Fish and Shellfish Immunology 87 (2019) 507–514

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# The pathogenicity characterization of non-O1 *Vibrio cholerae* and its activation on immune system in freshwater shrimp *Macrobrachium nipponense*

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ARTICLE INFO

*Keywords:*
*Macrobrachium nipponense*
Non-O1 *Vibrio cholerae*
Histopathological analysis
Immune related genes

ABSTRACT

Outbreaks of mass mortalities among cultured *Macrobrachium nipponense* occurred in a commercial hatchery during the autumn of 2017 in Jiangsu province, P. R. China, and non-O1 *Vibrio cholerae* was isolated and identified as causal agents of *M. nipponense*, with a $LD_{50}$ value $4.09 \times 10^4$ CFU/mL. Detection of virulence-associated genes by PCR indicated that XL1 was positive for *Mp*, *HlyA*, *RtxA*, *OmpU*, *Ace*, *Zot* and *T6SS*. Furthermore, the results of extracellular enzyme analysis revealed that the strain can produce lecithinase, amylase, gelatinase and hemolysin. Histopathological analysis revealed that the hepatic tubule lumen and the gap between the hepatic tubules became larger, and the brush border disappeared in the hepatopancreas. Quantitive real-time PCR (qRT-PCR) was undertaken to measure mRNA expression levels for thirteen immune related genes in *M. nipponense* after non-O1 *V. cholerae* infection. The transcriptional analysis of these immune related genes demonstrated that the expression levels of dorsal, relish, p38, crustin1, crustin2, crustin3, hemocyanin, i-lysozyme, anti-lipopolysaccharide factors 1, anti-lipopolysaccharide factors 2, prophenoloxidase were significantly up-regulated in hemolymph of *M. nipponense* post-infection. These results revealed varying expression profiles and clear transcriptional activation of these immune related genes in hemolymph, which will contribute to better understand the pathogenesis and host defensive system in non-O1 *V. cholerae* invasion.

## 1. Introduction

The oriental river prawn, *M. nipponense*, is widely distributed in freshwater and low-salinity regions of China and Japan [1]. This freshwater shrimp were favored by aquaculture farmers for its delicious meat, rich nutrition, short culture cycle and high market price [2]. According to the "*China Fisheries Yearbook*", in recent years, the annual production of *M. nipponense* is about $2 \times 10^5$ t, and the annual output value is nearly 10 billion yuan. It has become one of the important ways to increase agricultural efficiency and farmers' income [3,4]. However, with the expansion of the scale of cultivation, the increase of the density of breeding, various diseases are constantly emerging, especially bacterial diseases, which cause great harm, such as infected by *Vibrio mimicus* and *Aeromonas hydrophila* [5–7]. In September 2017, an outbreak of mass mortality break out at the river prawn farms in Yangzhou city, Jiangsu province, P. R. China, and the non-O1 *V. cholerae* was identified as one of the pathogens causing mass death in many farms by physiological, biochemical characteristics and molecular identification.

*V. cholerae* is a water-borne Gram-negative pathogen that divided into more than 200 serogroups depending on the O antigen [8]. The O1/O139 *V. cholerae* strains can cause cholera outbreaks and epidemics, *V. cholerae*, other than the O1/O139 serogroups, is collectively referred to as non-O1/O139 serogroups *V. cholerae* [9]. Non-O1/O139 *V. cholerae* is widely distributed in the environment, especially in water and seafood, and could cause mild gastroenteritis, cholera-like diarrhea, sepsis or other extraintestinal infections [10–12], but does not cause cholera outbreaks [13]. In the last decade, the non-O1/O139 *V. cholerae* were often ignored because its infection degree was not as serious as that of O1/O139 *V. cholerae*. However, the incidence of non-O1 *V. cholerae* was usually much higher than the O1/O139 *V. cholerae*, which has attracted more attention in recent years [14].

In this report, the strain non-O1 *V. cholerae* was isolated from the diseased *M. nipponense*. In order to explore the pathogenicity and virulence characteristics of non-O1 *V. cholerae*, the extracellular

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https://doi.org/10.1016/j.fsi.2019.01.050
Received 5 November 2018; Received in revised form 26 January 2019; Accepted 30 January 2019
Available online 31 January 2019
1050-4648/ 

**Table 1**
Sequence of primers used in this study.

| Gene | Primer sequences (5′-3′) | Product length (bp) |
|---|---|---|
| *Zot* | TCGCTTAACGATGGCGCGTTTT<br>AACCCCGTTTCACTTCTACCCA | 241 |
| *Ace* | TGATGGCTTTACGTGGCTTGTGATC<br>GCCTGTTGGATA AGCGGATAGATGG | 131 |
| *T6SS* | *vasA*-F-GTACGACCGATCCTGACGTT | 342 |
| | *vasA*-R-ATCTGAATGGTCGTGGCTTC | |
| | *vasK*-F-GCGTCAAATTCAGGAAGAGC | 399 |
| | *vasK*-R-CTGTCCCAGAACCCAACTGT | |
| | *vasH*-F-GTGGCACGCTATTTCTGGAT | 385 |
| | *vasH*-R-TTTCAGCTCACGCACATTTC | |
| *OmpU* | ACGCTGACGGAATCAACCAAAG<br>GCGGAAGTTTGGCTTGAAGTAG | 869 |
| *RtxA* | CTGAATATGAGTGGGTGACTTACG<br>GTGTATTGTTCGATATCCGCTACG | 417 |
| *NanH* | CTTCCTCCAATACGGTTCTTGTCTCTTATGC<br>TTCGGCTACCATCGGCAACTTGTATC | 314 |
| *HlyA* | *hlyA*-F1-GGCAAACAGCGAAACAAAAC | 738 |
| | *hlyA*-F2-GAGCCGGCATTCATCTGAAT | |
| | *hlyA*-R-CTCAGCGGGCTAATACGGTTTA | 481 |
| *Stn* | TCGCATTTAGCCAAACAGTAGAAA<br>GCTGGATTGCAACATATTTCGC | 172 |
| *Mp* | ACGTCCTCTGAATTGGTTAG<br>CTGTAACCGCGTAACATGAC | 1782 |
| *Stat* | TATATCCGAATGTGCCTAAG<br>ATAGTTTGTGGTGTGTTGGG | 195 |
| *Dorsal* | CATCAAAATGTTTGTTGCCCAG<br>CCATATCAGAAAATATCCAAA | 215 |
| *Relish* | GAGGAGGATGAAGAAGAAGAG<br>GCATAATCAAGTGGCGTGTAA | 186 |
| *p38* | CAGTATCCAACCGTCCACTG<br>GCTACCTTCTAGCCTTCTCAC | 165 |
| *ALF1* | GCCGATGGTGTCCTGGATG<br>TCCATGCGTCGTCCTCCG | 157 |
| *ALF2* | GGCACCAAACTCACTGGA<br>CTTAGCACATGCGACCCTG | 163 |
| *ALF3* | GAACTGCTGTCCAACCCTG<br>CCGGATGCTCCTCCGTTATC | 232 |
| *Cusrin1* | TGAAACTAACCTGTTCCAACG<br>GAATGCCCTGCGATCCGAAGAA | 165 |
| *Crustin2* | GTCCGGCCTTATCCCTGGAG<br>CATGGTTTGCAGACGGTGTC | 193 |
| *Crustin3* | GGCCTTGAGGTGGCGGGATT<br>GGTGTCGTGGGTTTGCCTAT | 223 |
| *Hemocyanin* | CAGTTTCGCAACTCTCTT<br>CACTCTCGAAATCTGTAAGT | 244 |
| *i-lysozyme* | GACCTTGCGTCATGCCAGAT<br>CCATGGGTTTATGTGCGTCTTC | 182 |
| *Prophenoloxidase* | GGTCTTCCCCTCCCGCTTC<br>CGCAGGTCCTTTGGCAGC | 166 |
| *18S* | TATACGCTAGTGGAGCTGGAA<br>GGGGAGGTAGTGACGAAAAAT | 182 |

enzyme activities, $LD_{50}$, and virulence genes of the isolates were tested. In addition, various immune parameters of *M. nipponense* in response to non-O1 *V. cholerae* infection were monitored at different point of time in hemolymph. The purpose of the present study is to provide a scientific reference for the breeding of *M. nipponense* and disease prevention.

## 2. Materials and methods

### 2.1. Experimental shrimp and bacteria preparation

Experimental *M. nipponense* (average weight was 2.66 ± 1.08 g) were provided by an aquaculture farm located at Gaoyou Country of Jiangsu Province, China, where no mortalities occurred, and they showed no clinical symptoms. The healthy *M. nipponense* acclimated in the aquarium and were fed three times daily with artificial food. The aquarium was supplied aerated freshwater at 26.0 ± 0.5 °C with flow-through water system.

The strain used in the present study was isolated from diseased *M. nipponense* in Yangzhou city, Jiangsu Province, China in 2017. The pure cultured bacteria were inoculated into liquid medium at 28 °C with shaking at 200 rpm for 24 h for amplification and the bacteria ($2.5 \times 10^8$ CFU/mL) was stored at 4 °C before usage.

### 2.2. Identification of bacteria

The morphological characteristic of non-O1 *V. cholerae* was observed by transmission electron microscope. Briefly, cell suspensions were fixed for 30 min by adding to the medium of 2.5% glutaraldehyde, and cooled at 4 °C for 2 h. Fixed cells were washed with 1% OsO4 for 30 min, washed 3 times with phosphate buffer (pH 7) for 10 min. The samples were dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranylacetate and lead citrate. All samples were examined in transmission electron microscope (Zeiss EM10).

Biochemical and physiological tests included oxidase and lactose, urease, tartrate utilization, xylose and so on. Standard plate and tube tests (Hangzhou Tian he Microorganism Reagent Co., Ltd., China) were used to examine the isolates phenotypic characteristics. In addition, the reactions were compared with the results from Bergey´s Manual of Systematic Bacteriology [15]. The 16S rRNA and *gyrB* gene sequences of the isolate XL1 was amplified by PCR according to Zhang et al. [16]. In addition, the isolate XL1 was further identified as non-O1 *V. cholerae* using a PCR assay by targeting the rfb-O1 and rfb-O139 genes.

### 2.3. Virulence gene detection

To study the pathogenicity of isolates for more detailed, total genomic DNA of the strain non-O1 *V. cholerae* was extracted using the EasyPure Genomic DNA Kit (Trans, China). Virulence genes, encoding the metalloproteinase (*Mp*), heat stable enterotoxin (*Stn*), hemolysin (*HlyA*), neuraminidase (*NanH*), repeat in toxin (*RtxA*), outer membrane protein (*OmpU*), auxiliary cholera enterotoxin (*Ace*), zonula occludens toxin (*Zot*) and *VI* secretion system (*T6SS*) were amplified by PCR using specific primers (Table 1) as recommended by Zhou et al. [17]. The PCR reaction system consisted of 10 μL of 2 × Easy Taq PCR Super® Mix, 0.5 μL of each of the primers (10 μM), 1 μL of DNA template and then $ddH_2O$ to 20 μL. PCR procedures are as follows: 1 cycle of 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. A final extension step of 72 °C for 5 min.

### 2.4. Determination of extracellular enzymes and hemolysin

The method of plate test was used to monitor lecithin activity, lipase activity, amylase activity, hemolytic activity and urease activity of isolated bacteria. The isolates were separately planted in LB nutrient agar that contained 10% egg yolk liquid, 1% tween 80, 1% starch, 7% rabbit erythrocytes, 2% urea (phenol red indicator) as substrate and incubated at 28 °C for 24 h. Egg yolk liquid, tween 80 and starch plate directly observe hydrolytic circle, which ring area shows enzymatic activity. If the hemolytic circle was observed on the LB nutrient agar containing rabbit erythrocytes, it showed that the isolate had hemolytic activity. If the bacterial lawn was red on the culture medium containing urea, it indicated that the strain had urease activity.

### 2.5. Bacterial virulence assay

After acclimatization, *M. nipponense* were divided into control and test groups. Prior to challenge, *M. nipponense* were randomly sampled and subjected to microbiological analysis, which indicated that they were free of pathogens. Groups of 75 shrimp were inoculated (50 μL) with different bacterial doses ranging from $2.4 \times 10^7$ to

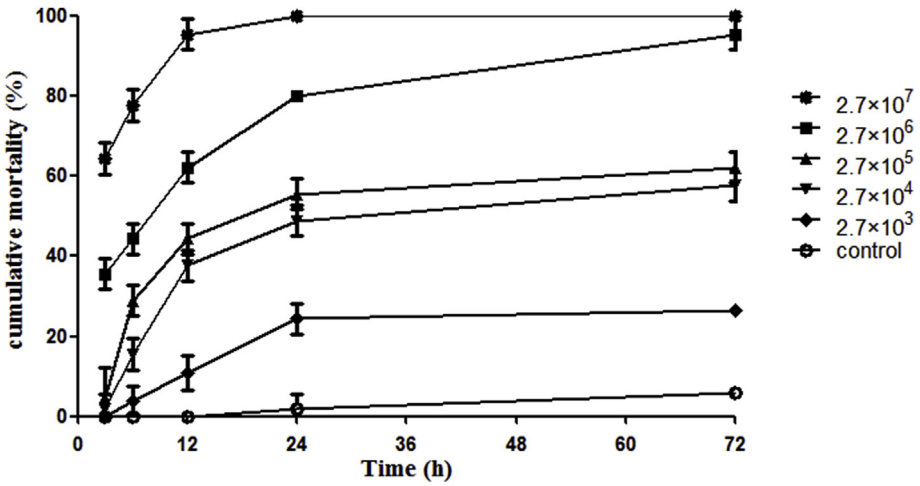


**Fig. 1.** Pathogenicity of *Non-O1 Vibrio cholerae* to *M. nipponense*.

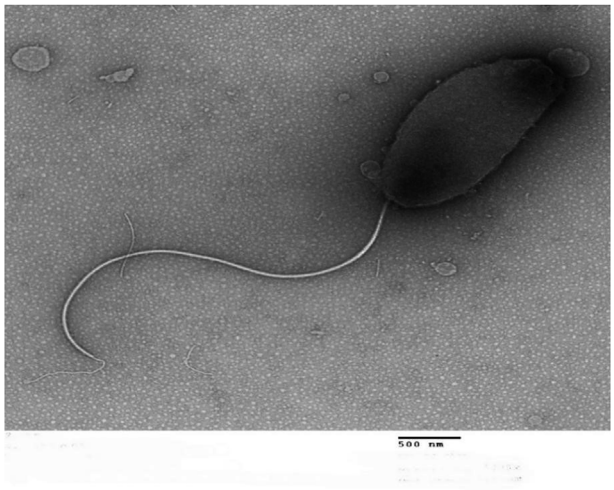


**Fig. 2.** Electron micrograph of XL1 showing polar single flagella (bar = 500 nm).

$2.4 \times 10^3$ CFU/mL for non-O1 *V. cholerae* in the aquarium, respectively. Control shrimp were exposed to the same experimental conditions without any bacterial inoculation. The mortality of shrimp was checked during this period. Dead shrimp were microbiologically analyzed for re-isolation of the inoculated isolate. The degree of virulence was expressed as lethal dose 50% ($LD_{50}$), calculated as described by Behreans and Karber [18]. All experiments were repeated three times.

### 2.6. Histological examination

Hepatopancreas from both infected and non-infected *M. nipponense* were fixed in 4% formaldehyde for histological examination. The formalin-fixed tissues were embedded in paraffin wax and processed by standard paraffin wax techniques. Sections stained with hematoxylin and eosin (HE) were processed according to a standard protocol [19].

### 2.7. Bacterial challenges and tissue collection

According to the results of 2.5, one group of shrimp (n = 50) were stimulated by the injection of $2.4 \times 10^3$ CFU/mL non-O1 *V. cholerae*, each prawn was injected with 50 μL through abdominal muscles, whereas the shrimp (n = 50) of control group were infected with the same dose of sterile PBS. After non-O1 *V. cholerae* infection, 3 shrimp were randomly taken from each group at 0, 3, 6, 12, and 24 h post-

**Table 2**
The characteristics of the strain XL1 in comparison with Non-O1 *V. cholerae*.

| Characteristics | XL1 | Non-O1 *V. cholerae* * | Characteristics | XL1 | Non-O1 *V. cholerae* * |
|---|---|---|---|---|---|
| Growth at 37 °C | + | + | Tartrate utilization | – | +/− |
| Oxidase | + | + | Malonate utilization | – | +/− |
| Arginine dihydrolase | – | – | Amylase | + | + |
| Acetate utilization | – | +/− | Citrate utilization | – | d |
| Melezitose | + | d | DNA enzyme | + | + |
| Lactose | – | – | D-Xylose | – | – |
| Maltose | + | + | Mushroom sugar | + | d |
| Mannitol | + | + | Raffinose | – | – |
| Mannose | + | +/− | Fructose | + | d |
| Sucrose | + | + | Melibiose | – | – |
| L-Arabinose | – | – | Cellobiose | – | – |
| D-Arabitol | – | – | Methyl red | – | +/− |
| D-Galactose | – | +/− | Inositol | – | – |
| D-Sorbitol | – | – | Urease | – | – |
| Erythritol | – | – | L-Rhamnose | – | – |
| Dextrin | + | d | Gelatin | + | + |
| Arginine dihydrolase | – | – | Nitrates | + | + |

Notes: +, positive; −, negative; +/−, positive and negative; d: data not described in Bergey's Manual of Systematic (Brenneret al., pp (2008) 520–528, second edition).

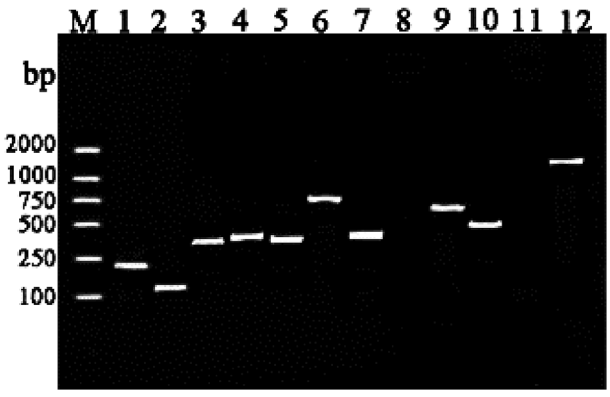


**Fig. 3. Electrophoresis of DNA products from our isolate bacteria**. M. Trans 2K DNA Marker; lane 1. *Zot* 241 bp; line 2. *Ace* 131 bp; line 3. *VasA* 342 bp; line 4. *Vask* 399 bp; line 5. *VasH* 385 bp; line 6. *OmpU* 865 bp; line 7. *RtxA* 417 bp; line 8. *NanH* 314 bp; line 9. *HlyA2* 738 bp; line 10. *HlyA1* 481 bp. Line 11. *Stn* 172 bp; line 12. *Mp* 1782 bp.

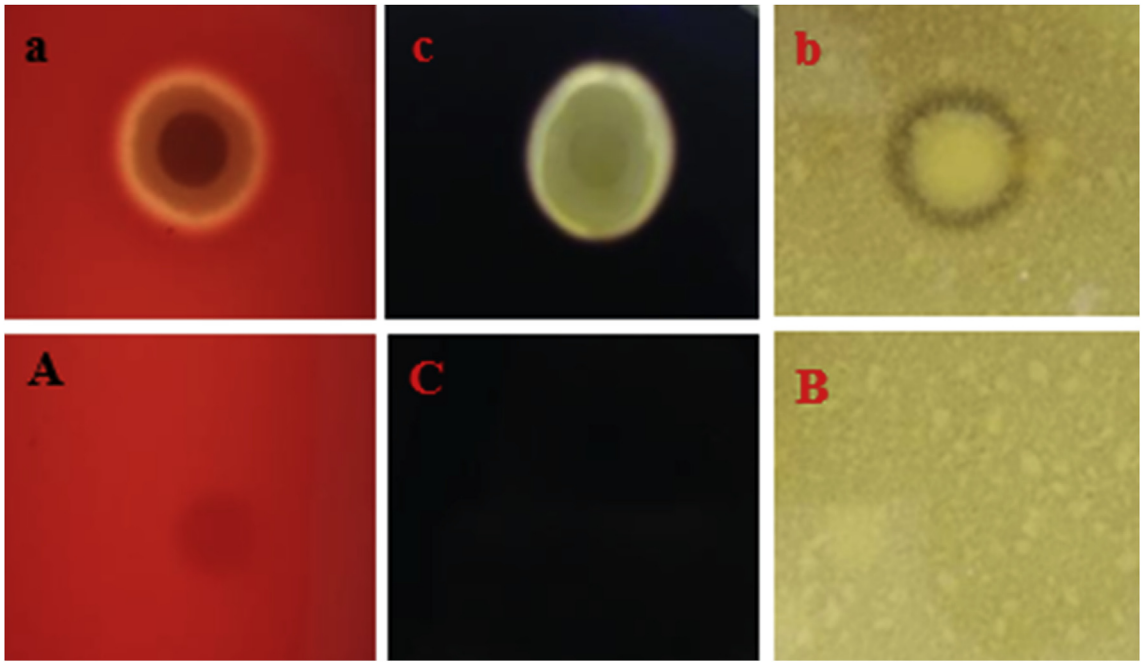


**Fig. 4. Determination of extracellular enzymes and hemolysin**. A(a): Hemolysin activities; A: Experimental group; a: Control group; B(b): Amylase activities; B: Experimental group; b: Control group; C(c): Lecithinase activities; C: Experimental group; c: Control group.

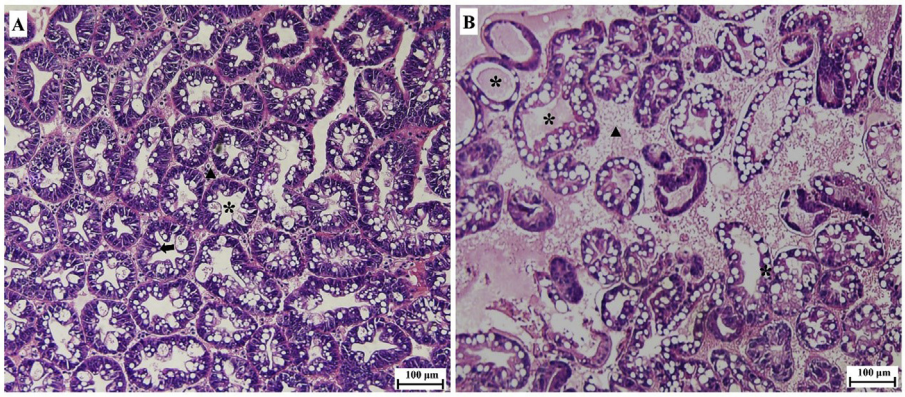


**Fig. 5. Change of hepatopancreas microstructure following non-O1 *V. cholerae* infection**. A represent the control group; B represent the experimental group; The asterisk indicates hepatic tubules; The arrow indicates brush border; The triangle indicates the connective tissue between the hepatic tubules.

injection, hemolymph was collected from the cardiocoelom, and immediately placed in liquid nitrogen for cryopreservation.

Total RNA of different tissues of *M. nipponense* were extracted by Trizol® Plus RNA Purification Kit, the specific extraction process was referred to the kit instructions (TianGen Biotech, Beijing). The concentration and purity of the extracted RNA were measured by an ultraviolet spectrophotometer, and the integrity of the RNA was detected by 1% agarose gel electrophoresis. The cDNA was synthesized using the PrimeScriptTM RT Reagent Kit with gDNA Eraser kit (TaKaRa). All cDNA samples were stored at −20 °C.

Real-time reactions were carried out in a 20 μL volume containing forward Primer (10 μM) 0.4 μL, reverse Primer (10 μM) 0.4 μL, 2 × SuperMix 10 μL, dd$H_2O$ 8.2 μL and 1 μL diluted cDNA template. Immune-related genes expression levels were detected by qRT-PCR using the primers listed in Table 1. The amplification protocol used was as follows: 94 °C for 5 min, 40 cycles of 10 s at 94 °C, 30 s at 60 °C. Each sample was run in triplicate. 18S gene was chosen as reference gene and the samples from control group were used as calibrator. The relative mRNA expression levels were calculated by the $2^{-\triangle\triangle CT}$ method.

### 2.8. Statistical analysis

Statistical differences were analyzed using ANOVA and Duncan's multiple comparison of the means were used in the present study. Statistical analysis was performed using SPSS 17.0 software. Significant differences were considered at $p < 0.05$.

## 3. Results

### 3.1. Pathogenicity of the strain XL1

The mortalities of *M. nipponense* after infection with varying levels of non-O1 *V. cholerae* XL1 are shown in Fig. 1. On the observation at 72 h, the strain caused 100% mortality at concentrations $2.7 \times 10^7$ CFU/mL. The mortality rate of shrimp decreased gradually as bacterial concentration from $2.7 \times 10^6$ CFU/mL to $2.7 \times 10^3$ CFU/mL. In addition, the bacteria from the experimentally dead shrimp were identified as non-O1 *V. cholerae* by physiological, biochemical characteristics and molecular identification. And the $LD_{50}$ of XL1 strain to *M. nipponense* was $4.09 \times 10^4$ CFU/mL. Meanwhile, the behaviour of *M.*

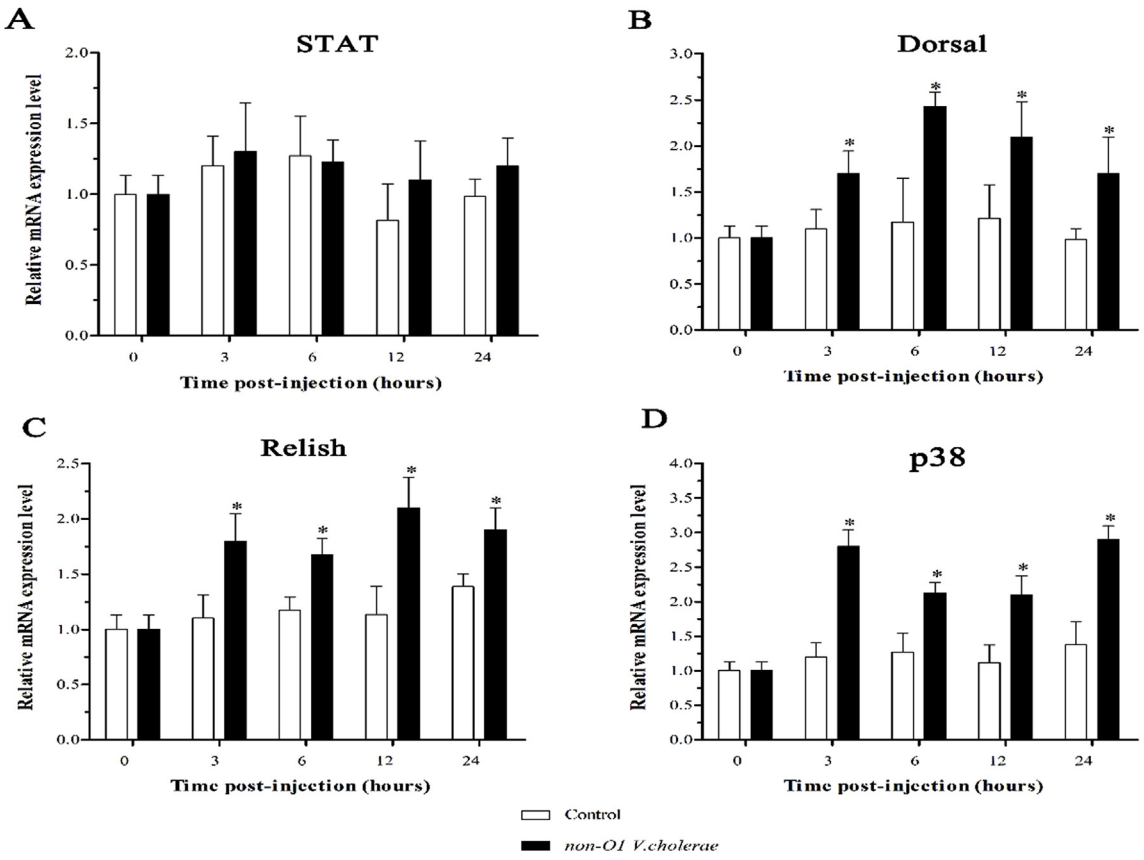


**Fig. 6. Variation in expression patterns of immune related genes in hemolymph at different time periods.** The relative expression of the genes in hemolymph of non-O1 *V. cholerae* -infected and uninfected mandarin fish at 0, 3, 6, 12, 24 h post-challenge. (A) STAT, (B) Dorsal, (C) Relish, (D) P38. Bars represent mean ± S.E. $^{**}P < 0.01$.

*nipponense* in control group remained normal, active and quickly responded to feeding. However, at 72 h, one of the control group's shrimp died, and the surface of the shrimp showed signs of being eaten, and no strain was found by bacterial isolation.

### 3.2. Identification and characterization of the isolate

The isolate XL1 were gram-negative, fermentative short rods with round-ends, approximately 0.5–1.0 μm long and 1.0–2.0 μm wide, which were motile by single polar flagella (Fig. 2). Convention physiological and biochemical tests showed that the strain XL1 can grow at 37 °C. Oxidase were positive, maltose, sucrose, fructose can be utilized, arginine dihydrolase was negative, nitrates were reduced, which was in common with *V. cholerae*. Other physical and chemical characteristics are showed in Table 2.

The 16S rRNA and *gyrB* gene sequences of the isolate XL1 were determined and deposited in GenBank under accession number MK421414 and MK421415, respectively. The similarity between the 16S rRNA gene sequence of the isolate XL1 and other *V. cholerae* strain in GenBank database was 99%, and the similarity between the *gyrB* gene sequences of the isolate XL1 and other *V. cholerae* in the GenBank database was 99%, strongly supporting the assignment of XL1 to *V. cholera*. In addition, the isolate XL1 was found not carrying rfb-O1 gene, so the isolate was identified as non-O1 *V. cholerae*.

### 3.3. Virulence genes and extracellular products of the isolates

As shown in Fig. 3, the strain XL1 can amplify out the metalloproteinase (*Mp*), hemolysin (*HlyA*), repeat in toxin (*RtxA*), outer membrane protein (*OmpU*), auxiliary cholera enterotoxin (*Ace*), zonula occludens toxin (*Zot*) and *VI* secretion system (*T6SS*) by PCR detection.

The results of enzyme activity experiments showed that the extracellular products of non-O1 *V. cholerae* XL1 had lecithinase, amylase, gelatinase and hemolysin activities, but did not have lipase and urease activity. The results are shown in Fig. 4.

### 3.4. Effect of non-O1 V. cholerae on the hepatopancreas microstructure

We have used the histological sections to observe the damage to hepatopancreas after infection of the non-O1 *V. cholerae* in *M. nipponense*. As shown in Fig. 5, compared with the control, the *M. nipponense* infected with non-O1 *V. cholerae*, the hepatic tubule lumen and the gap between the hepatic tubules became larger, and the brush border disappeared. These phenomena all indicate the physiological damage of non-O1 *V. cholerae* to *M. nipponense*.

### 3.5. Expression profiles of immune related genes in hemolymph after non-O1 V. cholerae infection

In order to detect the activation of the immune system of the *M. nipponense* in response to non-O1 *V. cholerae* infection, some immune-related genes were examined by the qPCR. The different expression profiles of these genes in hemolymph were shown in Fig. 6 and Fig. 7. As shown in Fig. 6, the vital transcription factor dorsal, relish, p38 mitogen-activated protein kinase, which were involved in Toll, Imd and MAPK signal pathways respectively, were significantly up-regulated compared with the control group during the infections, indicating the activation of these signal pathways. Some immune-related effector molecules, such as anti-lipopolysaccharide factors, crustins, i-lysozyme, hemocyanin and prophenoloxidase were detected. As shown in Fig. 7, some antimicrobial peptides antilipopolysaccharide factor 1 (ALF1), antilipopolysaccharide factor 2 (ALF2) and prophenoloxidase exhibit a

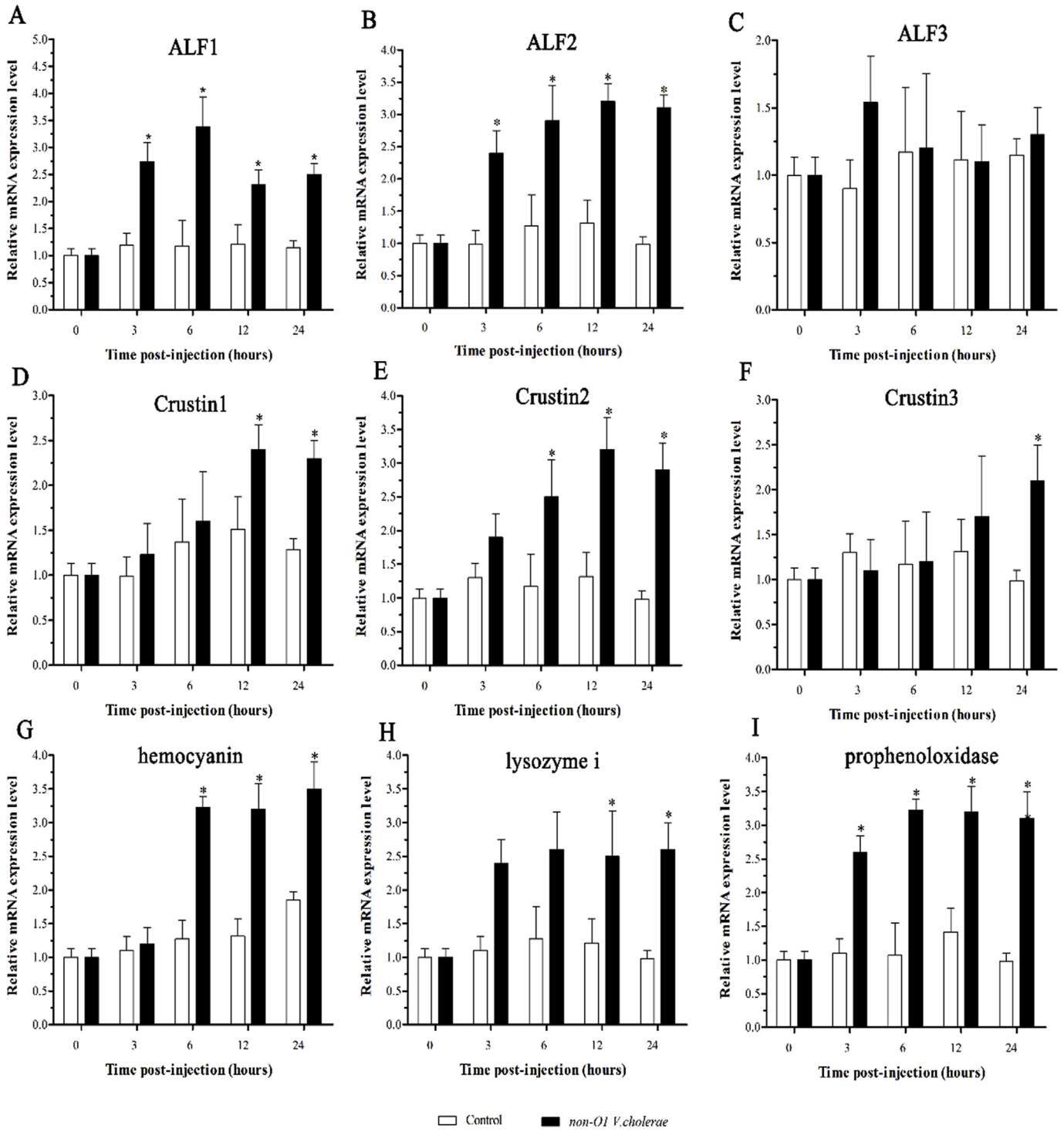


**Fig. 7. Variation in expression patterns of immune related genes in hemolymph at different time periods.** The relative expression of the genes in hemolymph of non-O1 *V. cholerae* -infected and uninfected mandarin fish at 0, 3, 6, 12, 24 h post-challenge. (A) ALF1, (B) ALF2, (C) ALF3, (D) Crustin1. (E) Crustin2, (F) Crustin3, (G) Hemocyanin, (H) lysozyme i, (I) Prophenoloxidase. Bars represent mean ± S.E. $P < 0.05$.

fast response to non-O1 *V. cholerae* infection by up-regulated of expression at 3 hpi. Crustin1, crustin2, crustin3, hemocyanin and i-lysozyme also significantly up-regulated during the infection at 6, 12 and 24 hpi. Compared with the control, the expression of ALF3 has no significant change during the infections.

## 4. Discussion

*M. nipponense* has developed into an important aquaculture freshwater shrimp species [20,21]. However, with the expansion of the farming, genetic retrogression and the pollution of the water environment, there were many diseases happened in the breeding process of *M. nipponense*, such as “soft-shell syndrome” causing by *Aeromonas veronii* [6], “red gill disease” causing by *Aeromonas hydrophila* [7], and spiroplasma infection [22]. In this study, a large number of epidemiological investigations were carried out, and non-O1 *V. cholerae* XL1 was isolated and identified. The pathogenicity of the non-O1 *V. cholerae* was confirmed by re-infection experiments, and the virulence characteristics

of the pathogen were analyzed.

Non-O1 *V. cholerae* is an important aquatic animal pathogenic microorganism that can infect various aquatic animals. Till present, the infection of non-O1 *V. cholerae* have been reported in *Plecoglossus altivelis* [23], *Mytilus galloprovincialis* [24], *Penaeus monodon* [25], *Ostrea gigas thunberg* [26], *Cyprinus carpio* [27], *Misgurnus anguillicaudatus* [28] and so on. Some report has demonstrated that fish could act as reservoirs and vectors of *V. cholerae* [29], it is one of the common species in aquatic animal pathogens. Through our epidemiological investigation, this is the first report that non-O1 *V. cholerae* could cause the death of *M. nipponense*. The pathogenic of bacteria is in connection with adhesion, flagella, biofilm formation, extracellular enzyme, hemolysin, etc [30,31]. The purified hemolysin caused increased vascular permeability of rabbit skin and rapid death of mice on intravenous injection and also lysed erythrocytes of various animal species [32]. It is also known that hemolysin is produced by most clinical isolates of non-O1 *V. cholerae* [33]. In the present study, the identified non-O1 *V. cholerae* could also exhibit hemolysin, lecithinase, amylase, gelatinase and possess flagella, indicating its potential pathogenic mechanisms. Though the specific characteristics of our identified non-O1 *V. cholerae* hemolysin is not well detected in this study, we can speculate the identified bacteria could destroy the hemocyte. Besides, the virulence gene expression is a key factor to felicitous colonization, invasion, in vivo growth [34]. *CTX* is the main virulence related component of *V. cholerae*, this study mainly tested its *Zot* and *Ace*. It has been clarified that the outer membrane protein *OmpU* is an important protective antigen of *V. cholera* [35]. However, Whether *OmpU* is a potential colonizing factor for the intestinal tract remains controversial [36]. Hemolysin (*HlyA*) and metalloproteinase (*Mp*) can cause vacuoles in cells and affect the tightness of epithelial cells, both of which, together with cytotoxic *rtxA*, can lead to cell separation and rounding [37–39]. *VI* secretion system T6SS involved in bacterial virulence and adaptability, giving *V. cholerae* relative to other bacteria in the intestines and environment of competitive advantage [40]. In our study, the *Mp*, *HlyA*, *RtxA*, *OmpU*, *Ace*, *Zot* and *T6SS* were amplified in the strain, further indicating its potential pathogenic mechanisms.

The crustaceans immune system is mainly based on innate immune defense, and it is generally considered that it does not have an acquired immune system [41]. The shrimp immune system and signaling pathways have been summarized in studies [42,43]. The Toll pathway and immune deficiency IMD pathway are thought to be the most important signaling pathways in the regulation of innate immune responses in shrimp [44]. In these two pathways, dorsal and relish are used as key nuclear transcription factors to regulate the expression of various downstream antimicrobial peptides [45]. In the present study, the up-regulation of *dorsal* and *relish* genes indicated the activation of signal pathways. However, the toll signal pathway was mainly activated by fungus and gram-positive bacteria, IMD pathway was activated by gram-negative bacteria [46]. As non-O1 *V. cholerae* was a kind of gram-negative bacteria, it mainly caused activation of the IMD pathway. The Jak/stat pathway, including three main cellular components: the receptor domeless, the Janus kinases and the stat transcription factor involves in regulating the immune response genes [47]. In Drosophila, the Jak/stat pathway is required but not sufficient for the antiviral response [48]. This pathway has been implicated in antiviral defense in insects [49]. The transcription of stat could be modulated after white spot syndrome virus (WSSV) infection, indicating its potential important functions to virus infection in shrimp [50]. In the present study, the unchanged expression of *stat* indicated Jak/stat pathway was not activated by non-O1 *V. cholerae*.

In shrimp, the humoral responses include the prophenoloxidase (proPO) system, the clotting cascade and a wide array of antimicrobial peptides [51]. Antilipopolysaccharide factor (ALF) is a kind of antimicrobial peptide and has been widely distributed among different crustaceans [52,53]. Different ALFs could exhibit different antibacterial and antiviral activities [54,55]. In *M. nipponense* after non-O1 *V. cholerae* infection, ALF1 and ALF2 could be significantly up-regulated, indicating their potential antimicrobial activities. The ALF3 may not participate in the antibacterial response for its unchanged expressions. Meanwhile, crustins, hemocyanin, i-lysozyme and prophenoloxidase were involved in the humoral immune in shrimp [56–58]. The up-regulations of these immune-related genes indicated their participation in anti-non-O1 *V. cholerae* infection.

In conclusion, in the present study, we identified a pathogenic non-O1 *V. cholerae* of *M. nipponense*, tested its lethality, analyzed its extracellular enzyme characteristics and hemolytic activity and virulence gene. During the infection experiment, non-O1 *V. cholerae* can activate the IMD signaling pathway and proPO system of *M. nipponense*, produce effector molecules such as antibacterial peptides that play an antibacterial role in humoral immunity.

## Ethics statement

All treatments of shrimp in this study 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.

## Acknowledgments

This work was supported by the Jiangsu Fisheries Research System-02, Fishery Science and Technology Innovation Projects of Jiangsu Province (D2017-3, D2017-4), Natural Science Foundation of Jiangsu Province (BK20180901, BK20160465).

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