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Identification of common antigens of three pathogenic *Nocardia* species and development of DNA vaccine against fish nocardiosis

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ABSTRACT

Fish nocardiosis is a chronic granulomatous bacterial disease and three pathogens have been reported so far, including *Nocardia asteroids*, *N. seriolae* and *N. salmonicida*. However, the absence of antigen markers is a bottleneck for developing effective vaccines against fish nocardiosis. In this study, the antigenicity of whole-cell protein of these three pathogenic *Nocardia* species were profiled by immunoproteomic analysis and 7 common immunogenic proteins were identified as follows: molecular chaperone DnaK (DnaK), molecular chaperone GroEL (GroEL), 30 S ribosomal protein S1 (RpsA), TerD family protein (TerD), FHA domain-containing protein (FHA), 50 S ribosomal protein L7/L12 (RplL) and PspA/IM30 family protein (PspA). Furthermore, the DNA vaccine encoding *FHA* gene against fish nocardiosis was developed and its efficacy was investigated in hybrid snakehead. The results suggested that it needed at least 7 d to transport pcDNA-FHA DNA vaccine from injected muscle to head kidney, spleen and liver and stimulate host's immune system for later protection. In addition, non-specific immunity paraments (serum lysozyme (LYZ), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase (AKP) and superoxide dismutase (SOD) activities), specific antibody (IgM) titers production and immune-related genes (*MHCIα*, *MHCIIα*, *CD4*, *CD8α*, *IL-1β* and *TNFα*) were used to evaluate the immune response induced in pcDNA-FHA vaccinated hybrid snakehead, it proved that all these mentioned immune activities were significantly enhanced after immunization. The results also showed hybrid snakehead vaccinated with pcDNA-FHA had higher survival rate (79.33%) compared with the controls after challenge with *N. seriolae*, indicating that the pcDNA-FHA DNA vaccine can supply immune protection against *N. seriolae* infection. Taken together, this study may warrant further development of these common immunogenic proteins as the antigens for vaccine or diagnosis and facilitate the prevention and treatment of fish nocardiosis.

1. Introduction

The genus *Nocardia* is Gram-positive, partially acid-fast, aerobic, catalase-positive, and non-motile pathogenic bacteria, belonging to the class: Actinobacteria, order: Actinomycetales, suborder: Corynebacteriaceae, Family: Nocardiaceae [1]. Nocardiosis is a zoonotic bacterial disease characterized by tissue suppuration, necrosis, or abscess formation, and it has been reported to infect humans and animals, such as fish, horses, cats and dogs [2]. In aquaculture, fish nocardiosis is a chronic granulomatous disease, commonly known as nodular disease. The immunocompromised fish are extremely susceptible to this disease through feeds, gills, and wounds. The typical clinical signs of infected fish include skin ulcers and numerous white nodular structures on gills, and in head kidney, trunk kidney, spleen, liver, etc [3]. So far, three pathogenic *Nocardia* species have been isolated from diseased fishes, including *Nocardia asteroids*, *N. seriolae* (formerly known as *N. kampachi*) and *N. salmonicida*. Notably, *N. seriolae* has been identified as the main pathogen of fish nocardiosis [4–6]. The infection of nocardiosis has been documented in many kinds of fish,

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including both freshwater and marine species, such as *Seriola quinqueradiata* in Japan, *Channa argus* in Korea, *Oncorhynchus tshawytscha* in Europe and America, and *Micropterus salmoides*, *Trachinotus ovatus*, *Larimichthys crocea* and *Terapon jarbua* in China [7–11]. In recent years, fish nocardiosis has frequently reported in global aquaculture industries and its incidence has been increasing yearly, which has caused substantial commercial losses in Southeast Asia, especially China [12].

Traditionally, pharmaceutical therapy to treat fish nocardiosis involves the various sensitive antibiotics, but shows only a limited efficacy and cannot prevent reinfection [13]. In addition, the frequent use of antibiotics promotes the spread of resistant bacteria and causes serious problems of food safety. Therefore, a vaccination strategy is a better choice to against fish nocardiosis. Previous studies shown that heat- and formalin-inactivated *Nocardia* were found to induce high antibody titers, but did not protect against artificial challenge of fish nocardiosis [14,15]. Furthermore, fish injected with live *N. soli* and *N. fluminea*, which are species genetically related to *N. seriolae* but not pathogenic, showed some resistance to artificial challenge with *N. seriolae* [16]. These observations suggest that antigen-specific cell-mediated immunity induced by live vaccines can play a major role in eliminating the bacteria. DNA vaccine is effective in aquaculture, which can express antigenic protein in animal tissues and then induce host immune response [17]. Cell-mediated immune responses are also induced by DNA immunization in fish [18]. In previous researches, DNA vaccines against viral and bacterial pathogens in aquaculture have been extensively analyzed and achieved promising results, and these vaccines have been shown to be effective against pathogen challenges, such as infectious pancreatic necrosis virus (IPNV) [19], infectious hematopoietic necrosis virus (IHNV) [20], *Vibrio alginolyticus* [21], *Aeromonas hydrophila* [22] and *Mycobacterium marium* [23]. In aquaculture, a DNA vaccine against IHNV was approved for clinical use in 2005 [24] and there was no reports about this disease outbreaks in vaccinated populations. Recently, a DNA vaccine against fish nocardiosis using an expression plasmid encoding Antigen 85-like (Ag85L) gene of *N. seriolae* was reported by Kato et al., and it conferred protective efficacy against *N. seriolae* infection in *Seriola dumerili* [25]. Considering the advantage of effective vaccines against fish nocardiosis, it is important to identify common antigens of three pathogenic *Nocardia* species (*N. seriolae*, *N. asteroids* and *N. salmonicida*), which could elicit a strong immune response in fish to analysis their capability to confer protective immunity. Furthermore, the identification and characterization of the common antigens will contribute to improve serological tests for detecting and monitoring the infections of fish nocardiosis.

With the rapid development and improvement of proteomics technology, a field of study that aims to characterize the entire complement of proteins expressed by a cell have been developed maturely [26]. Immunoproteomics is a frontier interdiscipline subject which combines proteomics and immunology and it has been widely applied in various aspects for candidate proteins screening, such as establishment of disease diagnostic markers, screening of drug targets, development of vaccines, promoting the understanding of pathogenesis and identification of common antigens. At present, it is a promising strategy for controlling co-infection of parasite, bacteria or virus to development a multivalent vaccine with common antigen of multiple homologous pathogens. Song et al. reported 5 common immunodominant antigens of *Eimeria tenella*, *E. acervulina* and *E. maxima* identified by immunoproteomics analysis, including elongation factor 2 (EF-2), 14-3-3 protein, ubiquitin-conjugating enzyme domain-containing protein (UCE) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [27]. Among them, the common antigen of GAPDH and 14-3-3 induced significant humoral and cellular immune response and provided effective protection against both one of *Eimeria* specie and the three of *Eimeria* species [28,29]. So far, fish nocardiosis is mainly caused by the infection of three pathogenic *Nocardia* species. Hence, exploring immunodominant antigens, especially common antigens of three pathogenic *Nocardia* species, is essential for developing practical and novel vaccine against fish nocardiosis.

In this study, the common antigens of three pathogenic *Nocardia* species (*N. seriolae*, *N. asteroids* and *N. salmonicida*) were identified by immunoproteomics analysis, which could function as important candidates of DNA vaccines against fish nocardiosis. Besides, the DNA vaccine encoding one of common antigen (Forkhead-associated (FHA) domain-containing protein) was developed and the immune response induce by the vaccine were investigated in hybrid snakehead.

2. Materials and methods

2.1. Bacterial strain, fish and ethics statement

N. seriolae strain ZJ0503 was isolated from diseased golden pompano (*T. ovatus*) in YangJiang city, Guangdong province, China [30], was used in the present study. Both *N. asteroids* (ATCC 19247) and *N. salmonicida* (ATCC 27463) were supplied by China microbe preservation management committee. These nocardial species were cultured in an optimized medium [glucose 20 g L^{-1}, yeast extract 15 g L^{-1}, K_2HPO_4 0.75 g L^{-1}, $CaCl_2$ 0.2 g L^{-1} (sterilized separately), and NaCl 5 g L^{-1}, pH 6.5 ± 0.2)] at 28 °C. Healthy hybrid snakeheads (*Channa argus* ♀ × *Channa maculate* ♂) weighing 30 ± 5 g were obtained from a fish farm in Gaozhou, Guangdong Province, China. They were fed twice daily with commercial feed and were acclimatized at 25 ± 0.2 °C for 2 weeks prior to initiating experiments. Fish were anaesthetized for handling with tricaine methanesulfonate (MS222) (Sigma, Beijing, China) prior to injection and blood collection. All animal experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of GuangDong Ocean University, and the animal facility was based on the National Institutes of Health guide for the care and use of Laboratory (NIH Publications No. 8023).

2.2. Preparation of whole-cell protein extracts

The whole-cell protein of *N. seriolae*, *N. asteroids*, and *N. salmonicida* were respectively extracted according to Coelho et al. [31] with modification. Briefly, the bacteria were grown on optimized medium agar plate at 28 °C for 2–3 d and the cells were harvested for preparation of bacterial suspension. Aliquots of 1 mL 1×10^9 CFU/mL bacterial suspension were spun down and then was washed three times with wash buffer (Tris-HCl 0.25 mol L^{-1}, $C_4H_6O_4Mg{\cdot}4H_2O$ 2.5 mmol L^{-1}). The cells were resuspended in 500 μL phosphate buffer saline (PBS) with lysozyme (1 mg mL^{-1}) and incubations were carried out for 2 h at 37 °C to digest cell wall, washed three times with wash buffer. Subsequently, the cells were spun down and resuspended in 500 μL lysis buffer (Ureophil 7.0 mol L^{-1}, Thiourea 2.0 mol L^{-1}, Chaps 65 mmol L^{-1}, DTT 65 mmol L^{-1}, PMFS 10 μg mL^{-1} (added just before use), IPG buffer 10 μg mL^{-1} (added just before use)). Incubations were carried out for 2 h at 4 °C (or incubations covered with ice), and cell lysis was confirmed by microscopy. Insoluble cellular debris were separated by centrifugation at 15,000 rpm min^{-1}, 4 °C for 1 h, and the clear supernatants containing whole-cell protein were transferred to a new collection tube. For preparative gels of protein identification, the cleanup and concentration determination of the whole-cell protein extracts from *N. seriolae*, *N. asteroids*, and *N. salmonicida* were performed by 2D Clean-Up Kit and 2D Quant Kit (GE Healthcare, Uppsala, Sweden), respectively.

2.3. Two-dimensional gel (2-DE) electrophoresis and western blot

The first-dimensional isoelectric focusing (IEF) and second-dimensional SDS-gel electrophoresis were performed according to the Amersham Biosciences manual [32]. IEF was performed using the IPGphor system (GE Healthcare, Uppsala, Sweden) with IPG strips (Immobiline DryStripTM, pH 4–7, 7 cm, GE Healthcare). Protein samples (approximately 120 μg/strip) were applied by in-gel rehydration (12 h) in a solution containing 7 mol L^{-1} Urea, 2 mol L^{-1} Thiourea, 1% w/v IPG buffer (pH 4–7), 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.15% w/v Dithiothreitol

Table 1
Primers used for gene cloning and expression detection.

Gene name	Primer name	Sequence 5′-3′	Restriction enzyme
FHA	pcDNA-FHA F	CGGGATCCATGAGCGAGAACAAGGACC	*Bam*HI
	pcDNA-FHA R	CCGCTCGAGCTATAGAGCCGAATCGCTCG	*Xho*I

Note: the restriction endonucleases cutting sites were shown with single underline.

(DTT) (GE Healthcare). The strips were focused initially for 1 h at 100 V, 30 min at 300 V, 1 h at 1000 V, 4 h at 5000 V. After IEF, the IPG strips were equilibrated for two intervals of 15 min in a solution containing 50 mmol L^{-1} Tris-HCl pH 6.8, 6 mol L^{-1} Urea, 30% v/v Glycerol, 1% w/v SDS, 1% w/v DTT and with Iodacetamide (2.5% w/v) in the second equilibration solution. Equilibrated strips were placed on 12.5% SDS-polyacrylamide gels, with a Bio-Rad Cell system (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed with an initial constant current of 80 V for 30 min, followed by 180 V until the tracking dye reached the gel bottom. And then, gels were processed for coomassie blue R-250 staining. The IEF and the 2D PAGE experiments of the whole-cell protein from *N. seriolae*, *N. asteroids*, and *N. salmonicida* were repeated three times, respectively.

The whole-cell protein preparations of *N. seriolae*, *N. asteroids*, and *N. salmonicida* were electrophoretically transferred to PVDF membrane (MILIPORE, Billerica, MA, USA) in Baygene BG-blot MINI (Baygene, San Francisco, CA, USA) for 2 h at 100 V, respectively. Western blot was conducted using rabbit anti-*N. seriolae* strain ZJ0503 antibody (Prepared in advance by our laboratory) as the primary antibody at a dilution of 1:1000 and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (BOSTER Biological Technology, China) as the secondary antibody at a dilution of 1:5000. Bound antibodies were detected by reaction in DAB (Diaminobenzidine). All experiments were performed in triplicate.

2.4. Gel analysis and LC-MS analysis

Gel analysis was processed according to the method of previous studies [33]. The immunogenic protein spots with the same isoelectric point and molecular weight of *N. seriolae*, *N. asteroids*, and *N. salmonicida* were respectively excised from the corresponding gels. Subsequently, protein samples were digested with trypsin and analyzed by LC-MS/MS analysis. All the peptide mass fragments of each protein spot were matched to the NCBI database using a MASCOT search engine (www.matrixscience.com) and NMPDR database (http://www.nmpdr.org/FIG/wiki/view,cgi). Protein identifications were considered significant if at least 2 different peptides were matched to the database. Mixture hits were ignored. The immunogenic proteins which were all identified in *N. seriolae*, *N. asteroids*, and *N. salmonicida* were considered as common antigens.

2.5. Bioinformatic analysis

The characteristic of these common antigens from three pathogenic *Nocardia* species were predicted by various online software. Wilkins et al. [34], ExPASy serve (http://www.expasy.org/) predicts the physical and chemical properties. Almagro Armenteros et al. [35], SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/), GPI (http://mendel.imp.ac.at/sat/gpi/gpi_server.html), cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and Loctree 3 (https://rostlab.org/services/loctree3/) improves predictions of signal peptide, membrane location site, nuclear localization signal and subcellular localization using deep neural networks. Jones et al. [36], InterProScan 5.0 program (http://www.ebi.ac.uk/Tools/pfa/iprscan/) predicted the protein family membership in a new java-based architecture.

2.6. Gene cloning and recombinant plasmids construction of FHA gene

FHA domain-containing protein (FHA) was one of the identified common antigens of *N. seriolae*, *N. asteroids*, and *N. salmonicida* and was chosen for following experiments. Genomic DNA was extracted from *N. seriolae* strain ZJ0503 using TIANamp Bacteria DNA Kit (Tiangen, Beijing) following the manufacture's instruction. According to the reference sequence of *FHA* gene from *N. seriolae* strain ZJ0503, the pair of primers were carefully designed with corresponding restriction enzyme sites using Primer 5.0 software (Table 1). The pcDNA-FHA F/R primers were used to amplify the *FHA* gene and the PCR were performed with KOD-plus-Neo DNA polymerase (Toyobo, Osaka, Japan) using the following PCR program, pre-denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 66.9 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 8 min. All PCR products of *FHA* gene were electrophoresed on 1% agarose gel and purified using EasyPure PCR Purification Kit (TRANSGEN, Beijing). The purified PCR products were digested by corresponding restriction enzymes, ligated into eukaryotic vectors pcDNA3.1-Flag (abbreviated herein as pcDNA). And then transformed into competent *Escherichia coli* DH5α cells. The construct was confirmed by corresponding restriction enzyme digestion and DNA sequencing by Guangzhou Sangon Biologic Engineering & Technology and Service Co. Ltd. The constructed recombinant plasmid was named as pcDNA-FHA. The positive clone was grown in LB medium broth with ampicillin and incubated at 37 °C overnight with shaking. The large quantity plasmids of pcDNA-FHA and pcDNA were prepared in advance using an endotoxin-free plasmid purification kit (Qiagen Inc., Chatsworth, CA) following the manufactures' instruction and the concentration was measured by the NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), conserving at −20 °C for the following experiments.

2.7. Vaccination and bacterial challenge in hybrid snakehead

According to previous studies, the plasmids pcDNA and pcDNA-FHA were diluted to 250 μg/mL with PBS [37], respectively. Healthy hybrid snakehead were divided into three random groups (one group with three replicate tanks (100 fish/replicate)) and injected intramuscularly with 100 μL of PBS, pcDNA and pcDNA-FHA, respectively. All the fish were maintained at 25 ± 0.2 °C for 35 d after vaccination. Three fish of each group were sampled to assess their immune response at 0, 1, 3, 5, 7, 14, 21, 28 and 35 d post vaccination (d.p.v.), respectively. Blood, head kidney, spleen, liver and muscle were collected from the injected fish for the various immunological analyses. At 35 d.p.v., the hybrid snakehead of three groups (one group with three replicate tanks (50 fish/replicate)) were challenged by intraperitoneal injection with 100 μL of *N. seriolae* strain ZJ0503 that resuspended in PBS to 3.31×10^6 CFU/mL which was the LD_{50} of *N. seriolae* infected with hybrid snakehead determined in our previous research. Mortality was monitored over a period of 14 d after the challenge, and dying fish were randomly selected for examination of bacterial recovery from head kidney, spleen and liver. Relative percent of survival (RPS) was calculated to the following formula: RPS = [1 – {Mortality (%) in immunized group/Mortality (%) in control group}] × 100%.

2.8. Detection of FHA gene expression in vaccinated hybrid snakehead

The injected muscle, head kidney, spleen and liver were taken from vaccinated hybrid snakehead at 3, 5, 7 and 35 d.p.v. Total RNA extraction and cDNA synthesis of these tissues were conducted using TransZol Up Plus RNA Kit and TransScript One-Step gDNA Removal and

cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), respectively. The RT-PCR assay was performed with the primer pcDNA-FHA F/R (Table 1) to detected the expression of *FHA* gene in hybrid snakehead.

2.9. Determination of immunological serum parameters

2.9.1. Detection of serum non-specific parameters

The blood samples from random fish in each group were collected from caudal vein with a sterile syringe at 0, 1, 3, 5, 7, 14, 21, 28 and 35 d.p.v., respectively. After coagulation, the blood was centrifuged and the serum samples collected from hybrid snakehead were used to measure lysozyme (LZM), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase activity (AKP) and superoxide dismutase (SOD), using the corresponding protease detection kit (Nanjing Jiancheng Bioengineering Institute, China).

2.9.2. Analysis of specific antibody (IgM) against N. seriolae by ELISA

The specific antibody (IgM) titers in hybrid snakehead serum was measured using enzyme linked immunosorbent assay (ELISA). The rabbit anti-hybrid snakehead IgM antibody were prepared in advance by our laboratory. During 35 d.p.v., three fish from each treatment group were assayed for specific antibody (IgM) response against *N. seriolae* every week. Briefly, 96-well microtiter plates were coated with 100 μL/well of *N. seriolae* strain ZJ0503 (1×10^8 CFU/mL) which were prepared by sonicating the cells at 30 Hz for 30 s. Two-fold serial dilutions of the hybrid snakehead serum samples were added to the microtiter plates, which had been blocked with 2% BSA. Antibody binding to the antigen was detected using rabbit anti-hybrid snakehead IgM antibody. Plates were incubated with goat anti-rabbit IgG HRP conjugate (BOSTER Biological Technology, China). The reaction was developed with a chromogenic reagent TMB (3,3,5,5-tetramethylbenzidine) (Nanjing Jiancheng Bioengineering Institute, China) and stopped by 2.0 mol L^{-1} H_2SO_4. Optical density was measured at 450 nm using a microplate reader (Bio-Rad, USA).

2.10. Detection of the expression of immune-related genes by qRT-PCR

Spleen was taken from the vaccinated fish at 0, 1, 3, 5, 7, 14, 21, 28 and 35 d.p.v., respectively. Total RNA extraction and cDNA synthesis were conducted using TransZol Up Plus RNA Kit and TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), respectively. The quantitatively real-time PCR (qRT-PCR) was carried out to investigate the effect of immunization on the expression of immune-related genes (see Table 2), including major histocompatibility complex class Iα (MHCIα), major histocompatibility complex class IIα (MHCIIα), Cluster of differentiation 4 (CD4), Cluster of differentiation 8α (CD8), interleukin 1β (IL-1β), and tumor necrosis factor α (TNFα), using real-time SYBR green PCR Master Mix on LightCycler® 96 SW 1.1 Real-Time PCR system (Roche, USA). Each assay was performed in triplicate with *β-actin* gene as the internal control. The PCR was performed in a 10 μL reaction volume containing 0.5 μL of each primer (10 μM), 0.3 μL cDNA, 3.7 μL PCR-grade water and 5 μL SYBR® Select Master Mix (ABI, USA) according to the manufacturer's protocol. The PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s, for the six immune-related and *β-actin* genes. Melt curve analysis amplification products was performed over a range of 60–95 °C at the end of each PCR reaction aiming to confirm single product generation. The relative expression levels of the immune-related genes were calculated using the comparative Ct $2^{(-\triangle\triangle Ct)}$ method.

2.11. Statistical analysis

Data were presented as the means ± standard deviation (SD). Statistical analysis was performed with one-way ANOVA with the SPSS statistics 21.0 software and the data were edited by GraphPad Prism software. Data represent the means for three independent experiments and statistically significant is highlighted with asterisks in the figures as follows: $p > 0.05$, not significant; $p < 0.05$ (*), significant; $p < 0.01$ (**), extremely significant.

3. Results

3.1. 2-DE profiles and immunoblot analysis of whole-cell protein

Whole-cell protein from *N. seriolae*, *N. salmonicida*, and *N. asteroids* were resolved by 2-DE and the three independent biological samples for each of 2-DE gels were performed, respectively. The same area of the three replicate gels for each experiment was selected for 2-DE profiles analysis. After Coomassie R-250 staining, 312, 371 and 357 spots (± 15 spots, standard deviation) were detected on the gels from *N. seriolae*, *N. salmonicida*, and *N. asteroids*, respectively (Fig. 1 SDS-PAGE). Most of the spots were found to be within the pH 4–7 range and had molecular masses of 20–100 kDa. Western blot analysis was performed on the whole-cell protein separated by 2-DE from *N. seriolae*, *N. salmonicida*, and *N. asteroids* using the rabbit anti-*N. seriolae* antibody, respectively. After western bolt analysis, the 11 immunoreactive protein spots with the same isoelectric point and molecular weight of *N. seriolae*, *N. salmonicida*, and *N. asteroids* were recognized, which were shown on the PVDF membrane (Fig. 1 Western blot).

3.2. Protein identification

Separately, the 11 immunogenic protein spots with the same isoelectric point and molecular weight of *N. seriolae*, *N. salmonicida*, and *N. asteroids* were cut from the gels for detection by LC-MS/MS analysis. The peptide masses were matched with the theoretical peptide masses of all proteins from the NCBI cog database or NMPDR database. The LC-MS/MS analysis showed 11 protein spots corresponded to 11 proteins could be identified in each experiment and their isoelectric point and molecular weight were in good agreement with theoretical predictions. In addition, 7 common proteins were confirmed among 11 immunogenic protein spots of *N. seriolae*, *N. salmonicida*, and *N. asteroids* and they were identified as follow, spot 1: Molecular chaperone DnaK (DnaK); spot 2: Molecular chaperone GroEL (GroEL); spot 3: 30 S ribosomal protein S1 (RpsA); spot 6: TerD family protein (TerD); spot 8: FHA domain-containing protein (FHA); spot 9: 50 S ribosomal protein L7/L12 (RplL); spot 10: PspA/IM30 family protein (PspA) (Table 3).

Table 2
Primes used for the expression of immune-related genes investigated by qRT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
MHCIα	TGCACTCATGGAAGGCATTTTACAC	GGGTAGCCTCTGAGAATGT
MHCIIα	ACTTTGGTACGCGGACTTCA	GAACTTTGGTACGCGGACTT
CD4	AATCTGTCTTCTGACCTCCAAC	CACCCATTTTCCGCTATCT
CD8α	TGGTCGGTTTCCTTGGTT	CTTTGTGCATGAATCCCCAT
IL-1β	GACAAAAGCATCCTGACGAC	GAAAATTGGCGGACCTGA
TNFα	CCGTTTTACACCGGATACTTTG	TACTCGCCCTTCATCACCAC
β-actin	ATGTCGCCCTGGACTTCG	CTGGGCAACGGAACCTCT

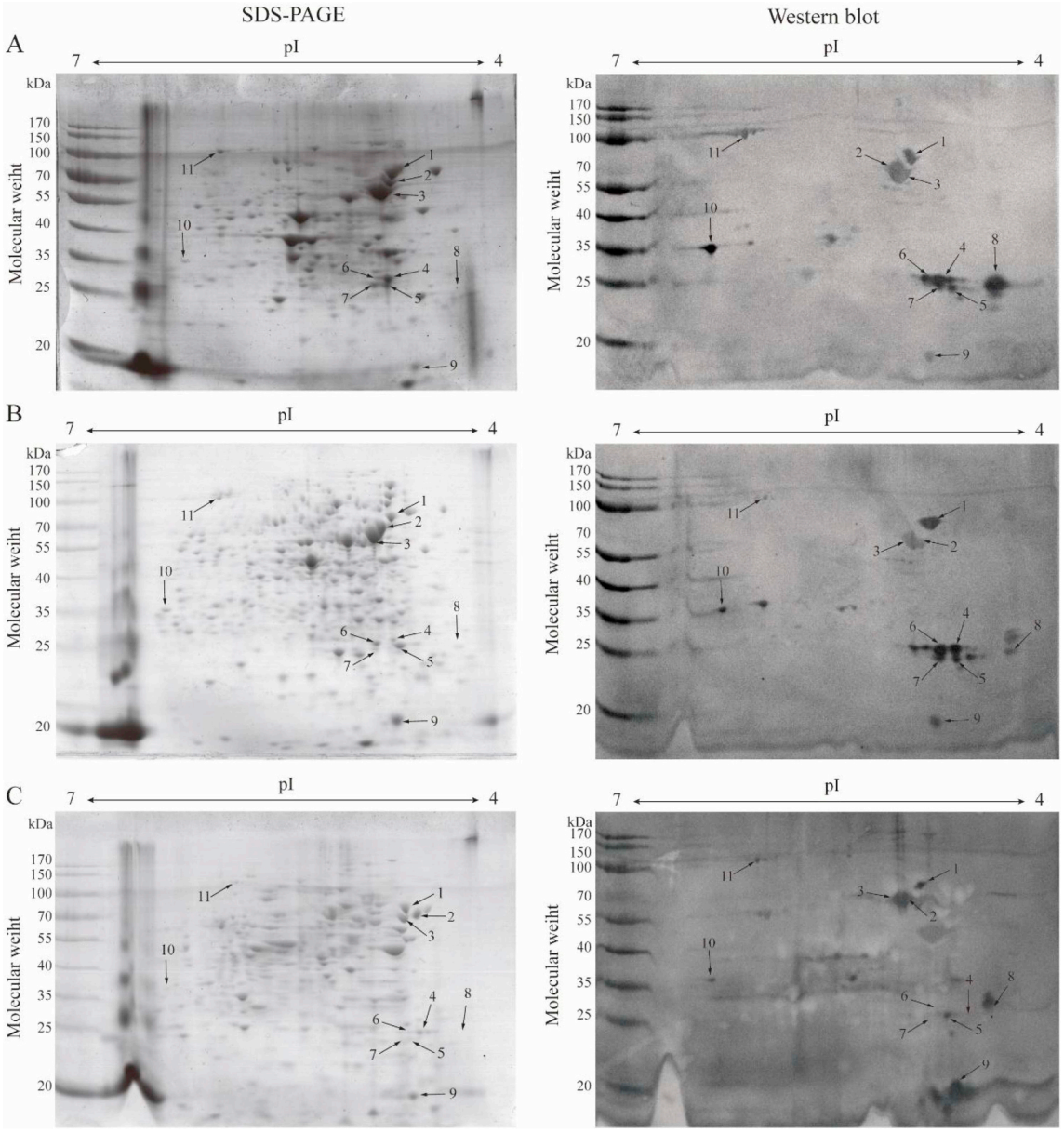

Fig 1. 2D maps (pH 4–7) of the whole-cell lysate of three pathogenic *Nocardia* species and identification of the common antigens by immunoblot analysis. The positions of molecular weight standards are indicated on the left. (A) The typical 2D gel (right) and western blot (left) of *N. seriolae*. (B) The typical 2D gel (right) and western blot (left) of *N. salmonicida*. (C) The typical 2D gel (right) and western blot (left) of *N. asteroids*.

Furthermore, the results of sequence analysis of DnaK, GroEL, RpsA, TerD, FHA, RplL and PspA were shown in Table 4.

3.3. Transcription analysis and expression of DNA vaccines

Transcription analysis of *FHA* gene in the injected muscle, head kidney, spleen and liver of the vaccinated fish was performed by RT-PCR at 3, 5, 7 and 35 d.p.v., respectively. In these tissues, the transcription of *FHA* gene was first detected in injected muscle at 3 d.p.v., in spleen at 5 d.p.v., in head kidney and liver at 7 d.p.v., and continuously detected within 35 d.p.v. in pcDNA-FHA group, but no detection in PBS and pcDNA groups (Fig. 2).

3.4. Analysis of immunological serum paraments

The serum non-specific paraments of LYZ, POD, ACP, AKP and SOD activities were significantly different ($p < 0.05$) between groups after vaccination with pcDNA-FHA. The LYZ value obtained in serum from hybrid snakehead vaccinated with pcDNA-FHA was higher than the corresponding controls at 5, 14, 21 d.p.v. with significantly different ($p < 0.05$) (Fig. 3A). Two time points where there was a statistical difference between groups were at 7 and 14 d.p.v. when the serum alternative POD activity was extreme significantly higher ($p < 0.01$) in pcDNA-FHA vaccinated group (Fig. 3B). The serum ACP activity in pcDNA-FHA vaccinated group was higher than the corresponding controls during 35 d.p.v. and there were significantly different ($p < 0.05$) at 1 and 3 d.p.v., and extreme significantly different ($p < 0.01$) at 5, 7, 14, 21, 28 and 35 d.p.v. (Fig. 3C). The AKP activities measured in serum from pcDNA-FHA vaccinated hybrid snakehead were extreme significantly higher ($p < 0.01$) than the corresponding controls at 5, 7, 14, 21 and 28 d.p.v. (Fig. 3D). The serum SOD activities in pcDNA-FHA vaccinated group were higher than the corresponding controls at 5, 7, 14, 21, 28 and 35 d.p.v. with significantly different ($p < 0.05$) (Fig. 3E).

During 1–35 d.p.v, the specific antibody (IgM) titers of hybrid snakehead was measure using ELISA technique and the statistical analysis was performed to assess the value of $\log_2$ (antibody titer) with ANOVA

Table 3
Immunoreactive proteins of three pathogenic *Nocardia* species by LC-MS analysis.

Spot	Protein name	Symbol	Score	Common peptide segment	Accession no.
1	Molecular chaperone DnaK	DnaK	503	AVGIDLGTTNSVIAVLEGGEPVVVANSEGSR EAGQIAGLNVLR DAGISVGDIDHVVLVGGSTR SETFTTADDNQPSVQIQVFQGER LLGSFELTGIPPAPR	gi|696557073
2	Molecular chaperone GroEL	GroEL	602	TIAYDEEAR KTDDVAGDGTTTATVLAQALVR GYISGYFVTDPER SALQNAASIAALFLTTEAVVADKPEK	gi|749286733
3	30 S ribosomal protein S1	RpsA	323	YFNDGDIVEGTIVK VDRDEVLLDIGYK VEEGIEGLVHISELAER	gi|696556195
6	TerD family protein	TerD	181	YLMALFDSK QHQALSQQAASVIGNQR MLEVQQASVQMAGHNR	gi|696556054
8	FHA domain-containing protein	FHA	210	FLLDQPTTSAGR HPDSDIFLDDVTVSR	gi|696553123
9	50 S ribosomal protein L7/L12	RplL	91	ELVSGLGLK DLVEGAPKPILEK	gi|696556548
10	PspA/IM30 family protein	PspA	509	FDSKIEEHADPKVQ EQSVEDLKVLHDQ HNRLEQIRASMRGDALPAGG	gi|696554183

Table 4
Sequence analysis of common antigens of three pathogenic *Nocardia* species.

Parament	Gene Symbol						
	GroEL	RpsA	DnaK	TerD	FHA	RplL	PspA
Open reading frame (bp)	1656	1467	1893	576	468	381	831
Number of amino acids (aa)	551	488	612	191	155	126	276
Molecular weight (KDa)	57.43	53.89	65.66	20.36	16.79	13.12	29.92
Isoelectric point	4.72	4.76	4.74	4.50	4.44	4.55	5.89
Instability index	28.70	42.75	30.29	24.70	38.52	24.17	46.80
Aliphatic index	104.88	90.66	90.21	80.21	73.48	105.48	81.92
Grand average of hydropathicity	−0.007	−0.388	−0.351	−0.229	−0.555	0.164	−0.516
Alpha helix (%)	52.63	39.55	42.48	21.47	16.13	58.73	80.80
Beta folding (%)	7.99	11.27	9.15	8.38	7.10	3.97	4.71
Random coil (%)	27.04	27.87	26.96	41.36	48.39	24.60	14.49
Signal peptides (SP)	–	–	+	–	–	–	–
GPI modification site	–	–	–	–	–	–	–
Nuclear localization signals (NLS)	–	–	+	–	–	–	–
Predicted subcellular localization	Cytoplasm	Cytoplasm	Cytoplasm	Nucleus	Nucleus	Cytoplasm	Cytoplasm
Molecular function	Antibiotic resistance	Ribosome constituent	Unfolded protein binding	DNA processing complexes	Signal transduction	Ribosome constituent	Transcription and transduction

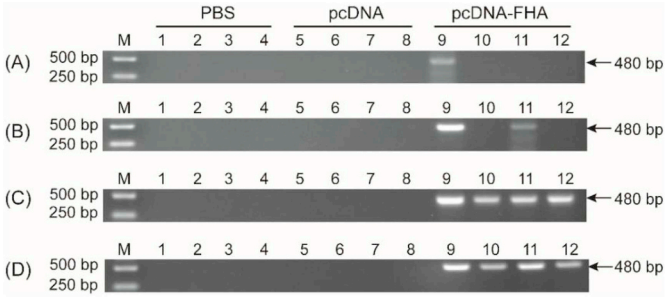

Fig. 2. RT-PCR analysis of transcription of *FHA* gene in different tissues of the vaccinated fish at 3, 5, 7 and 35 d post-vaccination. (A) 3 d post-vaccination; (B) 5 d post-vaccination; (C) 7 d post-vaccination; (D) 35 d post-vaccination. M: DL2000 DNA Marker; lane 1,5,9: muscle; lane 2, 6, 10: head kidney; lane 3, 7, 11: spleen; lane 4, 8, 12: liver.

approach. The specific antibody (IgM) production against *N. seriolae* in hybrid snakehead was shown in Fig. 3F and the result indicated that IgM titer against nocardiosis was detected in the sera of pcDNA-FHA vaccinated hybrid snakehead. From the first week to the fifth week post-vaccination, the values of $\log_2$ (antibody titer) in the sera of pcDNA-FHA vaccinated group stayed above 5.00, while those of the PBS and pcDNA vaccinated groups only kept 1.00–2.00 across all time point. Compared with PBS and pcDNA groups, IgM level in pcDNA-FHA group was higher with extreme significantly different ($p < 0.01$) from first week to the fifth week post-vaccination. In addition, the highest antibody level peaked at the third week post-vaccination and the values of $\log_2$ (antibody titer) was reached to 10.00 in pcDNA-FHA group.

3.5. Analysis of immune responses induced by DNA vaccine

Spleen from vaccinated hybrid snakehead were sampled at different time points after vaccination and immune responses induced by the pcDNA-FHA DNA vaccine were investigated by qRT-PCR. The results showed the expression levels of all investigated immune-related genes in pcDNA-FHA group were increased remarkably compared with that in PBS and pcDNA groups. Especially the expression levels of cluster of differentiation genes, *CD4* and *CD8α*, increased notably in pcDNA-FHA vaccinated group compared with that in PBS and pcDNA groups at

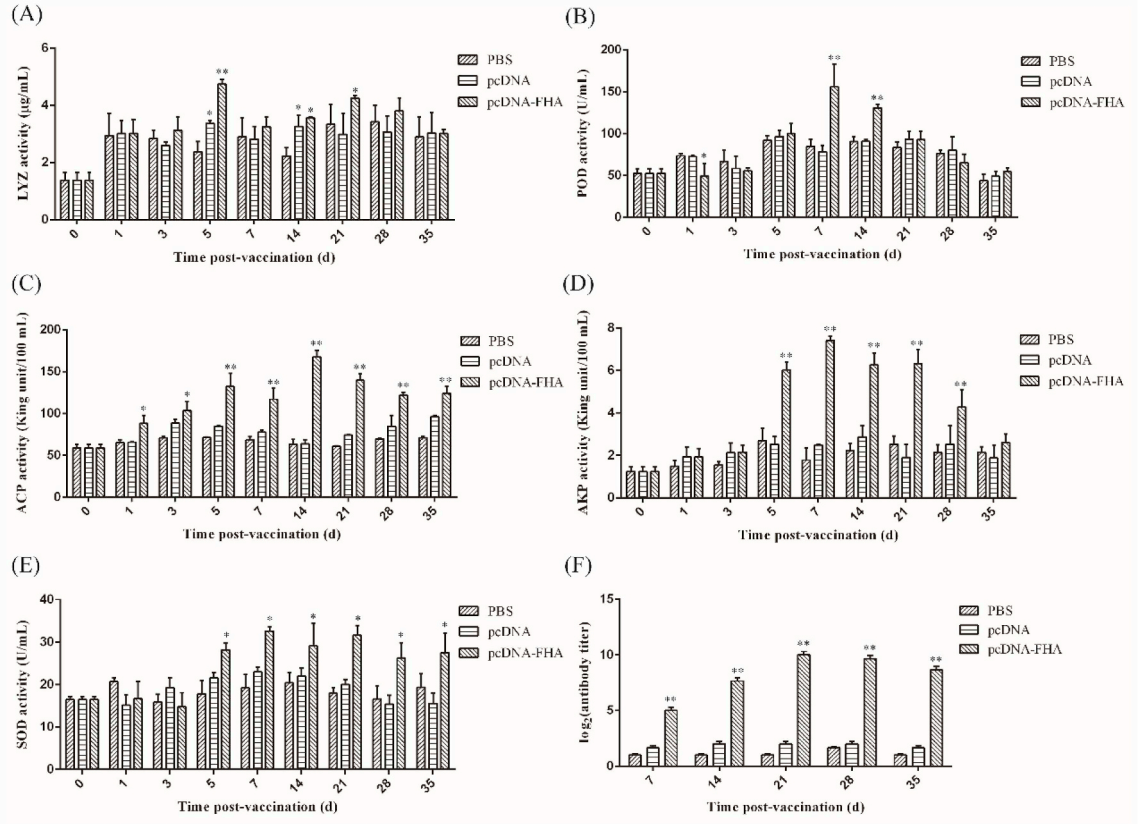

Fig. 3. The immunological serum parameters of vaccinated hybrid snakehead. (A) Serum LYZ activity. (B) Serum POD activity. (C) Serum ACP activity. (D) Serum APK activity. (E) Serum SOD activity. (F) Specific antibody titers against *N. seriolae* in hybrid snakehead by ELISA analysis. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. The different letters above the bars indicate the significant difference among different groups at the same time point ($*p < 0.05$, $**p < 0.01$).

1–35 d.p.v. Similarly, *MHCIα*, *MHCIIα*, *IL-1β* and *TNFα* genes were up-regulated significantly in pcDNA-FHA vaccinated group compared with that in PBS and pcDNA groups at 1–35, 3–21, 1–7 and 1–21 d.p.v., respectively (Fig. 4).

3.6. Vaccine efficacy against N. seriolae

After challenge with the pathogenic *N. seriolae* strain ZJ0503 at 35 d.p.v., cumulative survival rates of hybrid snakehead in each group were recorded during 14 d post-challenge. As shown in Fig. 5, the survival rate of hybrid snakehead in pcDNA-FHA vaccinated group was 79.33%, while the cumulative survival rates of hybrid snakehead vaccinated pcDNA and PBS groups were 45.33% and 44.67% at 14 d post-challenge by *N. seriolae* strain ZJ0503, respectively. Moreover, the RPS of hybrid snakehead vaccinated with pcDNA and pcDNA-FHA were 1.19% and 62.64%, respectively. The results indicated the survival rate of hybrid snakehead vaccinated in pcDNA-FHA vaccinated group was higher than that in corresponding control groups after challenge with *N. seriolae* strain ZJ0503 and the DNA vaccine encoding *FHA* gene had immune protection effect against *N. seriolae* in hybrid snakehead. The hybrid snakehead in PBS and pcDNA groups began to die at 4 d post-challenge, with a suddenly death increase at 5–10 d post-challenge, and then stay stable with no more dead fish at 11–14 d post-challenge. The dead hybrid snakehead in all groups showed typical signs of fish nocardiosis, including anorexic, skin ulcers and numerous white nodular structures in head kidney, trunk kidney, spleen, liver and no other pathogen except *N. seriolae* was isolated from the dead hybrid snakehead.

4. Discussion

Fish nocardiosis is a systemic bacterial disease and three pathogenic bacteria had been isolated from disease fish so far, including *N. samonicida*, *N. seriolae*, and *N. asteroids* [2]. Attempts to control fish nocardiosis are hampered by a lack of effective vaccines and the identification of common antigens of three pathogenic *Nocardia* species could facilitate the development of vaccine against fish nocardiosis. In this study, we systematically identified the common immunogenic proteins of *N. seriolae*, *N. samonicida*, and *N. asteroids* by immunoproteomic method and 7 common immunogenic proteins were identified as follows: DnaK, GroEL, RpsA, TerD, FHA, RplL and PspA. These common immunogenic proteins have already been revealed to antigenic in some pathogenic bacteria. For example, two heat shock proteins of *Francisella tularensis* (DnaK and GroEL) were found as antigens to induced long-lasting recall response in $CD4^+$ and $CD8^+$ alphabeta T cells [38]. Besides, previous study also showed that GroEL functioned as an antigen was actively secreted in *Bartonella bacilliformis* [39]. The fork-head associated (FHA) domain of ABC transporter was required for normal virulent infection by *M. tuberculosis* in mice, which formed parts of an important phosphor-dependent signaling pathway by interacting with a seine-threonine protein kinase in a kinase-dependent manner [40]. A putative phage-shock-protein A (PspA) homologue in *Burkholderia pseudomallei* carried out an extracytoplasmic response which was important for survival during the stationary phase, maintenance of the proton motive force across membranes and implicated in virulence [41]. *Yersinia pestis* tellurite resistance proteins TerD and TerE from the *terZABCDE* operon played an important role in filamentous cellular morphology during macrophage infection [42]. The 30 S ribosomal protein S1 (RpsA) protein of *M. tuberculosis* as antigenic target induced the specificity of the $CD4^+$ T cell response in the host cells [43] and the antibody had been raised in rabbit against 50 S ribosomal protein L7/L12 (RplL) of *Escherichia coli* [44]. Therefore, these common immunoreactive proteins might provide novel candidates as common

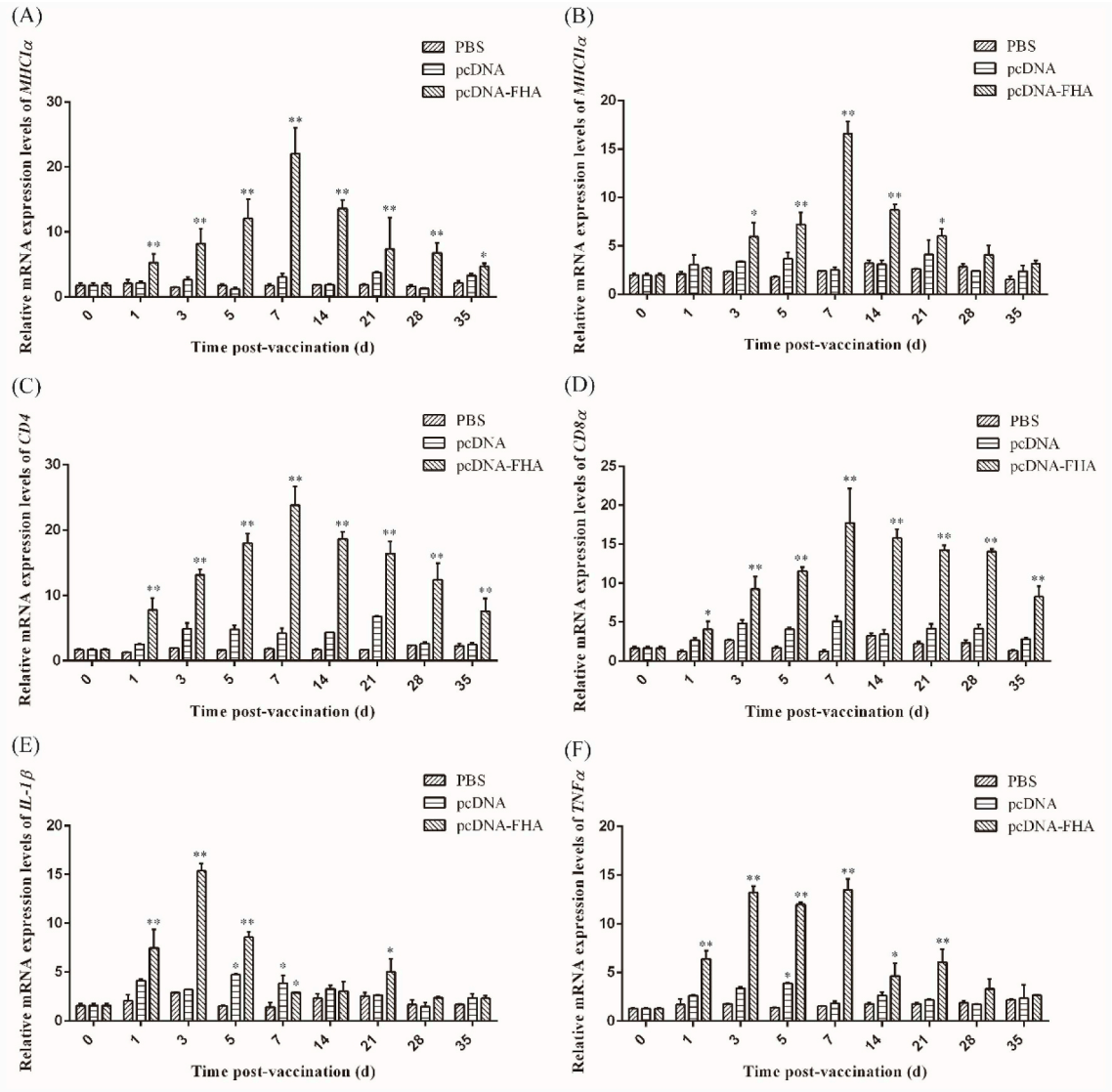

Fig. 4. qRT-PCR analysis of the expression of immune-related genes. The data were presented as the expression of *MHCIα* (A), *MHCIIα* (B), *CD4* (C), *CD8α* (D), *IL-1β* (E) and *TNFα* (F) genes. The mRNA level of each immune-related gene was normalized to that of *β-actin* and relative expression was calculated by deciding the 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. The different letters above the bars indicate the significant difference among different groups at the same time point (*$p < 0.05$, **$p < 0.01$).

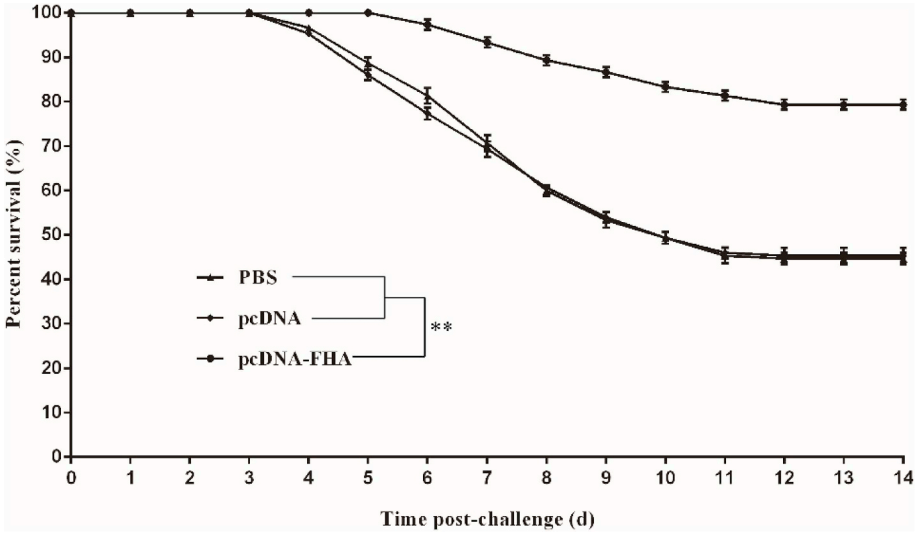

Fig. 5. Survival rate of vaccinated hybrid snakehead immunized with pcDNA-FHA, pcDNA or PBS following the challenging tests of 14 d by N. *seriolae* strain ZJ0503. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. The different letters above the bars indicate the significant difference among different groups at the same time point (**$p < 0.01$).

antigens for effective vaccine development against fish nocardiosis in the future. Among these 7 common immunoreactive proteins, the FHA domain-containing protein serves as a site for phosphorylated protein-protein interactions and conserved among bacterial species [45,46]. Previous studies have revealed that FHA protein of *M. tuberculosis* is associated with normal infection of macrophages, dormancy, and growth in the host [47,48]. And it was indicated that the common immunogenic protein of FHA also functioned as the potential virulence of three pathogenic *Nocardia* species, which would be a promising candidate for vaccine development. Hence, the protective efficacy of pcDNA-FHA DNA vaccine was performed firstly in hybrid snakehead against fish nocardiosis.

Normally, at the administration site, some DNA vaccine may be transferred to the circulatory system and distributed to other organs and tissues following cell migration after several days post-vaccination. This phenomena indicated that plasmid DNA could avoid local degradation at the administration site and in the blood plasma [49]. In our study, the intramuscular injection was chosen as vaccination strategies of pcDNA-FHA DNA vaccine, which could induce the persistent expression of exogenous antigen gene in the host cells [50]. When pcDNA-FHA DNA vaccine were injected into hybrid snakehead, the transcription of *FHA* gene in muscle, head kidney, spleen and liver were all detected from 7 to 35 d.p.v. It is suggested that the transfer of pcDNA-FHA DNA vaccine from the injected muscle to main immune organs took at least 7 d and stimulated host's immune system for following immune protection and this results were coincident with some other previous studies [37,51].

It has been shown that DNA vaccines stimulate innate, humoral, and cellular immune response, thereby more closely resemble to the balanced response in natural infection [52]. As lower vertebrates, fish mainly depend on non-specific immune to eliminate and kill invasive pathogenic bacteria in the first-line defense mechanism, such as opsonizing bacteria to enhance phagocytosis and activating the complement system by LYZ, utilizing oxidative radicals to produce hypochlorous acid to kill pathogens by POD, removing superoxide radicals to protect the reductive substances of host cells by SOD, activating phagocytes and increasing host's stress reaction by ACP, and changing the surface structure of pathogenic bacteria to enhance phagocytosis and degradation of phagocytes toward pathogenic bacteria [53–55]. Thus, it was necessary to detect the serum non-specific parameters to evaluate the immune effects of pcDNA-FHA DNA vaccine. In this study, the significant increase of serum non-specific parameters such as LYZ, POD, ACP, AKP and SOD activities were observed in pcDNA-FHA vaccinated group, while no significant changes were observed in corresponding control groups. These results might partly explain the improved immune response in hybrid snakehead vaccinated with pcDNA-FHA DNA vaccine.

In teleost fish, the spleen contains abundant B cells and is involved in trapping antigens from the blood. After stimulation with antigens, the B cells in spleen will differentiate into plasma cells and migrate to other immune related tissues by bloodstream [56]. Thus, the spleen of hybrid snakehead was chosen to analyze the immune response and the mRNA levels of immune-related genes (*MHCIα*, *MHCIIα*, *CD4*, *CD8α*, *IL-1β* and *TNFα*) were investigated by qRT-PCR. Among these genes, MHCIα and MHCIIα responsible for binding exogenetic peptides for the presentation to $CD8^+$ and $CD4^+$ T cells, respectively [56,57]. And a specific antibacterial response, especially the $CD8^+$ cytotoxic T lymphocyte (CTL) and $CD4^+$ Th cell responses will be elicited following the up-regulation of CD4 and CD8α [58]. As the important proinflammatory cytokines, IL-1β and TNFα function in initiation of the pro-inflammatory cytokine cascade, recruitment and activation of macrophages, and stimulation of the adaptive immune response [55]. In this study, the significantly increase on the mRNA levels of *MHCIα*, *MHCIIα*, *CD4* and *CD8α* indicated that the recognition and presentation of pcDNA-FHA DNA vaccine might involve in both MHCI-CD8 and MHCII-CD4 pathways. Meanwhile, the high expression of *IL-1β* and *TNFα* were also examined after vaccinated with pcDNA-FHA, which enhanced the inflammatory immune response of hybrid snakehead. These results further explained the improved immune response in hybrid snakehead vaccinated with pcDNA-FHA DNA vaccine.

Apart from the immune response mentioned above initiated by the DNA vaccine, antibody produced in response to vaccination are critical to the prevention of bacterial infection, and antibodies generated in response to infection were considered likely to play a major role in reducing mortality of bacterial challenge [59]. It is known that IgM as a major component of the humoral immune system of teleost fish, is regarded as the first antibody [54]. In this study, the specific antibody (IgM) titers generated against *N. seriolae* was assessed in the pcDNA-FHA vaccinated hybrid snakehead by ELISA. The result showed that the IgM expression increased significantly from the second week, persisting up to the third week of post-vaccination and maintaining high expression level within 35 d after immunization, which was consistent with the results of the antibody changes in the *Flavobacterium columnare* stimulated mandarin fish [60]. The survival rate of pcDNA-FHA vaccinated group was higher than that of control groups and the pcDNA-FHA DNA vaccine supplied immune protection against *N. seriolae* infection in hybrid snakehead with 62.24% RPS. Numerous studies have shown that the application of DNA vaccines can acquire preferable RPS against bacterial diseases in fish aquaculture, such as DNA vaccine encoding codon-optimized Ag85L against *N. serioale* in *S. dumerili* with RSP of 92.3% and 36.9% by low- and high-dose separate challenge [25], DNA vaccine encoding antigenic AcfA via addition of the molecular adjuvant Myd88 against *V. alginolyticus* in *Epinephelus coioides* with RPS of 83.3% by intramuscular injection [37], oral recombinant DNA vaccine SL7207-pVAX1-*sip* against *Streptococcus agalactiae* in *Nile tilapia* with RPS of 63% by mixed fodder administration and so on [55]. Therefore, in order to obtain highly effective vaccine for the treatment of fish nocardiosis, the immune effect induction of DNA vaccine encoding the other 6 novel common antigens of three pathogenic *Nocardia* species (DnaK, GroEL, RpsA, TerD, RplL and PspA) also need to be investigated in the future studies. Furthermore, DNA vaccine with addition of various adjuvants and chimeric DNA vaccine encoding multiple antigens will be performed for further study to improve their RPS against fish nocardiosis.

In summary, the immunogenic proteins of *N. seriolae*, *N. samonicida*, and *N. asteroids* were profiled by immunoproteomic analysis and 7 common antigens of these three pathogenic *Nocardia* species were identified systematically. Moreover, a DNA vaccine was developed by applying the common immunogenic protein of FHA as antigen, and pcDNA-FHA DNA vaccine induced significant immune response and supplied immune protection against *N. seriolae* infection in hybrid snakehead. Furthermore, the other 6 identified common antigens may be also applied to vaccine development against fish nocardiosis in further studies.

Declaration of competing interest

The authors declare no conflict of interest.

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