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Pathogenicity of non-O1/ O139 *Vibrio cholerae* and its induced immune response in *Macrobrachium rosenbergii*

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ABSTRACT

Outbreaks of mass mortalities occurred in *Macrobrachium rosenbergii* farms in Gaoyou county, Jiangsu Province of China. The bacterial isolates from *M. rosenbergii* exhibited the same phenotypic traits and biochemical characteristics, and were identified as non-O1/O139 *Vibrio cholerae* according to biochemical characteristics and molecular identification. In challenge test, *M. rosenbergii* infected with non-O1/O139 *V. cholerae* GXFL1-4 developed similar pathological signs to the naturally diseased prawns, and LD_{50} of the strain to *M. rosenbergii* was 4.5×10^6 CFU/mL at 96 h post-infection. Histopathological analysis revealed that hepatopancreas and intestines of diseased *M. rosenbergii* exhibited obvious inflammatory responses to non-O1/O139 *V. cholerae* infection. Detection virulence factors of the strain GXFL1-4 showed that the bacteria produced caseinase, lipase, amylase, lecithinase and hemolysin, and carried *toxR*, *hlyA*, *ompW*, *ompU*, *hap*, *rtxA* and *rtxC* virulence related genes, supporting the strong virulence to *M. rosenbergii*. Additionally, the immune related gene expression in *M. rosenbergii* evaluated by qRT-PCR analysis showed that *HSP70*, *Crustin*, *Lysozyme*, *TRL1*, *ALF1*, *Lectin*, *Peroxinectin*, *proPO* and *SOD* immune related genes were significantly up-regulated at 6 and 12 h after infection with GXFL1-4. The results of our study suggested that non-O1/O139 *V. cholerae* was an etiological element in the mass mortalities of *M. rosenbergii* and this study provided preliminary insights into the diversity in the immune response of *M. rosenbergii* to the bacterial invasion.

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* is an economically important aquaculture species for human consumption, and has been cultured commercially in many different countries, including China, Israel, Japan, India, Latin America, Caribbean and some countries in Africa [1,2]. There has been significant increase in the global production of *M. rosenbergii* which had exceeded 230, 333 tons in 2014 [3]. However, serious economic losses of *M. rosenbergii* industry have occurred due to epidemic infectious diseases, including *M. rosenbergii* nodavirus (MrNV), the extra small virus (XSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), *Lactococcus garvieae*, *Vibrio alginolyticus*, *Vibrio vulnificus*, *Aeromonas sobria*, *Aeromonas veronii*, *Enterobacter cloacae*, and spiroplasma [4–14]. Heavy mortalities occurred in *M. rosenbergii* farms in Gaoyou county of Jiangsu Province in the July of 2018, and one type of bacterial strain GXFL1-4 was isolated from the diseased *M. rosenbergii* that was subsequently identified as non-O1/O139 *Vibrio cholerae.*

V. cholerae is an important fish and human pathogen and lives naturally in brackish and estuarine ecosystems [15,16]. *V. cholerae* serotypes O1 and O139 have been well documented to be involved in the outbreak of epidemic cholera, mild gastroenteritis and septicemia in humans [17]. Non-O1/139 *V. cholerae* can infect some aquatic animals [18], and the diseases caused by *V. cholerae* are usually associated with its production of virulence factors such as hemolysin and protease [19]. Although most *V. cholerae* causing diseases of aquatic animals may be different to human, the toxin-producing *V. cholerae* isolated from diseased aquatic animals should still be considered to have a potential for zoonosis.

As invertebrates, *M. rosenbergii* lacks an acquired immune system and primarily depend on their innate immune system to resist invading pathogens including both cellular and humoral immune systems [20]. Cellular defense system includes encapsulation, nodule formation and phagocytosis, whereas humoral immune system possesses prophenoloxidase activating mechanism, clotting mechanism and production of antimicrobial peptides (AMPs) [21]. However, a better understanding of the molecular responses and defense mechanisms of prawns against invading pathogens is needed. The expression of immune-related genes

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is considered a potential marker of prawn's health status, as it provides relevant information about the activation and modulation of the immune system [22].

In this study, the pathogenicity of non-O1/O139 *V. cholerae* GXFL1-4 from diseased *M. rosenbergii* was investigated. Furthermore, to explore immune responses of *M. rosenbergii* after non-O1/O139 *V. cholerae* infection, the expression of immune-related genes of *M. rosenbergii* in response to non-O1/O139 *V. cholerae* challenge were investigated. These results will clarify the virulent characteristics of non-O1/O139 *V. cholerae* and provide a better understanding of the molecular responses and defense mechanisms of *M. rosenbergii* against non-O1/O139 *V. cholerae*.

2. Materials and methods

2.1. Disease outbreak description and bacterial isolation

An epizootic outbreak occurred in *M. rosenbergii* farms of Gaoyou county, Jiangsu Province, China. The water temperature fluctuated between 30 and 35 °C. Typically infected and dying prawns were randomly taken from the affected farms for pathogen examination.

Moribund *M. rosenbergii* was sanitized with 75% ethyl alcohol prior to being dissected. Hepatopancreas and muscle were sampled for bacterial isolation. Then the samples were streaked onto LB nutrient agar plates, and incubated at 28 °C for 18 h, the dominant uniform isolates were purified by streaking and re-streaking onto LB nutrient agar plates. The pure isolates were grown at 28 °C in LB broth medium for 18 h and preserved in 20% glycerol at −80 °C.

2.2. Bacterial virulence assay

Bacterial virulence of the obtained strain GXFL1-4 was examined by experimentally infecting healthy *M. rosenbergii* (1.02 ± 0.12 g). The *M. rosenbergii* was divided into six groups (each group with triplicate). Each group of the *M. rosenbergii* was maintained in a 10-L aquarium (30 prawns per aquarium) for a week at 28 ± 2 °C, and the farming water was aerated with an initial pH of 8.5, 5.0 mg/L of dissolved oxygen. Prior to the bacterial virulence assay, the strain GXFL1-4 was inoculated into 50 mL of LB broth medium, incubated at 28 °C with shaking at 180 rpm for 18 h, and enumerated by a 10-fold serial dilution in sterile phosphate buffer saline (PBS, pH 7.4) and spread on LB nutrient agar plates. The five groups were infected by exposure to 3.6×10^8, 3.6×10^7, 3.6×10^6, 3.6×10^5 and 3.6×10^4 CFU/mL of the bacteria, respectively, and the control group prawns were cultured in fresh water. The experimental *M. rosenbergii* were kept at 28 ± 2 °C and monitored for 96 h without feeding and water change. Mortalities of *M. rosenbergii* were observed at 6, 12, 24, 48, 72, and 96 h, the dead *M. rosenbergii* were immediately removed and sampled to re-isolate and confirm specific mortality by the challenge isolate. Average values were taken for calculating percentage of mortality, and the median lethal dose (LD_{50}) values were calculated as described by Behreans and Karber [23].

2.3. Histopathology

Hepatopancreas and intestine from dying *M. rosenbergii* were fixed with Bouin's fixative for histological examination. The fixed tissues were dehydrated in an ethanol series, and embedded in paraffin wax. After sectioning on a rotary, the samples were stained by hematoxylin and eosin (H&E) and examined with a light microscope.

2.4. Identification of bacteria

Transmission electron microscopy (TEM) was used to determine the morphological characteristic of the strain GXFL1-4 as previously described with minor modification [24]. The strain GXFL1-4 was fixed with 2.5% glutaraldehyde, post-fixed in osmium tetroside, dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranylacetate and lead citrate. The samples were then examined in transmission electron microscope. Besides, physiological and biochemical examinations were done on the bacterial culture using standard plate and tube tests (Hangzhou Tianhe Microorganism Reagent Co., Ltd., China) as described in Bergey's Manual of Systematic Bacteriology [25], including the following tests: arabitol, mannose, maltose, H_2S production, tartrate utilization, VP, xylose, mannitol, sucrose, galactosidase, aesculin, dulcitol, lactose, inositol, mushroom sugar, nitrate reduction, acetate, MR, galactose, sorbitol, O-F, erythrite, L-rhamnose.

Total genomic DNA of the bacteria for the PCR was extracted using the EasyPure Genomic DNA Kit (TransGen Biotech, Beijing, China), then the 16S rRNA and *gyrB* sequences of the strain GXFL1-4 was amplified by PCR according to Zhang et al. [26]. The universal PCR primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGG MTACCTTGTTACGACTT-3′) were used for amplification of the 16S rRNA gene, while universal PCR primers UP1 (5′-GAAGTCATCATGA CCG TTCTGCAYGCNGGNGGNAARTTYGA-3′) and UP2r (5′-AGCAGG-GTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3′) were used for amplification of the *gyrB* gene. Amplifications were performed in 25 μL reaction mixture containing12.5 μL of 2 × Easy Taq PCR Super® Mix (TransGen Biotech, Beijing, China), 0.5 μL of each of the primers (10 μM), 1 μL of DNA template and 10.5 μL of ddH_2O. The reaction mixture was pre-heated at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1min. A final extension step of 72 °C for 7 min was also used. Then a search was performed in the National Centre for Biotechnology Information (NCBI) database for sequence identity using the Basic Local Alignment Search Tool (BLAST) program. In addition, the strain GXFL1-4 was further identified as non-O1/O139 *V. cholerae* using a PCR based assay by targeting the *rfb-O1* and *rfb-O139* genes as described by Hoshino et al. [27].

2.5. Determination of extracellular enzymes and hemolysin

The strain GXFL1-4 was grown at 28 °C in LB broth medium for 18 h, and then 10 μL of the bacterial cultures were spot-inoculated onto LB nutrient agar that contained 1% gelatin (gelatinase test), 1% Tween-80 (lipase test), 2.5% skim milk (caseinase test), 2% starch (amylase test), or 10% egg yolk (lecithinase test) as substrate, respectively. Besides, the strain GXFL1-4 was also tested for β-hemolytic activity on nutrient agar supplemented with 7% rabbit erythrocytes. These plates were incubated for 24 h–48 h at 28 °C, the presence of the lytic halo around the colonies was observed, and then the diameter of the lytic halo around each well was measured.

2.6. Determination of virulence-related genes by PCR

The strain GXFL1-4 was screened for virulence determinants cholera toxin (*ctxA*, *ctxB*), toxin co-regulated pilus (*tcpA*), transcriptional regulatory proteins (*toxR*), hemolysin (*hlyA*), outer membrane protein (*ompW*, *ompU*), hemagglutinin protease (*hap*), repeat in toxin (*rtxA*, *rtxC*), accessory cholera toxin (*ace*), zonula occludens toxin (*zot*) to determine its pathogenic potential. Briefly, total genomic DNA of the isolate for the PCR was extracted using the EasyPure Genomic DNA Kit (TransGen Biotech, Beijing, China), and sterile PCR grade water was used as negative control. The specific primers used were given in Table 1. The following components were added into the mix to obtain 25 μL of PCR mixture: 12.5 μL of 2 × Easy Taq PCR Super® Mix (TransGen Biotech, Beijing, China), 0.5 μL of 10 mM forward primer, 0.5 μL of 10 mM reverse primer, 1 μL of DNA template (43.26 ng/μL) and 10.5 μL of ddH_2O. The amplification profile was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for

Table 1
Sequence of primers used for detection of virulence genes.

Target gene	Primer sequence (5′–3′)	Amplicon (bp)	Annealing (°C)	Reference
ctxA	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	302	55	[28]
ctxB	GGTTGCTTCTCATCATCGAACCAC GATACACATAATAGAATTAAGGATG	460	55	[29]
tcpA (Classical)	CACGATAAGAAAACCGGTCAAGAG GATCTGCAAGTGCTACTGCGC	466	55	[30]
tcpA (Eltor)	CACGATAAGAAAACCGGTCAAGAG GATCAGCGACAGCAGCGAAA	466	55	[30]
toxR	TTACTACTCACACACTTTGATGGCATCGTT TTAATGTTCGGATTAGGACACAACTCAAAAG	901	55	[31]
hlyA	CAATCGTTGCGCAATCGCG TTGACCTTCAGCATCACT	265	55	[30]
ompW	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	586	55	[28]
ompU	CCAAAGCGGTGACAAAGC TTCCATGCGGTAAGAAGC	655	55	[32]
hap	GTGAACAACACGCTGGAGAA CGTTGATATCCACCAAAGG	700	55	[33]
rtxA	GATTCTTCCGTTCAAGCTCCG TGGTTCAGGCTGTTGCACAC	2571	55	[34]
rtxC	CGACGAAGATCATTGACGAC CATCGTCGTTATGTGGTTGC	265	55	[35]
zot	CACTGTTGGTGATGAGCGTTATCG TTTCACTTCTACCCACAGCGCTTG	243	55	[36]
ace	GCTTATGATGGACACCCTTTA GTTTAACGCTCGCAGGGCAAA	284	55	[36]

1 min. A final extension step of 72 °C for 7 min was also used.

2.7. Bacterial challenge and tissue collection

To evaluate the immune responses after non-O1/O139 *V. cholerae* infection, 200 healthy *M. rosenbergii* (1.98 ± 0.29 g) were divided equally into two groups. One group were injected intramuscularly with 100 μL of the bacteria (concentration of 10^6 CFU/mL) per prawn and the other group were injected with PBS as the control. The hepatopancreas and hemolymph of 9 individuals from each group were sampled at 6, 12, 24, 48, 72 h post infection. Three prawns were pooled together as a sample. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.8. RNA isolation and gene expression analysis

Total RNA from hepatopancreas and hemolymph (n = 3) of each samples were isolated using the EasyPure RNA kit (TransGen Biotech, Beijing, China) and reverse-transcribed by TransScript One-Step gDNA Removal and cDNA Synthesis Supermix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Gene expressions including *HSP70* (heat shock protein 70), *Crustin* (crustin), *Lysozyme* (lysozyme), *TRL1* (toll like receptor), *ALF1*(anti-lipopolysaccharide factor), *Lectin* (C-type lectin), *Peroxinectin* (peroxinectin), *proPO* (prophenoloxidase), *SOD* (cytosolic manganese superoxide dismutase), were detected by quantitive real-time PCR (qRT-PCR) using the primers listed in Table 2. The RT-qPCR was performed with TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) in a ABI Prism 7500 Real-Time PCR System. The total reaction volume of 20 μL contained 10 μL 2 × TransStart Top Green qPCR SuperMix, 1 μL of cDNA, 0.4 μL of 10 mM forward primer, 0.4 μL of 10 mM reverse primer, 0.4 μL of ROX reference dye II and 7.2 μL of nuclease-free water. The amplification profile was as follows: denaturation at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 34 s. Each PCR assay was performed in triplicate for analysis. 18S rRNA gene was chosen as reference gene and the samples from control group were used as calibrator. Relative mRNA expression was determined using $2^{-\Delta\Delta CT}$ method, and significant differences were analyzed by one-way variance (ANOVA) analysis with a Tukey's HSD test using the SPSS 16.0 software ($P < 0.05$).

Table 2
Primers used for qRT-PCR.

Gene name	Primer sequence (5′–3′)	Genebank accession
18S rRNA	CTGTTACGGGTGACGGAGAA TCGGAAGAGTCCCGCATT	GQ131934
HSP70	ACAAGAAGGTTGGTGGAG GCAGAGGCTGAAAGAGTA	HG001455
Crustin	AAATCAATGTGCTATGCTCCAA GCAGAGGCTGAAAGAGTA	KX219626
Lysozyme	ACAGACTACGGTATCTTCCAG TTGTAGCGTTCGGTGTCG	AY257549
TRL1	ATACAGCAGCCATTCGTC TTCTTCTTCAGCCACAGC	KJ188410
ALF1	TCGTGACGCCAAGGTTAG GCAGACGCAGAAGGAAGG	FJ429306
Lectin	CAGAGTATTTCGTATCCACC ATGAGTCCCTCCTGTCCT	KX495216.
Peroxinectin	CACTGCTGCCTTCCGTTTC AGGGCTTGTGGATTATTCTG	AY606270
proPO	GCAACATTGGCGAACTGA GGGAAGGTCTCGACGACT	DQ182596
SOD	TGTCAGAAAGACCACGAA GATGCTGGCAACATAAGC	DQ073104

3. Results

3.1. Pathological symptoms

The diseased *M. rosenbergii* appeared clinical signs of weakness, poor appetite, empty stomach, red body and swimming alone. No virus including MrNV, XSV, IHHNV, white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome virus (TSV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) and Laem-Singh virus (LSNV) were found in the diseased *M. rosenbergii* by PCR assay using the specific primers [37,38].

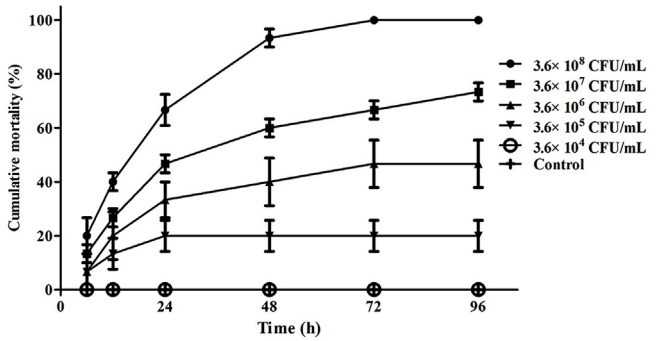

Fig. 1. Cumulative mortality of *M. rosenbergii* challenged with 3.6×10^8, 3.6×10^7, 3.6×10^6 and 3.6×10^5 CFU/mL of the strain GXFL1-4. Data are presented as mean ± SD (n = 3).

3.2. Pathogenicity of the isolate

The results of challenge experiment on *M. rosenbergii* were given in Fig. 1. All *M. rosenbergii* died at 96 h post-infection with 3.6×10^8 CFU/mL of GXFL1-4. The 3.6×10^7, 3.6×10^6 and 3.6×10^5 CFU/mL of GXFL1-4 could cause 73.3%, 46.7%, 20.0% mortality of *M. rosenbergii* within 96 h, respectively, while no *M. rosenbergii* died in the group of 3.6×10^4 CFU/mL and control group. The calculated LD50 of 96 h post-infection to *M. rosenbergii* was 4.5×10^6 CFU/mL. In addition, the re-isolated bacteria from the experimentally infected *M. rosenbergii* was confirmed phenotypically and molecularly as the strain GXFL1-4.

3.3. Histopathology

The histopathology of the control group (no infection) showed normal tissue characteristics in hepatopancreas and intestine of *M. rosenbergii* (Fig. 2A and C). However, the hepatopancreas of *M. rosenbergii* infected with the strain GXFL1-4 revealed rupture of the basal laminae of the hepatopancreatic tubules and severe necrosis, dilatation of

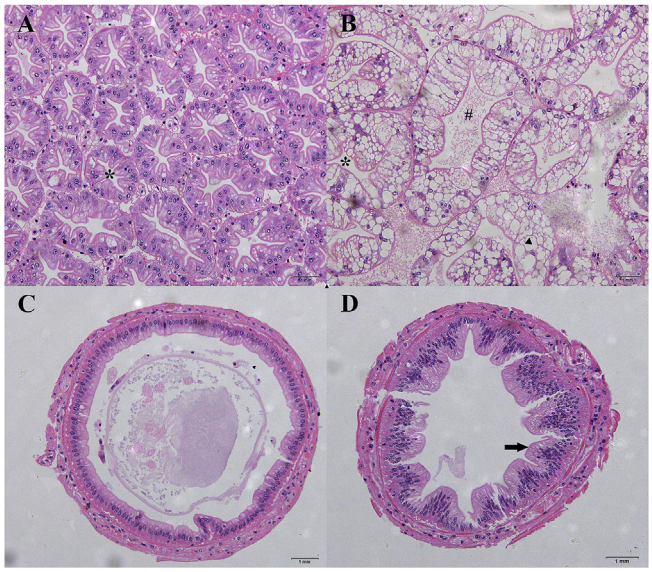

Fig. 2. Histological examination of *M. rosenbergii* hepatopancreas and intestine (magnification, 200×). (A) Hepatopancreas tissues from the control group; (B) Hepatopancreas tissues from the infected group showing severe necrosis, rounding and sloughing of cells; (C) Intestine from the control group; (D) Intestine tissues from the infected group showing disorganized of intestinal epithelial cells; The asterisk indicates hepatic tubules; The hash symbol indicates the connective tissue between the hepatic tubules; The triangle indicates vacuolation; The arrows indicates disorganized villi.

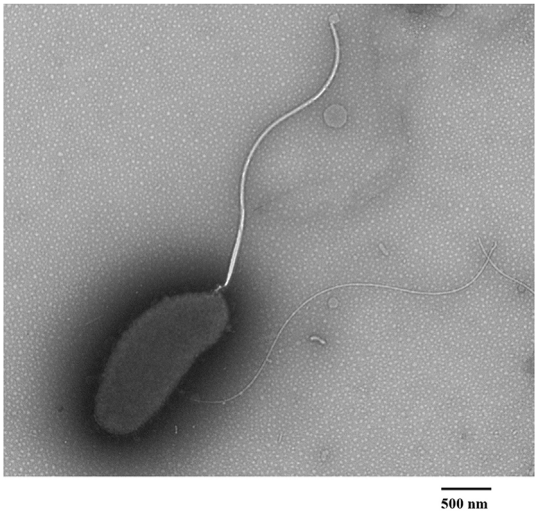

Fig. 3. Electron micrograph of GXFL1-4 showing polar single flagella (bar = 500 nm).

hepatopancreatic tubules gap, loss of the star shape of the lumen, loss of structure, atrophy, vacuolation and rounding into the lumen of tubular epithelial cells (Fig. 2B). In addition, the intestine of *M. rosenbergii* infected with the strain GXFL1-4 revealed disorganized of intestinal villus, disorganized of epithelial cells, severe necrosis of intestinal epithelial cells, and separation of intestinal epithelial cells from the basement membrane (Fig. 2D). These results suggested that the tissues integrity in *M. rosenbergii* was affected due to non-O1/O139 *V. cholerae* infection.

3.4. Characterization and identification of the isolate

Transmission electron microscopy showed that the cell was slightly curved rods with round-ends, approximately 0.5–1.0 μm long and 1.0–2.5 μm wide, which was motile by single polar flagella (Fig. 3). The biochemical tests showed that the strain GXFL1-4 was negative for erythritol, amygdatin, dulcitol, raffinose, melibiose, xylose, acetate utilization, gluconate utilization, citrate utilization, tartrate utilization, inositol, sorbose, salicin, adonitol, phenylalanine deaminnase, arginine dihydrolase, malonate utilization, H_2S production, cellobiose, mucate utilization, arabitol, arabinose, lactose, rhamnose. Additionally, the strain GXFL1-4 was positive for VP, ermentation, MR, motility, oxidase, catalase, indole, glucose, maltose, sucrose, mannose, galactose, mannitol, trehalose, glucose acid production, nitrate reduction, esculin, fructose, oextrin. All the characteristics of the strain GXFL1-4 were matched with the species description of *V. cholerae* in Bergey's Manual of Systematic Bacteriology.

The 16S rRNA and *gyrB* sequences of the strain GXFL1-4 were determined and deposited in GenBank under accession number MK045362, MK045363, respectively. The similarity between the 16S rRNA gene sequences of the strain GXFL1-4 and other *V. cholerae* isolates in the GenBank database was 100%, and the phylogenetic tree further demonstrated strain GXFL1-4 to be *V. cholerae* (Fig. 4a). Besides, the similarity between the *gyrB* gene sequences of the strain GXFL1-4 and other *V. cholerae* isolates in the GenBank database was 99%, and the phylogenetic tree further demonstrated that the strain GXFL1-4 was close to *V. cholerae* (Fig. 4b). Additionally, the strain GXFL1-4 was identified as non-O1/O139 *V. cholerae* for not carrying *rfb-O1* and *rfb-O139* genes.

3.5. Virulence factors and virulence related genes of the isolate

The extracellular enzymes and hemolysin activities of the strain GXFL1-4 were shown in Table 3, the bacteria produced caseinase,

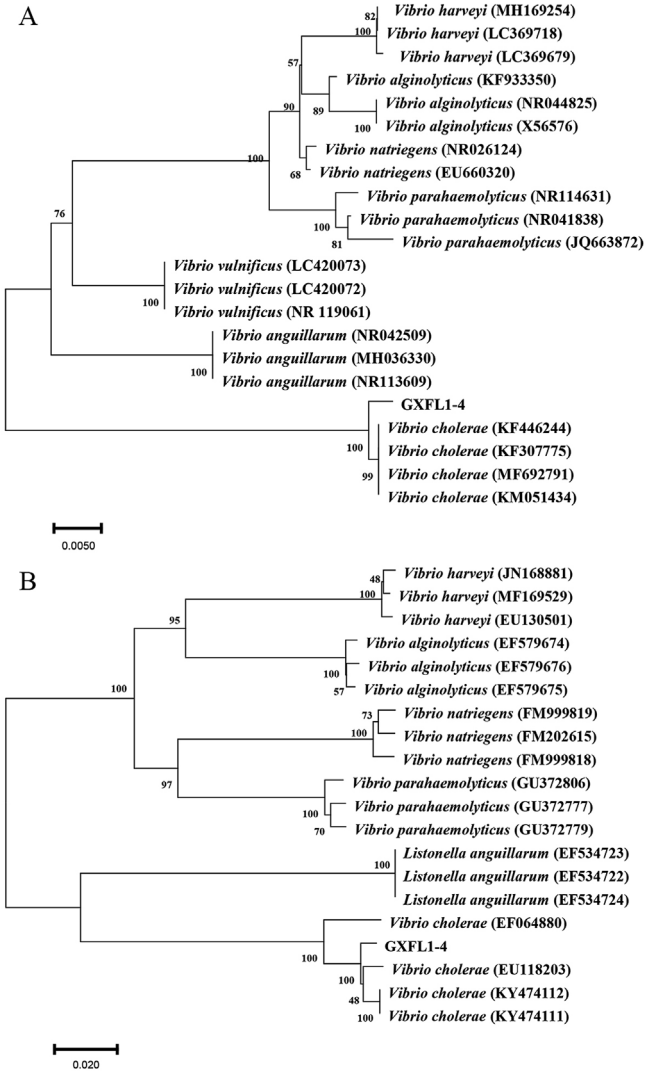

Fig. 4. (A) The neighbor joining (NJ) phylogenetic tree based on the partial 16S rRNA gene sequences. (B) The neighbor joining (NJ) phylogenetic tree based on the partial *gyrB* gene sequences. The bootstrap values are shown beside the clades. Accession numbers are indicated beside the names of bacteria, and scale bars represent distance values.

lipase, amylase, lecithinase and hemolysin, and did not produce gelatinase.

As shown in Fig. 5, the isolate carried *toxR*, *hlyA*, *ompW*, *ompU*, *hap*, *rtxA* and *rtxC* virulence related genes, genes of *ctxA*, *ctxB*, *tcpA*, *zot* and *ace* were not detected in the isolate. In addition, no fragments were present in the negative control (data not shown).

3.6. Expression of immune related genes after infection

After challenged with non-O1/O139 *V. cholerae* GXFL1-4, the different expression profiles of immune related genes in hepatopancreas and *hemolymph* of *M. rosenbergii* were shown in Fig. 6 and Fig. 7. Significant changes were observed in immune related genes of *M. rosenbergii* injected with non-O1/O139 *V. cholerae* when compared to *M. rosenbergii* injected with PBS. The enhanced mRNA expression of *HSP70*, *Crustin*, *Lysozyme*, *TRL1*, *ALF1*, *Lectin*, *Peroxinectin*, *proPO* and *SOD* in hepatopancreas at 6, 12 h post-infection was observed in Fig. 6. At 72 h post-injection, the expression changes of these immune related genes in infected *M. rosenbergii* returned back near to control *M. rosenbergii*. In addition, these immune related genes in *hemolymph* also exhibited a fast response to non-O1/O139 *V. cholerae* infection by up-regulating at 6, 12 h (Fig. 7).

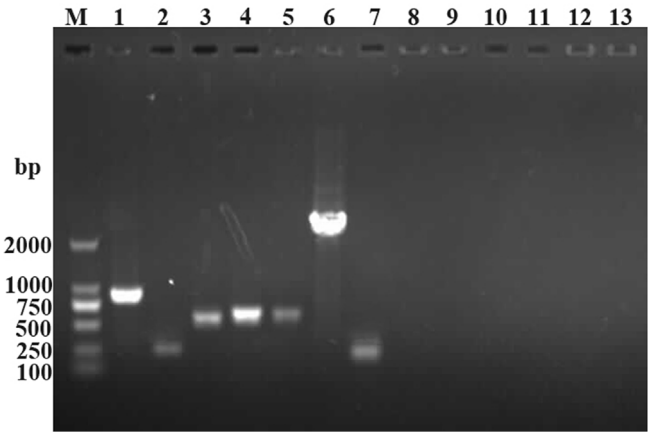

Fig. 5. Agarose (1%) gel electrophoresis of DNA products amplified from the strain GXFL1-4. M, Trans 2K DNA Marker; line 1, *toxR* (901 bp); line 2, *hlyA* (265 bp); line 3, *ompW* (586 bp); line 4, *ompU* (655 bp); line 5, *hap* (700 bp); *rtxA* line 6, (2571 bp); line 7, *rtxC* (265 bp); line 8, *ctxA* (302 bp); line 9, *ctxB* (460 bp); line 10, *tcpA* Classical (466 bp); line 11, *tcpA* Eltor (466 bp); line 12, *zot* (243 bp); line 13, *ace* (284 bp).

4. Discussion

Among the crustaceans, *M. rosenbergii* has played a prominent role in terms of its economic value and ease of captive farming. However, much viruses and bacteria limited the global production of *M. rosenbergii* and posed a great threat to commercially exploited *M. rosenbergii* [4]. In this study, the strain non-O1/O139 *V. cholerae* GXFL1-4 was isolated from moribund *M. rosenbergii* in this outbreak. Results of experimental infection trials showed that challenged *M. rosenbergii* developed clinical signs of weakness and red body as well as the naturally infected *M. rosenbergii*. Then, the same bacteria were re-isolated from dead prawns, and serious necrosis, vacuolization and inflammation in the hepatopancreas and intestine occurred in challenged *M. rosenbergii*. These results suggested that non-O1/O139 *V. cholerae* is the etiological agent of the enzootic.

Vibriosis is known to cause significant economic damage in aquaculture industry, and *Vibrio* species are the most frequently reported bacteria that are implicated as pathogens of shrimp and fish [39,40]. *V. cholerae* is an autochthon flora in brackish water and estuarine systems, and an important aquatic animal and human pathogen. Studies have shown more than 200 serogroups of *V. cholerae* have been identified so far and epidemic cholera has been confined only to isolate within serogroups O1 and O139. However, there are only limited reports of infection due to *V. cholerae* in aquatic animals. Previous studies showed that non-O1/O139 *V. cholerae* was pathogenic to *Penaeus monodon*,

Table 3
The extracellular enzyme and hemolysin activity of the strain GXFL1-4.

Extracellular products	Caseinase	Lipase	Amylase	Lecithinase	Hemolysin	Gelatinase
Halo (mm)	18 ± 1.2	22 ± 1.5	6 ± 1.2	14 ± 1.5	20 ± 2.0	0

Data are presented as mean ± SD (n = 3).

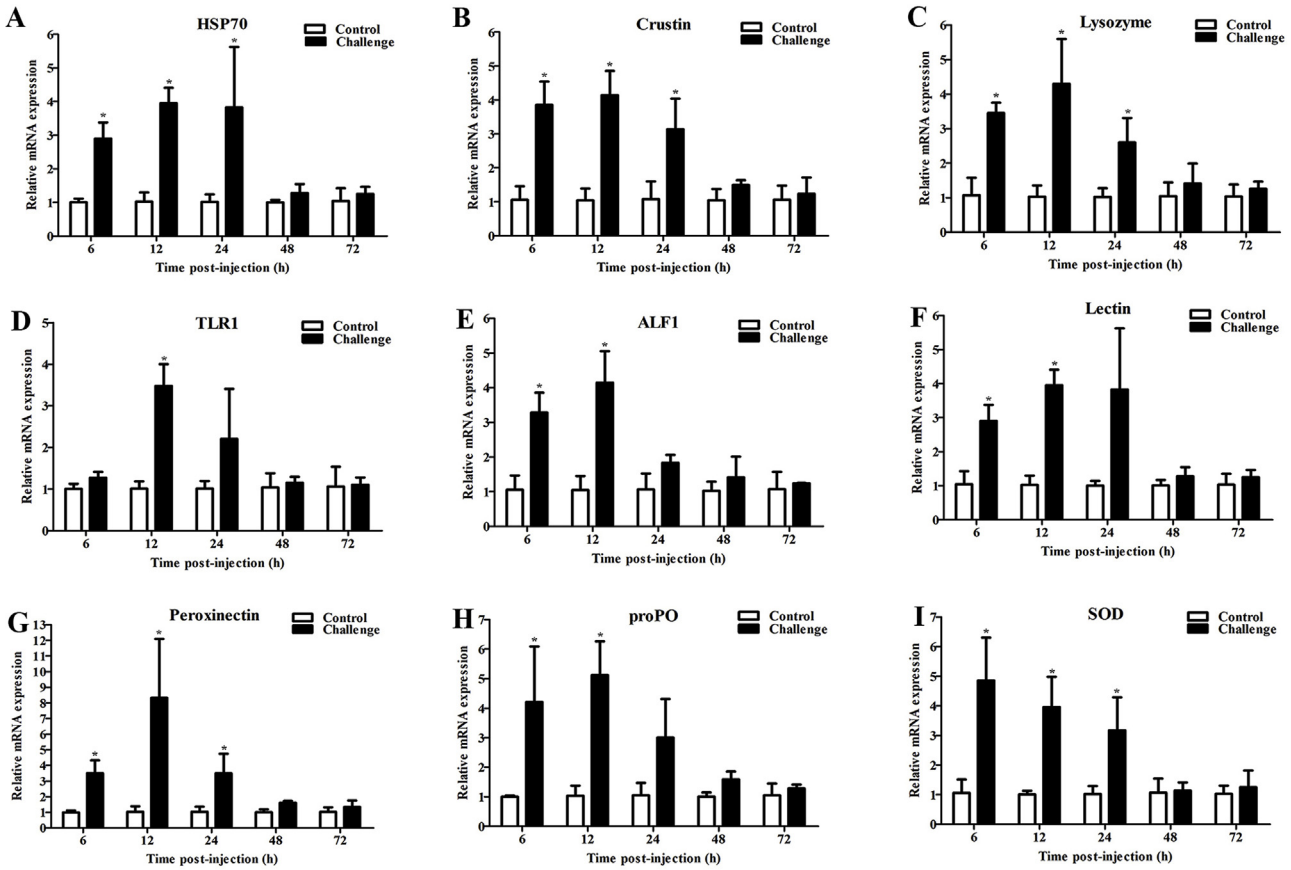

Fig. 6. Analysis of immune-related genes expression in *M. rosenbergii* hepatopancreas in response to non-O1/O139 *V. cholerae* challenge by real-time PCR at 0, 6, 12, 24, 48, 72 h post-injection. Data are presented as mean ± SD (n = 3). The asterisk indicated significant differences between the control group and the experimental group at each time point ($P < 0.05$).

Penaeus vannamei, *Misgurnus anguillicaudatus* and *Cyprinus carpio* [41–44]. Joseph et al. reported that *V. cholerae* O139 could cause high mortalities in *P. monodon*, *Fenneropenaeus indicus* and *Litopenaeus vannamei* [45]. Notably our isolate was the strain which caused noted mortalities in *M. rosenbergii* immersion infection. The pathogenesis of non-O1/O139 *V. cholerae* infection in prawns may be due to its various virulence factors.

Vibrio strains have numerous virulence factors including e.g. proteases, phospholipases, lipases, hemolysin, cytolysin, cytotoxin and enterotoxin, and are known pathogens for shrimp and fish [46–48]. Previous studies showed that extracellular products (ECPs) could play an important role in the pathogenesis of various aquatic pathogens. ECP of aquatic pathogens such as *Vibrio anguillarum*, *Vibrio harveyi*, *Streptococcus uberis* and *Aeromonas hydrophila* have been reported to be toxic in some cases [49,50]. Therefore the ECPs, e.g. proteases, phospholipases, lipases and hemolysin, are considered to be one of the most important determinants of virulence in *V. cholerae*. In this study, the strain GXFL1-4 produced caseinase, esterase, amylase, lecithinase and hemolysin, supporting the strong virulence of the strain GXFL1-4 to *M. rosenbergii*. Virulence-related genes are epidemic markers for identifying pathogenicity of *V. cholerae*. Although non-O1/O139 *V. cholerae* generally lack several major virulence factors such as cholera toxin and toxin-coregulated pilus [51], a number of other synergistic factors which play roles in the infection process have been identified. These factors include the transcriptional regulatory proteins, repeats-in-toxin, hemolysins, outer membrane proteins and so on [52]. The *ompW* gene, a most important epidemic marker, was reported to contribute to the virulence of the *V. cholerae* by self-protection and promotion in the adherence to and colonization of host cells [53]. The *toxR* gene was first discovered as the regulatory gene of the cholera toxin operon, but it was later shown to be involved in the regulation of many other genes in *V. cholerae* [31]. The *hlyA* gene is one generally accepted virulence gene in many bacterial pathogens, especially in a variety of bacteria belonging to *Vibrio* sp [30].Though the strain GXFL1-4 in this study were found to be non-toxigenic because of lacking major enteric toxin encoding genes like *ctxA* (encoding cholera toxin A subunit), *ctxB* (encoding cholera toxin B subunit), *tcpA* (encoding toxin-coregulated pilus for classical/ElTor), *zot* (encoding zonula occludens toxin) and *ace* (encoding accessory cholera enterotoxin), the strain harbored pathogenicity related genes like *rtxA* (encoding repeat toxin A subunit), *rtxC* (encoding repeat toxin C subunit), *toxR* (toxin regulatory gene), *hapA* (encoding hemagglutinin protease), *hlyA* (encoding hemolysin) and *ompW* (encoding the outer membrane protein specific for *V. cholerae*). These results indicated that the virulence genes might be the virulence markers of non-O1/O139 *V. cholerae*, might play certain roles in the virulence of the bacteria to *M. rosenbergii*.

To understand the immune response of *M. rosenbergii* infected with non-O1/O139 *V. cholerae*, the expression levels of nine immune-related genes were determined. Previous studies showed that Toll-like receptors (TLRs) have been recognized as a bridge between innate and acquired immunity, and the Toll-pathway is involved in responses against fungi, Gram-positive, and Gram-negative bacteria by activating signaling pathways that lead to expression of defense molecules [54,55]. Srisuk et al. reported that TLR of *M. rosenbergii* was found to be putatively involved in the innate defense against *Aeromonas caviae* [56]. Anti-lipopolysaccharide factor (ALF) is also a kind of antimicrobial peptide and has been widely distributed among different crustaceans [57]. Previous studies showed that different ALFs could exhibit different antibacterial and antiviral activities [58]. Recently, many studies have emphasized the possible role of C-type lectins as non-self-

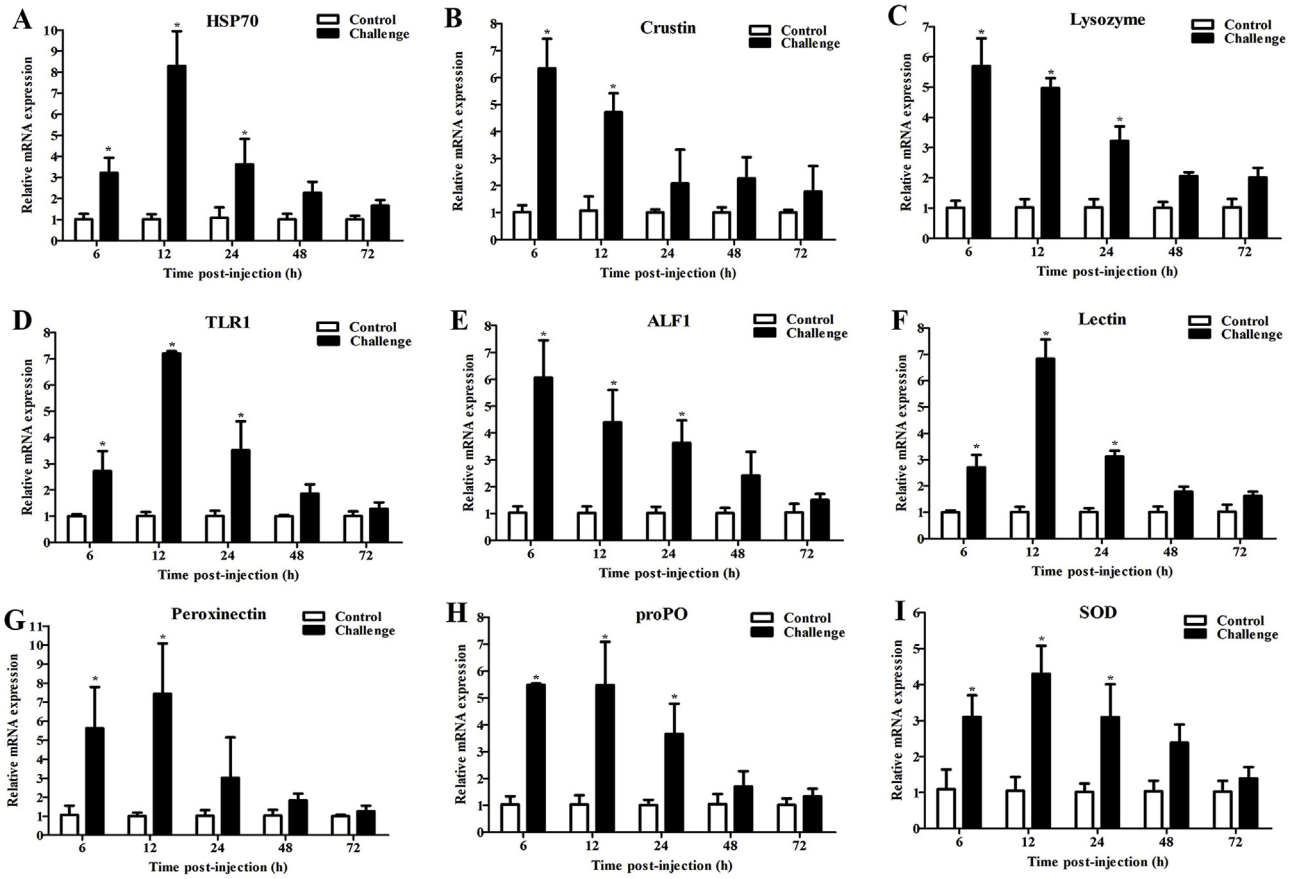

Fig. 7. Analysis of immune-related genes expression in *M. rosenbergii* hemolymph in response to non-O1/O139 *V. cholerae* challenge by real-time PCR at 6, 12, 24, 48, 72 h post-injection. Data are presented as mean ± SD (n = 3). The asterisk indicated significant differences between the control group and the experimental group at each time point ($P < 0.05$).

recognition molecules in shrimp immunity, these C-type lectins were reported to be involved in activation of proPO, antibacterial activation, encapsulation and phagocytosis and many other various biological responses in invertebrates [59,60]. In this study, compared to the control group, the expression level of *HSP70*, *Crustin*, *Lysozyme*, *TRL1*, *ALF1*, *Lectin*, *Peroxinectin*, *proPO* and *SOD* at 6 and 12 h was significantly increased after infection with non-O1/O139 *V. cholerae*, and the most prominent expression of these immune related genes subsided after 24 h. Therefore, it can be assumed that non-O1/O139 *V. cholerae* can activate some signaling pathways of *M. rosenbergii* that lead to expression of defense molecules against non-O1/O139 *V. cholerae*.

In summary, the present study identified non-O1/O139 *V. cholerae* as the bacterial pathogen causing red body syndrome and mass mortality of *M. rosenbergii*. Regarding immune response, changes were found in gene expression of *HSP70*, *Crustin*, *Lysozyme*, *TRL1*, *ALF1*, *Lectin*, *Peroxinectin*, *proPO* and *SOD* of *M. rosenbergii* during the time-course of a successful immune response to the pathogen non-O1/O139 *V. cholerae*.

Ethics statement

All animals' treatments were strictly in accordance with the guidelines of Animal Experiment Ethics Committee of Yangzhou University. The protocol was approved by Animal Experiment Ethics Committee of Yangzhou University.

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