2, muscle injected with PBS; 3–4, muscle injected with vaccine DNA at 10 days p.i.

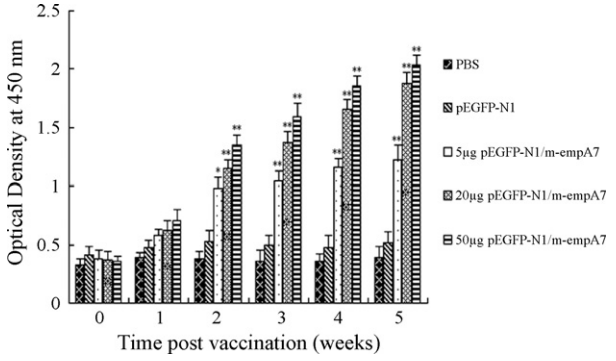

Fig. 6. ELISA detection of anti-m-EmpA7 antibody in serum from fish immunized with plasmid DNA vaccines (pEGFP-N1-m-*empA7*) and control fish injected with PBS and plasmid control (pEGFP-N1). Each column represents the mean optical density (OD) among replica groups measure data at a serum dilution of 1:50 OD. Statistically significant differences (* and **) were found between treatment and control (*$p < 0.05$; **$p < 0.01$).

Table 2
Protective effect of DNA vaccine (pEGFP-N1/m-*empA*7) on Japanese flounder.

Fish injected with:	Cumulative mortality (death/total)	RPS (%)[a]	χ^{2}[b]	*p*-Value
PBS	87.5% (7/8)	–	–	–
pEGFP-N1	75% (6/8)	14.2	0.410	0.522
5 μg pEGFP-N1/m-*empA*7	37.5% (3/8)	57.5	4.267	0.039
20 μg pEGFP-N1/m-*empA*7	25% (2/8)	71.4	6.349	0.012
50 μg pEGFP-N1/m-*empA*7	12.5% (1/8)	85.7	9.000	0.003

[a] Relative percentage survival (RPS) = {1 − [% mortality (vaccinated)/% mortality (PBS control)]} × 100.
[b] χ^{2} of each treatment was calculated based on the values of each treatment and the PBS control.

reached already at 5 weeks post-vaccination. The group injected with 50 μg of pEGFP-N1/m-*empA*7 demonstrated the most notable progress in antibody response and produced highest level of specific antibody ($p < 0.01$) (Fig. 6). Antibody levels against m-EmpA7 were statistically significant at the serum dilution of 1:50 in vaccinated fish in relation to PBS buffer or pEGFP-N1 negative controls; however, no statistical differences were observed between the antibody levels at dilutions of 1:500 or higher (data not shown). No antibodies were detected in the control fish or fish immunized with pEGFP-N1.

3.6. Protection of flounder from *V. anguillarum* challenge

At 4 weeks post-vaccination, Japanese flounder injected with different DNA vaccines were challenged by i.m. injection with *V. anguillarum* W-1 cell suspension. Mortality was first observed at 2 days after the challenge among fish injected with PBS and pEGFP-N1, followed by fish in groups injected with 5, 20 and 50 μg of pEGFP-N1/m-*empA*7 at days 7, 10 and 13, respectively.

As shown in Table 2, there were significant differences in the cumulative mortality and RPS among fish groups. Fish injected with pEGFP-N1 or PBS experienced onset of mortalities early in the experiment, reaching 75% and 87.5% cumulative mortality, respectively, at 10 days after inoculation. In contrast, the groups immunized with 5, 20 and 50 μg of pEGFP-N1/m-*empA*7 showed considerably delayed onset of mortalities and the cumulative mortalities were significantly lower, respectively at 37.5% ($p < 0.05$), 25.0% ($p < 0.05$) and 12.5% ($p < 0.01$), resulting in the RPSs of 57.1, 71.4, and 85.7, respectively.

Histopathological analyses of vaccinated and non-vaccinated control fish those survived from the bacterial challenges revealed signs of tissue lesions resulting from *V. anguillarum* infection (data not shown). Major lesions were observed in the kidney, intestine, liver and spleen, including severe necrosis and infiltration of macrophages. Percentages of fish that presented the histological alterations were 100, 100, 40, 33, and 28 for fish vaccinated with PBS buffer, pEGFP-N1, 5, 20 and 50 μg of pEGFP-N1/m-*empA*7, respectively.

4. Discussion

The extracellular zinc metalloprotease is an important virulence factor of the fish pathogen *V. anguillarum* [23,24,31–34], and the toxin has also been indicated to be a potential candidate antigen for DNA vaccine [26,27]. In this study, we selected the gene of a mutant of EmpA, m-*empA*7 (with the $Glu^{347} \rightarrow Lys$ mutation), to construct the DNA vaccine—pEGFP-N1/m-*empA*7, of which the expressed protein had no proteolytic activity or cytotoxicity but still retained its antigenicity.

The pEGFP-N1/m-*empA*7 was transfected in CHO and HEK293T cells. The expression of m-EmpA7 was confirmed by fluorescence microscopy observation and Western-blot analysis. The expressed protein was about 36 kDa, which corresponded to the molecular weight of the stable derivative of m-EmpA7, but much smaller than the m-EmpA7-EGFP fusion protein. It could be the result of a general incomplete proteolysis at C-terminus of m-EmpA7 depending on the protein itself during preparation of samples by heat treatment, as we previously mentioned [26,27]. Since the expressed EGFP reporter protein was fused with C-terminus of m-EmpA7, degradation at C-terminus of the enzyme resulted in the cleaving away of ~27 kDa EGFP. The pEGFP-N1/m-*empA*7 was further observed to express in the muscle of the injected Japanese flounder by Western-blot at 10 days after immunization, and the specific bands corresponded well to the molecular weight of m-EmpA7. These results suggested that the recombinant m-EmpA7 might be expressed and processed correctly in vitro and in vivo, which may be prerequisite for DNA vaccination to successfully elicit immune responses.

Significant specific antibody responses were observed in the Japanese flounder immunized with the m-EmpA7 DNA vaccine, and the titers of the specific antibody were dose-dependent. The degree of the elicited immune response appeared to be proportional to protection efficiency. Moderate differences of vaccine efficacy were found in the three vaccinated groups (5, 20 and 50 μg of pEGFP-N1/m-*empA*7 i.m. groups), with the RPSs of 57.1%, 71.4% and 85.7%, respectively, as well as the reduction in the number of fish with histopathological alterations compared to the control. Similar results have also been observed in other fish vaccinated with DNA vaccines for bacterial proteins [6,20,35] and viral proteins [9,36,37]. Kumar reported that the DNA vaccine using the outer membrane protein (OMP38) gene of *V. anguillarum* had a moderate level of protection to experimental challenge with *V. anguillarum*, with a RPS of 55.60% [6]. Rainbow trout vaccinated with a DNA vaccine against VHSV had the RPSs of 78–97%, depending on the vaccine dose, after homologous and heterologous challenge, respectively [38].

The plasmid pEGFP-N1/m-*empA*7 injected to fish was detectable in the muscle tissues at 3 days after injection. It was stable, with relatively little degradation being detected by the polymerase chain reaction. The plasmid could be detected in injected muscle even at the latest time point tested, which is 40 days after injection (data not shown). Our results also demonstrated that high levels of transcription and expression of the m-*empA*7 gene could be detected in muscle tissues of Japanese flounder 10 days post-injection respectively by RT-PCR and Western-blot analysis. This course persisted for 45 days after injection and eliminated slowly thereafter (data not shown). Anderson observed that the luciferase activity in rainbow trout (*Oncorhynchus mykiss*) peaked 5–7 days post-injection, and then declined over time. However, the expression of the enzyme could be detected even at 115 days after injection [7]. In goldfish, the cells number expressing β-galactosidase in fish muscle was found to decrease slowly between 21 and 70 days after injection of *lacZ* gene [39]. With the G gene of VHSV, there was indirect evidence that the number of cells expressing the viral protein at day 84 after injection was reduced by 90% compared to the maximum number that is found between 2.5 and 14 days [40]. The persistence of the plasmid might be associated with long-lasting transcription and expression of the encoded antigen.

The specific antibodies might play a role in the prevention of pathogen from adhering and penetrating epithelium of gills, skin and fins of fish during the initial stages of infection, and in the

opsonization of the pathogen for elimination by phagocytosis [41]. The pathogenicity of *V. anguillarum* was partly due to the extracelllular products. These secreted proteins are readily processed by antigen-presenting cells and presented for immune system detection, even before the breakdown of the *V. anguillarum* cells themselves [23,24]. Therefore, secreted proteins such as EmpA are crucial to early immunostimulation and protection against acute *V. anguillarum* infections. The initial success of this vaccine offers promise for the development of a protective vaccine for vibriosis.

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