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Comparison of protective efficacy between two DNA vaccines encoding DnaK and GroEL against fish nocardiosis

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ABSTRACT

Fish nocardiosis is a chronic granulomatous bacterial disease mainly caused by three pathogenic bacteria, including *Nocardia seriolae*, *N. asteroids* and *N. salmonicida*. Molecular chaperone DnaK and GroEL were identified to be the common antigens of the three pathogenic *Nocardia* species in our previous studies. To evaluate the immune protective effect of two DNA vaccines encoding DnaK or GroEL against fish nocardiosis, hybrid snakehead were vaccinated and the immune responses induced by these two vaccines were comparatively analyzed. The results suggested it needed at least 7 d to transport *DnaK* or *GroEL* gene from injected muscle to head kidney, spleen and liver and stimulate host's immune system for later protection after immunization by DNA vaccines. Additionally, non-specific immunity parameters (serum lysozyme (LYZ), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase (AKP) and superoxide dismutase (SOD) activities), specific antibody (IgM) production and immune-related genes (*MHCIα*, *MHCIIα*, *CD4*, *CD8α*, *IL-1β* and *TNFα*) were used to evaluate the immune responses induced in vaccinated hybrid snakehead. It proved that all the above-mentioned immune activities were significantly enhanced after immunization with these two DNA vaccines. The protective efficacy of pcDNA-DnaK and pcDNA-GroEL DNA vaccines, in terms of relative percentage survival (RPS), were 53.01% and 80.71% respectively. It demonstrated that these two DNA vaccines could increase the survival rate of hybrid snakehead against fish nocardiosis, albeit with variations in immunoprotective effects. Taken together, these results indicated that both pcDNA-DnaK and pcDNA-GroEL DNA vaccines could boost the innate, humoral and cellular immune response in hybrid snakehead and show highly protective efficacy against fish nocardiosis, suggesting that DnaK and GroEL were promising vaccine candidates. These findings will promote the development of DNA vaccines against fish nocardiosis in aquaculture.

1. Introduction

Fish nocardiosis is a widespread chronic granulomatous disease in aquatic environment and its infection of fish leads to skin ulcers and numerous white nodular structures on gills, and in head kidney, trunk kidney, spleen, liver, etc [1]. 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* [2–4]. Notably, *N. seriolae* was the most frequently isolated pathogen of fish nocardiosis recently and has been identified as the main pathogen of fish nocardiosis [5]. The infection of fish nocardiosis has been documented in more than 39 kinds of fish, both freshwater and especially marine species [6]. 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 [7]. Traditionally, pharmaceutical

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therapy to treat fish nocardiosis involves the various sensitive antibiotics, nevertheless this practice shows only a limited efficacy along with serious problems of environmental pollution, microbial resistance, and drug residues [8,9]. In contrast, vaccines have advantages in safety, environmental friendliness, and long-term efficacy protection in aquaculture.

An ideal practical field vaccine should include common protective antigens among three pathogenic *Nocardia* species (*N. seriolae*, *N. asteroids* and *N. salmonicida*), which could elicit a strong immune response in fish to analyze their capability to confer protective immunity. In our previous research, molecular chaperone DnaK and GroEL were shown to be the common immunodominant antigens from *N. seriolae*, *N. asteroids* and *N. salmonicida* by immunoproteomics analysis (Not published). DnaK and GroEL belong to the heat shock family of proteins (HSPs), also known as Hsp70 and Hsp60 respectively. Homologs of DnaK and GroEL are present in almost all species of bacteria, which play critical roles in modulating protein fold within cells to guarantee their normal functions [10]. In addition, accumulating evidences have demonstrated that extracellular DnaK and GroEL proteins from different pathogenic bacteria play protective and regulatory roles in host's immune response. For example, the secreted protein of DnaK from *Mycobacterium tuberculosis* acts as an immunomodulator with putative virulence role in bacterial infections, polarizing macrophages to M2 phenotype, with production of IL-10 [11]. The secretion of GroEL protein has been previously reported in *Helicobacter pylori*, in which the GroEL protein can be found extracellularly in culture supernatants and serve to protect a secreted urease enzyme [12,13]. Moreover, the GroEL may locate on the surface and also in the secretome of several pathogenic bacteria, which play an important role in the adherence and uptake of bacteria into the host cells [14,15]. These features, at least in part, explain the role of DnaK and GroEL proteins in pathogenic bacterial virulence.

Being the conserved proteins and having a pivotal role in pathogenic microbes, bacterial DnaK and GroEL have been considered to provide a novel candidate for vaccine development. Moreover, it have been reported that DnaK and GroEL have abilities to stimulate a memory T cell response and are potent stimulator of immune response even in the absence of CD4 cells [16]. Therefore, DnaK and GroEL proteins have being applied as antigens for vaccine development against various pathogenic bacteria, such as a subunit vaccine of DnaK protein against *Salmonella enterica* [17], a tetra-antigen vaccine by conjugating OmpA, DnaK, Tul4 and SucB proteins against *Francisella tularensis* [18], a DNA vaccine encoding GroEL against *Edwardsiella tarda* [10], a chimeric DNA vaccine based on SopB and GroEL against *S. enterica*, and so on. So far, fish nocardiosis is mainly caused by the infection of *N. seriolae*, *N. asteroids* and *N. salmonicida* and the common protective antigens of DnaK and GroEL from these three pathogenic *Nocardia* species could be good candidates to develop effective vaccines against fish nocardiosis.

DNA vaccine is effective in aquaculture, which can express antigenic protein in animal tissues and then induce host immune response [19]. Additionally, cell-mediated immune responses are also induced by DNA immunization in fish [20]. A lot of researches have extensively analyzed DNA vaccines, achieved promising results and showed DNA vaccines can supply effective protection against pathogen challenges. Besides, a DNA vaccine against IHNV was approved for clinical use in 2005 [21] 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* [22]. Therefore, the application of common protective antigens of DnaK and GroEL as candidates of DNA vaccine is a reasonable strategy to against fish nocardiosis.

In the present study, the secreted proteins of DnaK and GroEL were identified from extracellular products of *N. seriolae* by shotgun mass spectrometry (MS). Furthermore, two DNA vaccines encoding DnaK and GroEL were developed and the protective efficacy were investigated and compared 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 [23] and was 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 snakehead (*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. Bioinformatic analysis, sequence alignments, and phylogenetic analysis

According to the whole genome sequence data of *N. seriolae* strain ZJ0503 (accession no. NZ_JNCT01000022), sequence analysis was performed with the BLAST program using NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The physical and chemical properties were predicted using ExPASy software (http://www.expasy.org/). The location of domains was predicted by SMART (http://smart.embl-heidelberg.de/) and IntroPro (http://www.ebi.ac.uk/interpro/). The signal sequence was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) online software. Nuclear localization signal was predicted by cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). Protein family membership was predicted by the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The typical structures were predicted by SWISS-MODEL online software (https://swissmodel.expasy.org/interactive) and modified by PyMOL software. Protein multiple sequence alignments were performed by ClustalX 2.0 program with the default parameters and edited by the GeneDoc software. The phylogenetic tree was generated based on the deduced amino acid sequence with the neighbor-joining method using MEGA 6 program, in which the poisson distribution substitution model and bootstrapping procedure with 1000 bootstraps were applied.

2.3. Preparation and identification of the N. seriolae extracellular products

The extracellular products of *N. seriolae* strain ZJ0503 were obtained by cellophane overlay method [24,25]. In brief, *N. seriolae* were grown on optimized medium agar plate at 28 °C for 2 d, and a single colony was prepared for bacterial suspension. Then, 100 μL of the bacterial suspension was spread closely on optimized medium plates covered with sterile cellophane sheet and incubated at 28 °C for 3–5 d. The *N. seriolae* cells grown on the cellophane sheet were stripped away from the optimized medium plates, and the extracellular products were washed down with sterilized PBS, subsequently. The harvested suspension was centrifuged at 8000 × *g*, 4 °C for 20 min, and the supernatant containing extracellular products was filter sterilized with a 0.2 μm membrane filter. Then, the sterilized supernatant was transferred into a dialysis tubing (3.5 k MW) and dialyzed in ultrapure water at 4 °C for 16–24 h. During dialysis, the ultrapure water was changed 3–4 times. The purified supernatant was transferred into a centrifuge tube after dialysis and frozen under −80 °C. Finally, it was lyophilized

Table 1
Primers used for gene cloning and expression detection.

Gene name		Primer name	Sequence 5′-3′	Restriction enzyme
DnaK		pcDNA-DnaK F	GAAGATCTATGGCTCGTGCGGTCGGTATC	*Bgl*II
		pcDNA-DnaK R	GGAATTCTCACTTCTTCTCAGGCTCCTCGACGACCTC	*Eco*RI
GroEL	pcDNA-GroEL F		CGGGATCCATGCCCATCCCTGATCTGGAG	*Bam*HI
	pcDNA-GroEL R		GGAATTCCTAGAAGTCCATGCCGCCCATG	*Eco*RI

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

using a vacuum freeze dryer to obtain the protein dry powder which was identified using shotgun MS.

2.4. Cloning and recombinant plasmids construction of DnaK and GroEL genes

Molecular chaperone DnaK and GroEL were the identified common antigens of *N. seriolae*, *N. asteroids*, and *N. salmonicida* in our previous research 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 *DnaK* and *GroEL* genes from *N. seriolae* strain ZJ0503, two pairs of primers were carefully designed with corresponding restriction enzyme sites using Primer 5.0 software (Table 1). The pcDNA-DnaK F/R and pcDNA-GroEL F/R primers were used to amplify the *DnaK* and *GroEL* genes, respectively. 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, 67.5 °C (DnaK)/63.9 °C (GroEL) for 90 s, 72 °C for 60 s and a final extension at 72 °C for 8 min. All PCR products of *DnaK* and *GroEL* genes 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 plasmids were named as pcDNA-DnaK and pcDNA-GroEL, respectively. 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-DnaK, pcDNA-GroEL 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.

2.5. Vaccination and bacterial challenge in hybrid snakehead

According to previous studies, the plasmids pcDNA, pcDNA-DnaK and pcDNA-GroEL were diluted to 250 μg/mL with PBS [26], respectively. Healthy hybrid snakeheads were divided into four random groups (one group with three replicates tanks (100 fish/replicate)) and injected intramuscularly with 100 μL of PBS, pcDNA, pcDNA-DnaK and pcDNA-GroEL, 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 replicates 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%.

2.6. Detection of the expression of DnaK and GroEL genes by RT-PCR

The injected muscle, head kidney, spleen and liver were taken from vaccinated hybrid snakehead at 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-DnaK F/R and pcDNA-GroEL F/R (Table 1) to detect the expression of *DnaK* and *GroEL* genes in hybrid snakehead, respectively.

2.7. Investigation 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).

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

The specific antibody (IgM) production in hybrid snakehead serum was measured using enzyme linked immunosorbent assay (ELISA) at 7, 14, 21, 28, and 35 d post-vaccination. The rabbit anti-hybrid snakehead IgM antibody were prepared in advance by our laboratory (Not published). 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 as the primary antibody at a dilution of 1: 1000. Plates were incubated with goat anti-rabbit IgG HRP conjugate (BOSTER Biological Technology, China) as the secondary antibody at a dilution of 1:5000. 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).

2.9. 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

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

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 (CD8α), 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.

2.10. 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. Sequence analysis and characterization of DnaK and GroEL

The *DanK* and *GroEL* genes were obtained from genomic DNA of *N. seriolae* strain ZJ0503 by PCR. Sequence analysis revealed that the open reading frame (ORF) of *DnaK* gene was 1893 bp and encoded a polypeptide of 612 amino acids (aa) with an estimated molecular mass of 65.66 kDa and a theoretical isoelectric point (pI) of 4.74. The structure of DnaK protein was composed of a signal peptide (resides 1–29 aa, MARAVGIDLGTTNSVIAVLEGGEPVVVAN) at N-terminate, a nuclear location signal (resides: 64–88 aa) and a heat shock protein 70 family signature, which contained five kinds of functional sites, including tyrosine kinase phosphorylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, N-glycosylation, and N-myristoylation site (Fig. 1A). Three-dimensional structure of DnaK protein of *N. seriolae* was predicted by SWISS-MODEL and its was closed to that of DnaK protein of *E. coli* [27] (Fig. 1B). Protein family membership of DnaK was predicted and it belonged to heat shock protein 70 homologous superfamily. Protein BLAST showed that the deduced amino acid sequence of DnaK protein from *N. seriolae* displayed high homology among bacterial species, range from 97.88% identity with *N. niigatensis* to 60.54% identity with *E. coli* (Fig. 1C). A phylogenetic tree was constructed to identify the evolutionary relationships between *N. seriolae* with other bacterial species based on DnaK protein homologs and the results showed the DnaK protein of *N. seriolae* was closely related to DnaK protein of *N. niigatensis* (Fig. 1D).

Sequence analysis revealed that the open reading frame (ORF) of *GroEL* gene was 1656 bp and encoded a polypeptide of 551 aa with an estimated molecular mass of 57.43 kDa and a predicted pI of 4.72. The structure of GroEL protein was preferably consistent with a chaperonin Cpn60/TCP-1 domain (resides: 33–533 aa), which contained three kinds of functional sites, including N-myristoylation site, protein kinase C phosphorylation site, and casein kinase II phosphorylation site (Fig. 2A). Three-dimensional structure of GroEL protein of *N. seriolae* was predicted by SWISS-MODEL and it was closed to that of GroEL protein of *Thermus thermophilus* [28] (Fig. 2B). Protein family membership of GroEL was predicted and it belonged to heat shock protein 60 homologous superfamily. Protein BLAST showed that the deduced amino acid sequence of GroEL protein from *N. seriolae* displayed high homology among bacterial species, range from 99.07% identity with *N. niigatensis* to 60.72% identity with *E. coli* (Fig. 2C). A phylogenetic tree was constructed to identify the evolutionary relationships between *N. seriolae* with other bacterial species based on GroEL protein homologs and the results showed the GroEL protein of *N. seriolae* was closely related to GroEL protein of *N. niigatensis* (Fig. 2D).

3.2. Identification of DnaK and GroEL as secreted proteins

Preparation and shotgun MS of the extracellular products of *N. seriolae* strain ZJ0503 showed that the characteristic peptides of DnaK and GroEL proteins were detected with confidence greater than or equal to 99% (Table 3), which confirmed that DnaK and GroEL were secreted proteins of *N. seriolae*. Notably, the identified coverage rates of DnaK (63.07%) and GroEL (65.88%) proteins were quite high and ranked top two of all identified secreted proteins of *N. seriolae*.

3.3. Transcription analysis and expression of DNA vaccines

Transcription analysis of *DnaK* and *GroEL* genes in the injected muscle, head kidney, spleen and liver of the vaccinated fish were performed by RT-PCR at 7 and 35 d.p.v., respectively. In these tissues, the transcription of *DnaK* and *GroEL* genes were all detected for the first time at 7 d.p.v. and continuously detected within 35 d.p.v. in pcDNA-DnaK and pcDNA-GroEL groups respectively, but no detection in PBS and pcDNA groups (Fig. 3).

3.4. Analysis of serum non-specific parameters

The LYZ value obtained in serum from pcDNA-DnaK group at 14, 35 d.p.v. and pcDNA-GroEL group at 5–21 d.p.v. were higher than the corresponding control groups with significantly different ($p < 0.05$) (Fig. 4A). Two time points at which there was a statistical difference between groups were at 7, 14 d.p.v. when the serum alternative POD activity was both extreme significantly higher ($p < 0.01$) in pcDNA-DnaK and pcDNA-GroEL groups. Moreover, the serum POD activity of

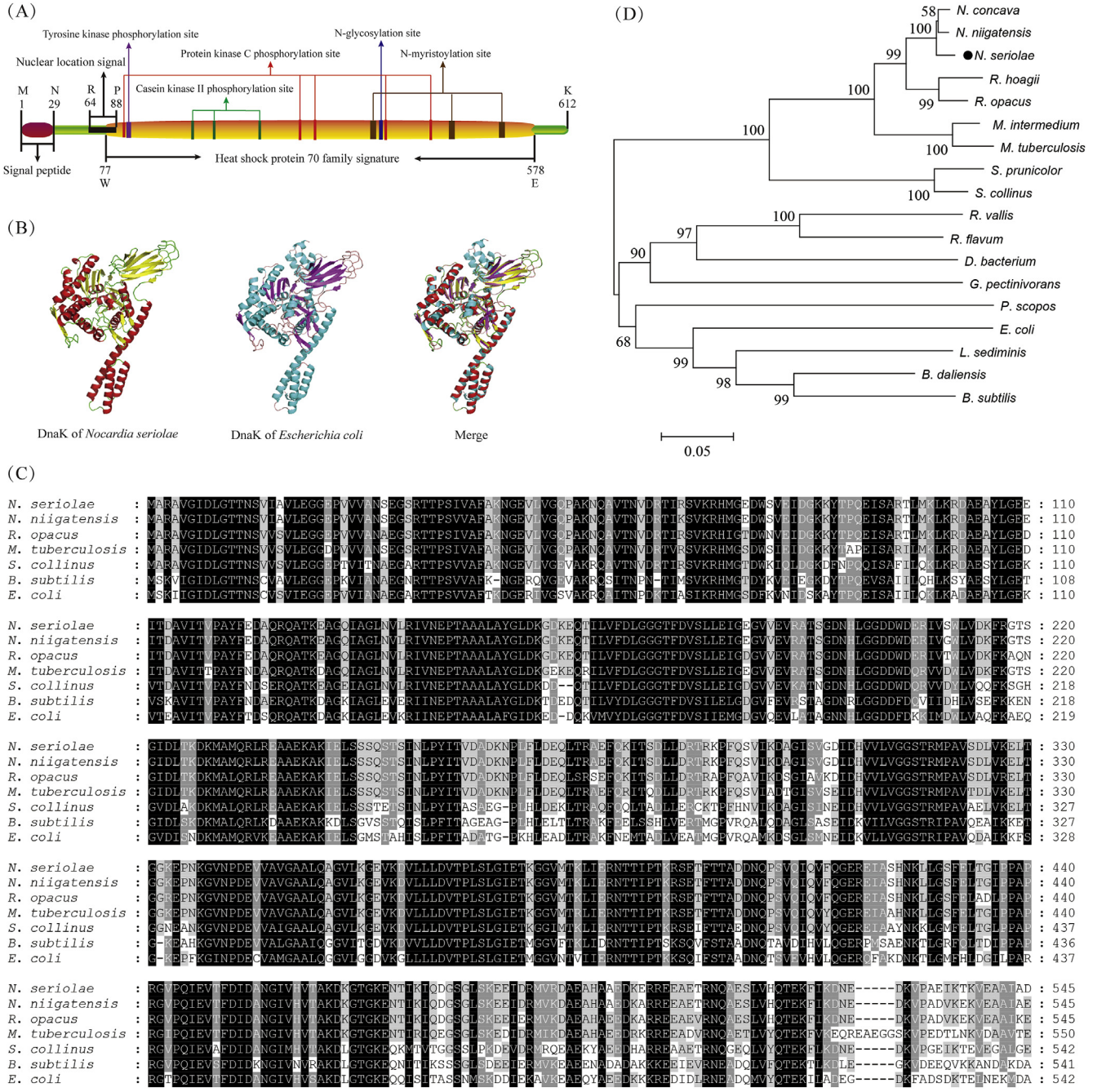

Fig. 1. Sequence and structure analysis of DnaK from *N. seriolae*. (A) The predicted schematic representation of the domain topology of protein DnaK. The protein DnaK was comprised of a signal peptide at N-terminus (residues 1–29) and heat shock protein 70 family signature (residues 77–578). (B) The predicted three-dimensional structure of protein DnaK from *N. seriolae* (Left), *E. coli* (Middle) and the overlapped three-dimensional structure of protein DnaK from them (Right). (C) Multiple alignment of the deduced amino acid sequences of protein DnaK among different bacterial species. The shadowed regions indicate residues sharing homology: black regions indicate 100% homology, and gray regions indicate homology above 80%. (D) Construction of phylogenetic tree among *N. seriolae* and other bacterial species with protein DnaK homologous sequences. Protein sequences were aligned with Clustal W, and the nonrooted neighbor-joining tree was generated by MEGA 6.0 program. Numbers at branch points indicate bootstrap support. GenBank accession numbers are shown as followed: *Nocardia seriolae* (WP_033089209.1), *Nocardia concava* (WP_040814026.1), *Nocardia niigatensis* (WP_040862332.1), *Rhodococcus opacus* (WP_064080339.1), *Rhodococcus hoagii* (WP_081121128.1), *Mycobacterium tuberculosis* (WP_031698445.1), *Mycobacterium intermedium* (WP_069419922.1), *Streptomyces collinus* (WP_020941253.1), *Streptomyces prunicolor* (WP_019062991.1), *Bacillus subtilis* (WP_120363467.1), *Bacillus daliensis* (WP_090840861.1), *Escherichia coli* (PWL89645.1), *Granulicella pectinivorans* (WP_089843510.1), *Deltaproteobacteria bacterium* (RLB80240.1), *Prevotella scopos* (WP_065367718.1), *Rhizobium vallis* (WP_126922350.1), *Laceyella sediminis* (WP_106341464.1) and *Rhizobium flavum* (WP_077548522.1).

pcDNA-DnaK group was also observed significantly different ($p < 0.05$) at 21, 35 d.p.v., compared with the corresponding control groups (Fig. 4B). The serum ACP activity in pcDNA-DnaK group at 3–35 d.p.v. and pcDNA-GroEL group at 3–28 d.p.v. were higher than the corresponding control groups with significantly ($p < 0.05$) or extreme significantly difference ($p < 0.01$) (Fig. 4C). The AKP activities measured in serum from pcDNA-DnaK group at 3–28 d.p.v. and pcDNA-GroEL group at 7–28 d.p.v. were up-regulated with significantly ($p < 0.05$) or extreme significantly difference ($p < 0.01$) compared with the corresponding control groups (Fig. 4D). The serum SOD

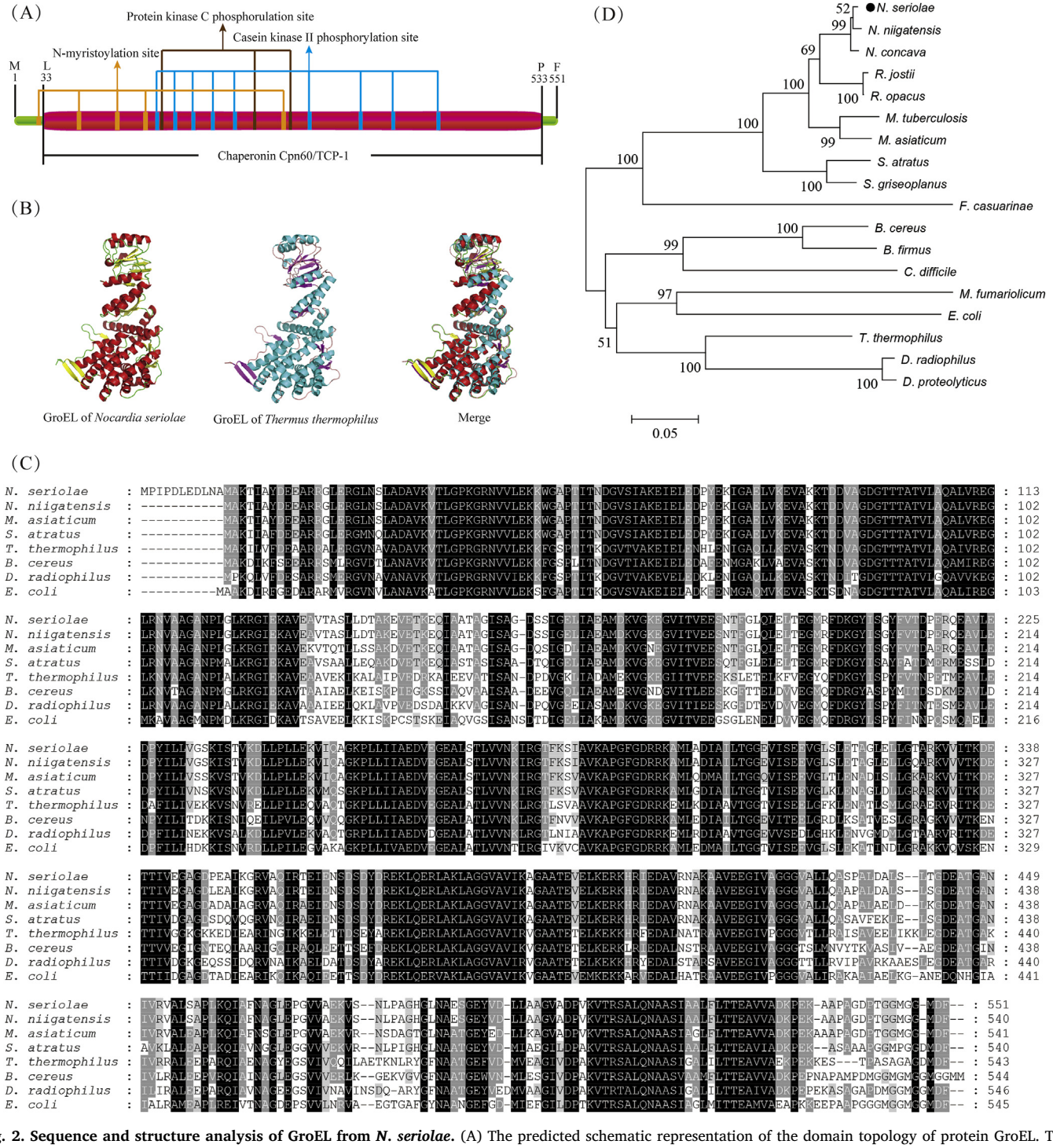

Fig. 2. Sequence and structure analysis of GroEL from *N. seriolae*. (A) The predicted schematic representation of the domain topology of protein GroEL. The protein GroEL was comprised of a chaperonin Cpn60/TCP-1 signature (residues 33–533). (B) The predicted three-dimensional structure of protein GroEL from *N. seriolae* (Left), *T. thermophilus* (Middle) and the overlapped three-dimensional structure of protein GroEL from them (Right). (C) Multiple alignment of the deduced amino acid sequences of protein GroEL among different bacterial species. The shadowed regions indicate residues sharing homology: black regions indicate 100% homology, and gray regions indicate homology above 80%. (D) Construction of phylogenetic tree among *N. seriolae* and other bacterial species with protein GroEL homologous sequences. Protein sequences were aligned with Clustal W, and the nonrooted neighbor-joining tree was generated by MEGA 6.0 program. Number at branch points indicate bootstrap support. GenBank accession numbers are shown as followed: *Nocardia seriolae* (GAM50754.1), *Nocardia concave* (WP_040813948.1), *Nocardia niigatensis* (WP_040862266.1), *Rhodococcus jostii* (SED13772.1), *Rhodococcus opacus* (ANS30321.1), *Mycobacterium tuberculosis* (WP_116395620.1), *Mycobacterium asiaticum* (WP_036352057.1), *Streptomyces atratus* (WP_037692815.1), *Streptomyces griseoplanus* (WP_055586559.1), *Bacillus cereus* (WP_098613191.1), *Bacillus firmus* (WP_061794077.1), *Deinococcus radiophilus* (WP_126351251.1), *Deinococcus proteolyticus* (WP_013615453.1), *Clostridioides difficile* (WP_003420909.1) and *Methylacidiphilum fumariolicum* (WP_009061558.1), *Frankia casuarinae* (WP_063579210.1), *Thermus thermophilus* (WP_096411521.1) and *Escherichia coli* (YP_002894488.1).

Table 3
Characteristic peptides of DnaK and GroEL proteins identified in the *N. seriolae* extracellular products by shotgun MS.

Parameters	Protein name	
	DnaK	GroEL
Characteristic peptides	AVGIDLGTTNSVIAVLEGGEPVVVANSEGSR	GLNSLADAVK
	NGEVLVGQPAK	KWGAPTITNDGVSIAKEIELEDPYEK
	HMGEDWSVEIDGKK	KTDDVAGDGTTTATVLAQALVR
	DAEAYLGEEITDAVITVPAYFEDAQR	AVEAVTASLLDTAK
	EAGQIAGLNVLRIVNEPTAAALAYGLDKGDK	EQIAATAGISAGDSSIGELIAEAMDK
	ATSGDNHLGGDDWDERIVSWLVDK	GYISGYFVTDPERQEAVLEDPYILLVGSK
	IELSSSQSTSINLPYITVDADKNPLFLDEQLTR	DLLPLLEK
	KPFQSVIKDAGISVGDIDHVVLVGGSTRMPAVSDLVK	KVVITKDETTIVEGAGDPEAIK
	GVNPDEVVAVGAALQAGVLK	AGAATEVELK
	DVLLLDVTPLSLGIETK	AFNAGLEPGVVAEK
	RSETFTTADDNQPSVQIQVFQGER	FLTTEAVVADKPEKAAPAGDPTGGMGGMDF
	LLGSFELTGIPPAPR	
	IQDGSGLSKEEIDR	
	NQAESLVHQTEKFIKDNEDKVPAEIK	
	IADANEALEGTDIAAIK	
Identified number	105	135
Coverage rates	63.07%	65.88%

activities in pcDNA-DnaK group at 1, 3, 5, 7, 21 d.p.v. and pcDNA-GroEL group at 3–7 d.p.v. were higher than the corresponding control groups with significantly ($p < 0.05$) or extreme significantly difference ($p < 0.01$) (Fig. 4E). The increased serum non-specific parameters of LYZ, POD, ACP, AKP and SOD activities indicated that the non-specific immune response were both induced by pcDNA-DnaK and pcDNA-GroEL DNA vaccines in hybrid snakehead.

3.5. Specific antibody (IgM) response of immunized hybrid snakehead

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 approach. The results indicated that every week IgM titers against *N. seriolae* were detected in the serums of pcDNA-DnaK and pcDNA-GroEL vaccinated hybrid snakehead, respectively. From the first week to the fifth week post-vaccination, the values of $\log_2$(antibody titer) in the serums of pcDNA-DnaK vaccinated group stayed above 5.00 across all time points and a peak level was reached to 10.00 at the fourth week post-vaccination, lasting to fifth week post-vaccination with no decline. Meanwhile, the values of $\log_2$(antibody titer) in the serums of pcDNA-GroEL vaccinated group stayed above 6.00 across all time point and a peak level was reached to 13.00 at the fourth week post-vaccination. In the controls, those of the PBS and pcDNA vaccinated groups only kept 1.00–2.00 across all time points. Compared with PBS and pcDNA groups, IgM level in pcDNA-DnaK and pcDNA-GroEL groups were both higher with extreme significantly different ($p < 0.01$) from first week to the fifth week post-vaccination. Furthermore, the peak level of specific antibody (IgM) induced by pcDNA-GroEL DNA vaccine was higher than that of pcDNA-DnaK DNA vaccine (Fig. 5).

3.6. Analysis of immune responses induced by DNA vaccines

Spleen from vaccinated hybrid snakehead were sampled at different time points after vaccination and immune responses induced by the pcDNA-DnaK and pcDNA-GroEL DNA vaccine were investigated by qRT-PCR, respectively. The results showed the expression levels of all investigated immune-related genes in pcDNA-DnaK and pcDNA-GroEL group were increased remarkably compared with that in PBS and pcDNA groups, respectively. With regard to cellular immunity, the mRNA level of *MHCIα*, *MHCIIα*, *CD4* and *CD8α* were increased notably both in pcDNA-DnaK and pcDNA-GroEL vaccinated group compared with that in PBS and pcDNA groups within 35 d.p.v. However, there was no significant difference between pcDNA-DnaK and pcDNA-GroEL groups after immunization. Similarly, *IL-1β* and *TNFα* genes were up-regulated significantly in pcDNA-DnaK and pcDNA-GroEL vaccinated group compared with that in PBS and pcDNA groups within 35 d.p.v. and no significant difference were found between pcDNA-DnaK and

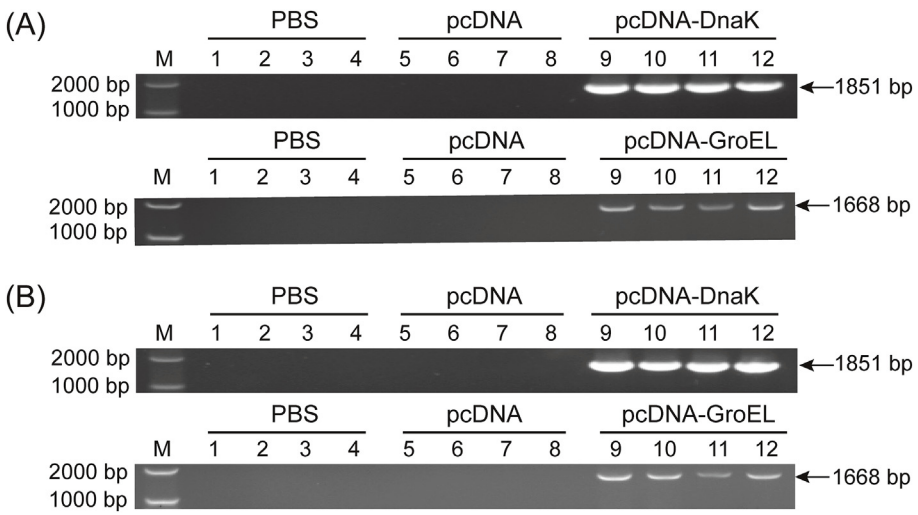

Fig. 3. RT-PCR analysis of transcription of *DnaK* and *GroEL* genes in different tissues of the vaccinated fish at 7 and 35 d post-vaccination. (A) Transcription of *DnaK* (Up) and *GroEL* (Down) genes at 7 d post-vaccination. (B) Transcription of *DnaK* (Up) and *GroEL* (Down) genes at 35 d post-vaccination. 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.

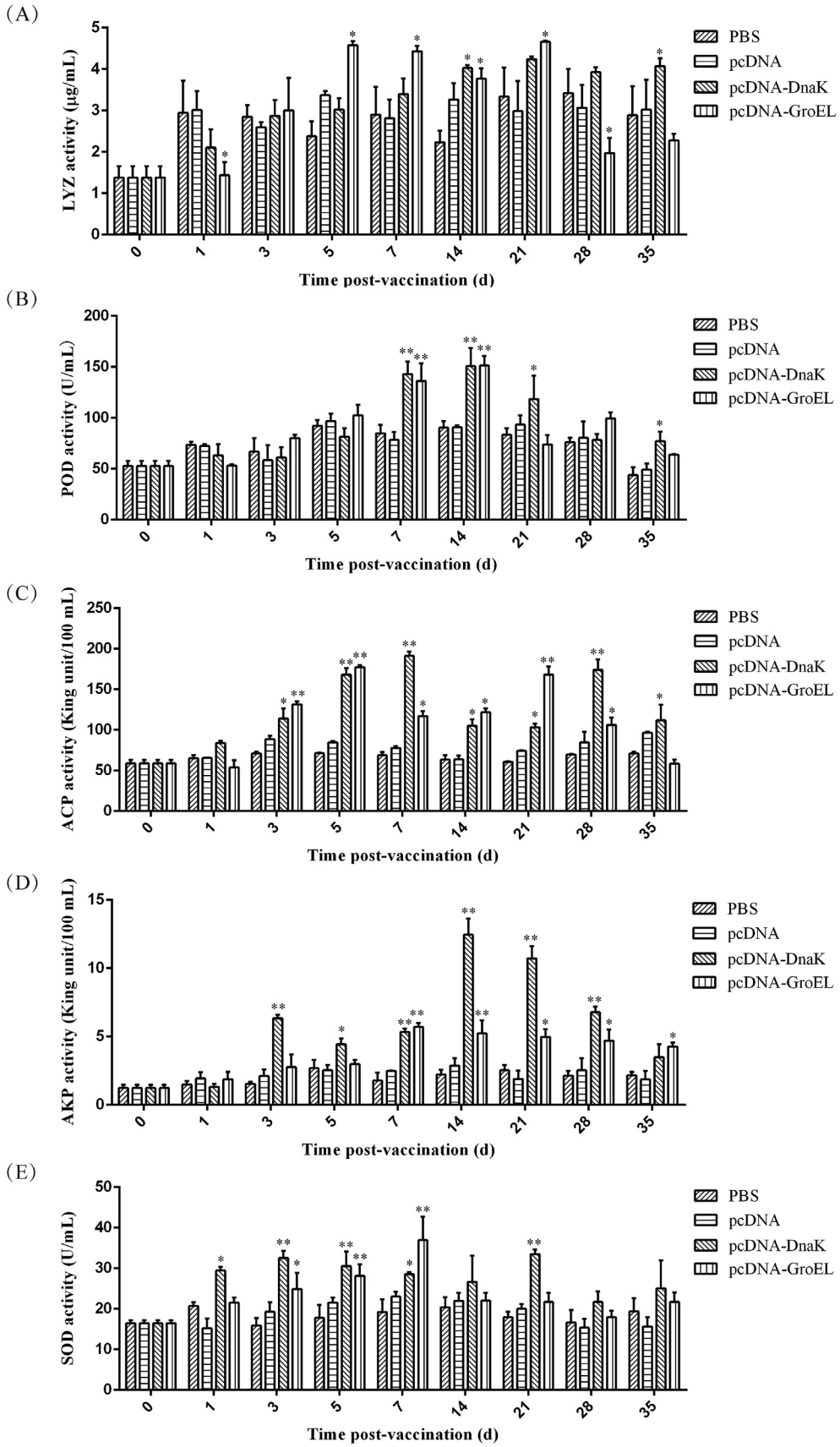

Fig. 4. The serum non-specific parameters in vaccinated hybrid snakehead. (A) Serum LYZ activity. (B) Serum POD activity. (C) Serum ACP activity. (D) Serum APK activity. (E) Serum SOD activity. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. The different letters above the bars indicate the significant difference among different groups at the same time point ($*p < 0.05$, $**p < 0.01$).

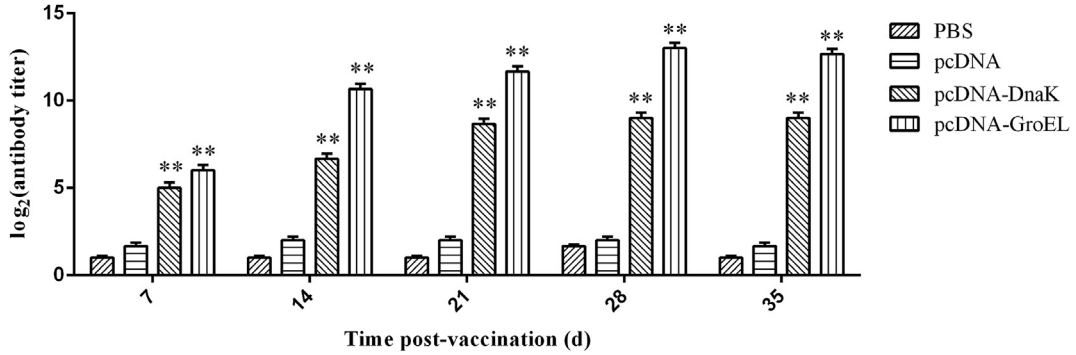

Fig. 5. Analysis of serum specific antibody (IgM) levels against *N. seriolae* in hybrid snakehead by ELISA. Serums were collected at 7, 14, 21, 28 and 35 d post-vaccination from the hybrid snakehead vaccinated with PBS, pcDNA, pcDNA-DnaK and pcDNA-GroEL. Data are presented as means ± SE (n = 5). Each bar represents mean $\log_2$ value ± SE of the highest dilution of the serum when P/N ≥ 2.1 (P/N = OD_{450} of the inspected serum/OD_{450} of the negative serum). The different symbols above the bars indicate the significant difference among different groups at the same time point (*$p < 0.05$, **$p < 0.01$).

pcDNA-GroEL groups, which suggest that inflammatory response were triggered after immunization by two DNA vaccines (Fig. 6).

3.7. 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. 7, the survival rates of hybrid snakehead in pcDNA-DnaK and pcDNA-GroEL vaccinated groups were 74.00% and 89.33%, while the cumulative survival rates of hybrid snakehead vaccinated pcDNA and PBS groups were 45.33% and 44.67% at 14 d post-challenge, respectively. Moreover, the RPS of hybrid snakehead vaccinated with pcDNA, pcDNA-DnaK and pcDNA-GroEL were 1.19%, 53.01% and 80.71%, respectively. The results indicated that these two DNA vaccines increased the survival rates of hybrid snakehead after challenge with *N. seriolae* strain ZJ0503. Furthermore, the pcDNA-DnaK and especially pcDNA-GroEL DNA vaccine had effective immune protection effect against infection of *N. seriolae*. The hybrid snakehead in PBS and pcDNA groups began to die at 4 d post-challenge, with a sudden 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

In this study, the immune responses induced by pcDNA-DnaK and pcDNA-GroEL DNA vaccines were investigated and the protective efficacy between these two DNA vaccines were compared in hybrid snakehead against *N. seriolae* infection. Fish nocardiosis is a systemic bacterial disease and three pathogenic bacteria had been isolated from diseased fish so far, including *N. samonicida*, *N. seriolae*, and *N. asteroids* [6]. In order to control fish nocardiosis, the identification of virulence factors of these three pathogenic *Nocardia* species can provided effective candidate for vaccine development. The secretome studies of pathogenic actinomycetes have shown that secreted proteins are closely related to their pathogenicity and involved in bacterial pathogen-host interaction which affecting the physiology and innate immune response of the target cells [29,30]. Experimentally, the MS analysis of the extracellular products from *N. seriolae* strain ZJ0503 proved that both Dnak and GroEL were secreted proteins. Combining our previous study, both DnaK and GroEL proteins were identified to be the common immunogenic proteins of *N. seriolae*, *N. samonicida*, and *N. asteroids* by immunoproteomic analysis, which might indicate that the secreted proteins of DnaK and GroEL were potential virulence factors, acting effective antigens for vaccine development against fish nocardiosis. Furthermore, numerous studies have shown that DnaK and GroEL of microbial origin play an important role in pathogenesis and host immune response. For example, two heat shock proteins of *F. tularensis* (DnaK and GroEL) were found as antigens to induced long-lasting recall response in $CD4^+$ and $CD8^+$ alphabeta T cells [31]. DnaK of *M. tuberculosis* induced protective mucosal immunity against tuberculosis in CD4-depleted mice when administered by DNA vaccine [32]. Besides, previous study also showed that GroEL functioned as an antigen which located in the soluble and insoluble fractions (including inner and outer membranes) of cells and was actively secreted in *Bartonella bacilliformis* [33]. Definitely, these studies revealed that induction of microbial genes encoding DnaK and GroEL might provide a novel strategy to boost the immune response of individuals, which make them as candidates of choice for vaccine development against pathogenic microorganisms.

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 [34]. In our study, the intramuscular injection was chosen as immunization route of pcDNA-DnaK and pcDNA-GroEL DNA vaccines, which could induce the persistent expression of exogenous antigen gene in the host cells [35]. When these two DNA vaccines were injected into hybrid snakehead, the transcription of *DnaK* and *GroEL* genes in muscle, head kidney, spleen and liver were all detected from 7 to 35 d.p.v., respectively. It is suggested that the transfer of these two DNA vaccines 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 [26,36].

Additional studies have also shown that both DnaK and GroEL were recognized as immunodominant antigens in several pathogenic bacteria and induced strong humoral and cellular immune response in host by DNA vaccines [10,32]. 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 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 [37–39]. Apart from the non-specific immune response in humoral immune system, the specific immune response of antibodies generation is also critical to the prevention of bacterial infection. Besides, it is known that IgM is a

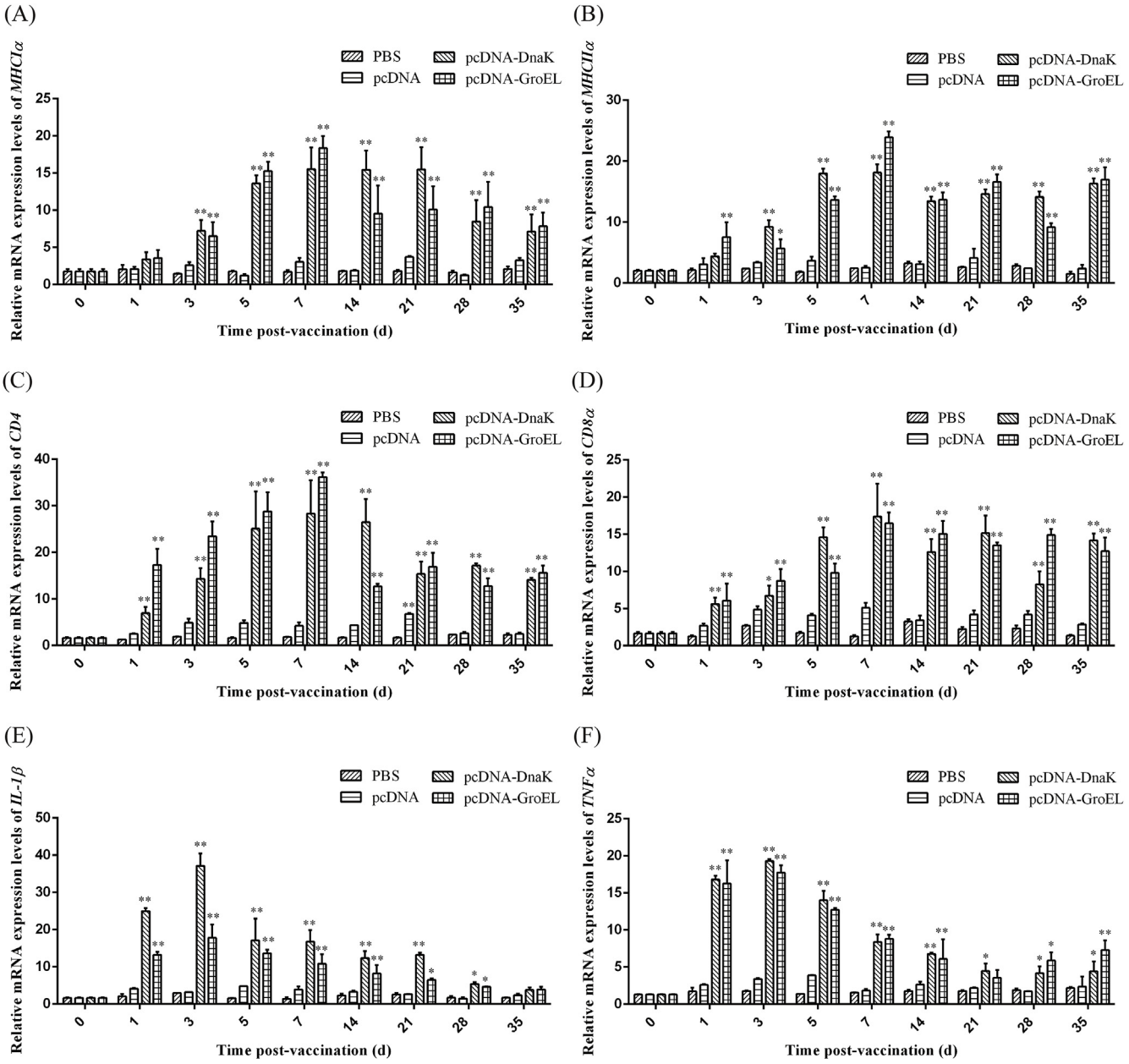

Fig. 6. qRT-PCR analysis of the expression of immune-related genes. The data were expressed as the expression of *MHCIα* (A), *MHCIIα* (B), *CD4* (C), *CD8α* (D), *IL-1β* (E) and *TNFα* (F) genes. The mRNA expression level of each immune-related gene was normalized to that of *β-actin* and relative expression 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 symbols above the bars indicate the significant difference among different groups at the same time point (*$p < 0.05$, **$p < 0.01$).

major component of the humoral immune system of teleost fish and regarded as the first antibody [38]. Therefore, it was necessary to assess the serum non-specific parameters and specific antibody (IgM) production to evaluate the immune effects of pcDNA-DnaK and pcDNA-GroEL DNA vaccines. In our study, the significant increase of serum non-specific parameters such as LYZ, POD, ACP, AKP and SOD activities were both observed in vaccinated hybrid snakehead by these two DNA vaccines, while no significant changes were observed in corresponding control groups. In addition, both the IgM expression of pcDNA-DnaK and pcDNA-GroEL vaccinated groups increased significantly from the first week, persisting up to the third week of post-vaccination and maintaining high expression level within 35 d after immunization. The results about non-specific parameters and specific antibody (IgM) titters indicate that the humoral immune responses are both induced to enhance the immunological competence in hybrid snakehead vaccinated with pcDNA-DnaK and pcDNA-GroEL DNA vaccines.

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 through bloodstream [40]. 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 [40,41]. And a specific antibacterial response, especially the $CD8^+$ cytotoxic T lymphocyte (CTL) and $CD4^+$ Th cell responses will be induced following the up-regulation of CD4 and CD8α [42]. As the important

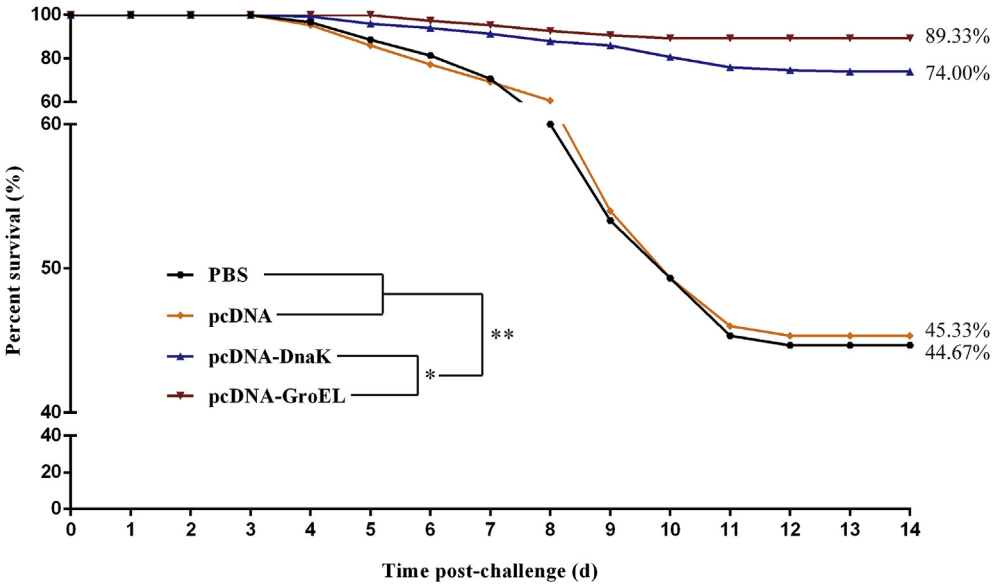

Fig. 7. Survival rate of vaccinated hybrid snakehead immunized with pcDNA-DnaK, pcDNA-GroEL, pcDNA or PBS following the challenging tests of 14 d by N. *seriolae* strain ZJ0503. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. The different letters above the bars indicate the significant difference among different groups at the same time point (**$p < 0.01$).

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 [39]. In this study, the significantly increase on the mRNA levels of *MHCIα*, *MHCIIα*, *CD4* and *CD8α* indicated that the recognition and presentation of pcDNA-DnaK and pcDNA-GroEL DNA vaccines 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 these two DNA vaccines, which enhanced the inflammatory immune response of hybrid snakehead.

In this study, the survival rate of pcDNA-DnaK and pcDNA-GroEL vaccinated fish were higher than that of corresponding control groups and the pcDNA-GroEL DNA vaccine supplied immune protection against *N. seriolae* infection in hybrid snakehead with 80.71% RPS, which was much higher than that of pcDNA-DnaK vaccine with 53.0% RPS. Relevant researches showed that specific antibodies probably play a major role in reducing mortality of bacterial challenge [43]. In this study, the peaked level of $\log_2$(antibody titer) in pcDNA-GroEL vaccinated hybrid snakehead reached to 13.00, which was obviously higher than that of pcDNA-DnaK DNA vaccine (9.00) and it might cause the different RPS between these two DNA vaccines. Besides, the different RPS might also attribute to the difference in functional properties and immune-related activating pathways of DnaK and GroEL proteins which needed to be examined in the future. Interestingly, the DnaK and GroEL were also reported as effective adjuvant molecules. For example, leptospiral surface proteins fused with DnaK provided an enhanced immune response in mice comparing with non-fusion proteins [44]. Besides, the percent survival rate of IpaB-GroEL fusion protein immunized group (90–95%) were higher than that of IpaB and GroEL proteins co-immunized group (80–85%) against lethal challenge with *Shigella flexneri*, *S. boydii* and *S. sonnei* in mice [45]. Therefore, it provided some ideas for developing fusion vaccines and improving vaccine protective effects by using DnaK and GroEL as adjuvant molecules. In summary, both the pcDNA-DnaK and pcDNA-GroEL DNA vaccines could boost innate response, humoral immune response and cellular immune response, which demonstrate that DnaK and GroEL are promising vaccine candidate against fish nocardiosis, and these findings will promote the vaccine development against fish nocardiosis.

Author contributions

LX and YL designed the experiments. LX, JC, and BL performed the experiments. BL, WW, SH and WF collected clinical data. JC, BL, SH, and WW contributed analysis. JC and LX wrote the paper. YL polished the paper.

Declaration of competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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