doi:10.21077/ijf.2026.73.2.173448-13
Available online at: epubs.icar.org.in/index.php/IJF

Characterisation of virulence factors, biofilm formation, and antimicrobial peptide gene (AMP) expression in the Indian white shrimp *Penaeus indicus* (H. Milne Edwards, 1837), following immersion challenge with *Vibrio parahaemolyticus*

Naveenkumar Radhakrishnan[1], Akshaya Panigrahi[2], Megha Kadam Bedekar[1*], Saranya Chakrapani[2], Prasitha Krishnakumar[2], Arun Nagarajan[2], Sathish Kumar Thangaraj[2] and C. Siva[2]

[1]ICAR-Central Institute of Fisheries Education, Versova, Mumbai-400 061, Maharashtra, India
[2]ICAR-Central Institute of Brackishwater Aquaculture, Chennai-600 028, Tamil Nadu, India

Abstract

Vibrio parahaemolyticus is a major bacterial pathogen in shrimp aquaculture, possessing inherent virulence factors such as the haemolysin secretion system and biofilm forming ability, which enhance its pathogenic potential. In shrimp, antimicrobial defences play a central role in combating bacterial infections. This study investigated the pathogenicity factors of a *Vibrio parahaemolyticus* isolate from an infected early-larvae of *Penaeus indicus* (H. Milne Edwards, 1837). The virulence and biofilm-related genes were identified through PCR amplification, and biofilm formation ability was quantified using the crystal violet assay at different temperatures. Following immersion challenge with *V. parahaemolyticus* (1×10^6 CFU ml^{-1}), the relative expression of antimicrobial peptide (AMP) genes in circulating haemocytes of juvenile shrimp (2.0±0.5 g; n=30 per tank) was examined at 0, 12, 24, 48, 72, and 96 hours post-infection (hpi). Biofilm production was higher at 32°C than at 27°C. PCR analysis detected the presence of *toxR*, *trh*, *aphA*, *motX*, *flig*, *mcp*, and *puvA*, whereas *tdh*, *tlh*, *luxO*, and *pomA* genes were not detected. Enhanced expression of biofilm-related genes was expressed at levels of *V. parahaemolyticus* was observed at 32°C after 24 and 48 h. The relative expression of AMP genes, such as crustin, penaeidin, anti-lipopolysaccharide factor (ALF), and stylicin, showed significant upregulation ($p<0.05$) at 96 hpi. Notably, this study provides the first eicdence of stylicin expression in the haemocytes of *P. indicus* during *V. parahaemolyticus* infection, underscoring its potential role in the shrimp innate immune response and emphasising the importance of AMPs in host defence against the pathogen.

*Correspondence e-mail:
megha.bedekar@cife.edu.in

Keywords:
Haemocytes, Host defence, Immersion challenge, Innate immunity virulence factors, Pathogenicity

Received : 22.11.2025
Accepted : 02.04.2026

Introduction

Vibrio species are ubiquitous in aquatic environments and include several opportunistic pathogens responsible for vibriosis, one of the most prevalent bacterial disease affecting shrimp larvae and juveniles. The disease poses a major threat to shrimp hatcheries and aquaculture farms (Karunasagar *et al.*, 1994; Kumar *et al.*, 2014). Opportunistic pathogenic *Vibrio* sp. can infect shrimp through multiple routes and include species such as *V. parahaemolyticus, V. alginolyticus, V. harveyi, V. campbelli, V. angullarum, V. vulnificus, V. fluvialis, V. mimicus, V. splendidus, V. owensii,* and *V. orientalis*. Infected shrimp exhibit clinical signs such as abnormal swimming behaviour, empty gut, soft shell, brown gills and body discolouration (Jayasree *et al.*, 2006; de Souza Valente and Wan, 2021). Among the *Vibrio* spp., *V. parahaemolyticus* is of particular concern as certain strains carrying PirA and PirB toxin genes are the causative agents of acute hepatopancreatic necrosis disease (AHPND), that lead to high mortality in cultured shrimp (Tran *et al.*, 2013; Joshi *et al.*, 2014; Han *et al.*, 2015). In addition to PirAB toxins, these strains possess

© 2026 Indian Council of Agricultural Research

several other virulence factors, including haemolysin production and biofilm formation, which play key role in the pathogenesis and survival within the host environment (Han *et al.,* 2016; Li *et al.,* 2019a).

Naturally, all crustaceans, including shrimp, depend on non-specific immune mechanisms for protection against invading pathogens, as they lack an adaptive immune system. Haemocytes are the mediators of these innate immune responses and participate in cellular immune processes such as phagocytosis, encapsulation, nodulation, and activation of the prophenoloxidase cascade system, which leads to melanin synthesis. In contrast, humoral responses include the secretion of clotting proteins, enzyme inhibitors, antioxidant enzymes and antimicrobial peptides (Tassanakajon *et al.,* 2013; Kulkarni *et al.,* 2021; Panigrahi *et al.,* 2022). Antimicrobial peptides (AMPs) play important role in shrimp immunity by exhibiting broad spectrum antimicrobial activity against invading pathogens, such as bacteria, parasites, fungi, and viruses (Seyfi *et al.,* 2020). Beyond their direct microbicidal effects, AMPs contribute to pathogen recognition, immune signalling and modulation of innate immune responses, thereby enhancing host defence, in the absence of adaptive immunity (Tassanakajon *et al*., 2011). AMPs are generally low-molecular-weight proteins (<10 kDa) with diverse structural and biochemical characteristics. They commonly exhibit amphipathic structure with cationic or anionic charges that selectively interact with the negatively charged membranes of microbial pathogens (Essig *et al.,* 2014). Their antimicrobial mechanisms include bacterial membrane disruption, inhibition of protein and cell wall synthesis, interference with enzymatic activities, and DNA replication, as well as antibiofilm activity (Raheem and Straus, 2019; Talapko *et al.,* 2022; Chakrapani *et al.,* 2024).

Several studies have described the structural characteristics of AMPs in penaeid shrimp species, including *P. monodon, P. setiferus, P. japonicus, P. vannamei* and *P. indicus* (Afsal *et al.*, 2016; Saucedo-Vazquez *et al.,* 2022). The classification and molecular characteristics of several AMP families in shrimp, such as crustin, penaeidin, anti-lipopolysaccharide factors (ALFs), stylicin, hemocyanin-derived peptides and lysozymes have been well documented (Tassanakajon *et al.,* 2011; Radhakrishnan *et al.,* 2026). In penaeid shrimps, crustin expression is predominantly observed in immune-associated tissues, including the epithelial layer, stomach, intestine, and particularly haemocytes, especially in response to pathogenic infection (Li *et al.,* 2019b). The expression and distribution of penaeidins have been reported in haemocytes and during the early larval stages of *P. vannamei* (Muñoz *et al.,* 2002, 2003). ALF peptides exhibit lipopolysaccharide binding properties and are capable of inhibiting bacterial endotoxin activation. The first ALFs were identified in haemocytes through transcriptome analysis in *P. vannamei* (Gross *et al.,* 2001) and *P. monodon* (Supungul *et al.,* 2002). ALFs exhibit broad-spectrum antibacterial activity against both Gram positive and negative bacteria (Matos *et al.,* 2018), specific anti-vibrio activity (Tharntada *et al.,* 2008; Zhou *et al.,* 2019), as well as antiviral (Jiang *et al.,* 2022), and antifungal activities (Tassanakajon *et al.,* 2011). In 2010, a novel stycilin peptide was first identified from *Litopenaeus stylirostris* (Rolland *et al.,* 2010). The expression of stylicin peptides (Lvan-Stylicin 1 and 2) has been observed in haemocytes and in the midgut of *P. vannamei* after *Vibrio* infection (Farias *et al.,* 2019). Previous studies have shown that stylicin peptides display potent antimicrobial activities against *V. penaeicidae* (Rolland *et al.,* 2010) and *V. parahaemolyticus* (Chakrapani *et al.,* 2024).

Although Indian white shrimp, *P. indicus* has considerable potential as a candidate species for diversified shrimp aquaculture (Das *et al.,* 2022), studies on antimicrobial peptides (AMPs) in this species remain limited. While numerous investigations have explored the biochemical aspects of immune defence in shrimp, relatively few have examined the gene expression of AMPs in *P. indicus* in response to *V. parahaemolyticus* infection. Therefore, the present study aimed to characterise the virulence factors of *V. parahaemolyticus* and to elucidate the relative expression patterns of AMP genes in the haemocytes of *P. indicus* following an immersion challenge.

Materials and methods

Isolation of *Vibrio parahaemolyticus* strain

Pathogenic *V. parahaemolyticus* was isolated from the infected mysis larvae stage of *P. indicus* at MES shrimp hatchery, ICAR-Central Institute of Brackishwater Aquaculture. The tissue homogenate was serially diluted and cultured on Thiosulphate Citrate Bile Salt agar (TCBS) (Himedia, India) plates, and maintained at 32°C under aseptic conditions. Large blue-green colonies observed were purified on Zobell Marine agar (Himedia, India) plate, and further molecular identification was performed to confirm the species identity.

Molecular characterisation and phylogenetic analysis

Molecular identification of *V. parahaemolyticus* isolate was confirmed by 16S rRNA gene sequencing employing earlier reported primers (Kumar *et al.,* 2021). Bacterial genomic DNA was isolated using a HiPura Bacterial genomic DNA purification kit (Himedia, India). Further a PCR reaction was performed (95°C, 3 min; 95°C, 30 s; 60°C, 30 s; 72°C, 60 s; 72°C, 10 min; 30 cycles) and the amplified PCR product was purified using a GeneJET PCR product purification kit (Thermo Fisher Scientific, USA), and sequenced at Symbiont Life Science, India. The forward and reverse sequences were assembled in BioEdit-version: 5.0.9 (Hall, 1999). Homology search and similarity findings were performed using NCBI-BLASTN (http://www.ncbi.nih.gov/BLAST). The 16S rRNA gene sequences were aligned with those of closely related *Vibrio* spp. Phylogenetic tree was constructed using maximum likelihood algorithms with a bootstrap value of 1000 in MEGA 11 software-version 11.0.13 (Tamura *et al.,* 2021).

Virulence gene identification

Molecular characterisation of *V. parahaemolyticus* using species-specific virulence gene, Vp-toxR (368 bp) was confirmed by PCR amplification (Kim *et al.,*1999). Further, presence of haemolysin-related virulence genes such as tdh, trh, and tlh as well as biofilm-related genes aphA and Mox-t were confirmed (Raja *et al.,* 2017; Chakrapani *et al.,* 2024). The details of primers used are presented in Table 1. Bacterial genomic DNA amplification was performed in 13 µl of PCR reaction mixture containing 6.5 µl of master mix [containing Taq 2x master mix and 1.5 mM $MgCl_2$ (AMPLICON (Denmark)], 0.5 µl (10 pmol $µl^{-1}$) each of forward and reverse primers, 0.5 µl of bacterial DNA and 5 µl of nuclease-free water. The amplified DNA fragments were resolved by

Table1. List of primers of virulence and biofilm-related genes

Primer name	Sequence 5'-3'	Annealing temperature	Amplicon size (bp)	Reference
V.para toxR	toxRF-GTCTTCTGACGCAATCGTTG toxRR-ATACGAGTGGTTGCTGTCATG	60°C /30 s	368	Kim *et al*. (1999)
V.para Tlh	tlhF-AAAGCGGATTATGCAGAAGCACTG tlhR-GCTACTTTCTAGCATTTTCTCTGCG	60°C/30 s	450	Bej *et al*. (1999)
V.para Tdh	tdhF-GTAAAGGTCTCTGACTTTTGGAC tdhR-TGGAATAGAACCTTCATCTTCACC	60°C/30 s	269	Bej *et al*. (1999) Raja *et al*. (2017)
V.para Trh	trhF-TTGGCTTCGATATTTTCAGTATCT trhR-CATAACAAACATATGCCCATTTCCG	60°C/30 s	500	Bej *et al*. (1999) Raja *et al*. (2017)
aphA	aphAF- ACACCCAACCGTTCGTGATG aphAR- GTTGAAGGCGTTGCGTAGTAAG	58°C/30 s	200	Chakrapani *et al*. (2024)
Mot-X	Motx F-ATGTGTCGAGCAAGATGTCG MotX R-TACGCGCGTTTAAATTACCC	58°C/30 s	210	Chakrapani *et al*. (2024)
FliG	F-GGTTCAGCGTGTTGGTAGTGC R-GCGTGGGTCCATCCATTT C	60°C/30 s	250	Xiu *et al*. (2017)
LuxS	F-GGATTTTGTTCTGGCTTTCCACTT R-GGGATGTCGCACTGGTTTTTAC	58°C/30 s	300	Guo *et al*. (2019)
Mcp	F-GGTTGAGGGCTCTTCTGTGGTC R-TGTACGAACTTCATCAGCAACGAC	60°C/30 s	99	Ji *et al*. (2011)
OpaR	F-TGTCTACCAACCGCACTAACC R-GCTCTTTCAACTCGGCTTCAC	60°C/30 s	177	Lu *et al*. (2021)
PomA	F-CGGATGAACCCGAAGACCT R-ATCGCAACAAGACCAACCAA	58°C/30 s	75	Sato *et al*. (2000)
pvuA (Reference gene)	F-CAAACTCACTCAGACTCCA R-CGAACCGATTCAACACG	60°C/30 s	200	Ma *et al*. (2015)

toxR - Transcriptional activator; tlh - Thermolabile haemolysin; tdh - Thermostable direct haemolysin; Trh - Thermostable direct haemolysin related haemolysin; aphA - Acid phosphatase; MotX- Na+ driven flagellar motor protein; Flig - Flagellar motor switch protein; LuxS - Quorum sensing regulator; mcp - Methyl-accepting chemotaxis protein; OpaR - DNA binding transcriptional regulator; PomA - Polar flagellar motor; pvuA - TonB-dependent siderophore vibrioferrin receptor

agarose gel electrophoresis, stained with ethidium bromide (0.5 µg ml^{-1}), and visualised under a UV transilluminator (BioRad, USA).

Biofilm formation assay

Crystal violet staining assays were employed to quantify biofilm formation, as previously described (Chakrapani *et al.,* 2024), with some modifications. Briefly, *V. parahaemolyticus* was cultured for 4-5 h and adjusted to an OD_{600} = 0.2 in sterile ZMA broth. In a 24 well microtitre plate, 0.2 ml of bacterial culture was added in each well, and the total volume was adjusted to 2 ml with ZMB broth. Wells without bacterial inoculum were taken as a control. The plates were then incubated at 27°C and 32°C for 24-48 h. The contents were discarded, and the wells were washed with phosphate-buffered saline (PBS, pH 7.4), followed by fixation with 99% methanol for 15 min. Subsequently, the samples were stained with 1.5 ml of 0.1% (w/v) crystal violet and rinsed with deionised water. Then, 1.5 ml of glacial acetic acid was added, and plates were read at 570 nm using an ELISA Reader (Biotek Instruments Inc USA, Version- Gen5: 3.05.11).

Biofilm-related gene expression analysis

In brief, total RNA was isolated from *V. parahaemolyticus* grown at 32°C for 24 h and 48 h. Subsequently, RNA was converted into cDNA using the iScript cDNA Synthesis Kit (BioRad, Applied Biosystem's, USA). Real-time amplification of biofilm-related gene expression was performed on a thermal cycler (Applied Biosystem's Real-Time PCR system Step One Plus®) using SYBR Green dye. Briefly, the cycling parameters included an initial holding stage of 10 min at 95°C, 45 cycles of denaturation at 95°C for 00.15 s and isothermal annealing and extension at 60°C for 1 min. At the end of each cycle, melt curve analysis (60-95°C) was carried out to ensure the specificity of the primer. A 20 µl PCR reaction was prepared with each tube containing 10 µl of 2X SYBR® Green qPCR master mix (Bio-Rad, USA), 1 µl of each forward and reverse primers (10 pmol), 1 µl of template DNA (30–60 ng) and 7 µl of nucleus-free water. All the samples were analysed in triplicate, and the relative expression was calculated by the comparative threshold value (CT) and $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Bacterial challenge study

For the experimental challenge, healthy juvenile *P. indicus* were transferred to 500 l circular FRP tank and acclimated for 2 weeks under laboratory conditions. Immersion challenge was conducted in triplicate with 20 shrimps per tank, following the methods described by Saulnier *et al.* (2000). To confirm the susceptibility of *P. indicus* to *V. parahaemolyticus,* challenge study was conducted in healthy juvenile *P. indicus* (2-3 g) by immersing shrimps for 2 h in seawater containing *V. parahaemolyticus* at a concentration of $1x10^8$ cfu ml^{-1}). The challenged shrimps were transferred to 60 l tanks containing filtered seawater and maintained under standard culture conditions. Unchallenged shrimp were maintained in separate 60 l tanks as control groups. At each sampling point, six shrimps per group were randomly collected for subsequent analysis.

© 2026 Indian Council of Agricultural Research

Haemolymph collection

Haemolymph was collected from the rostral and ventral sinuses of shrimp as per de Lorgeril *et al.* (2008), using a sterile 1 ml syringe (25 gauge) containing 300 µl of chilled anticoagulant solution (RNase-free 10% sodium citrate, pH 7.0). Haemolymph was collected from the control group shrimps prior to experimental infection, and designated as the uninfected control group (0 h). In the challenged groups, haemolymph was collected at 12, 24, 48, 72, and 96 hpi and the haemolymph samples were centrifuged at 800 *g* for 15 min at 4°C. The pellets of haemocytes were collected, and 500 µl of TRI reagent (Sigma, USA) was added for RNA extraction. The samples were then stored at -80°C until further analysis.

Total RNA isolation and reverse transcription

Total RNA was extracted from haemocytes of both infected and uninfected group, following the manufacturer's instructions (TRI Reagent (Sigma, USA). Total RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm using Nanodrop 2000C spectrophotometer (Thermoscientific, USA). The total RNA samples with absorbance ratios (A260:A280) greater than 1.8 were selected for subsequent reverse transcription step. First-strand cDNA was generated in a 20 µl reaction volume containing 5 µg total RNA, 5x iScript Reaction Mix (4 µl), iScript reverse transcriptase (1 µl) and nuclease-free water (10 µl). The reverse transcription protocol comprised the first step of priming at 25°C for 5 min, the reverse transcription (cDNA synthesis) step at 46°C for 20 min, the RT enzyme inactivation step at 95°C for 1 min, and a final hold at 4°C following the Bio-Rad iScript cDNA synthesis kit protocol.

Quantitative RT-PCR analysis of antimicrobial peptide genes expression

The relative expression levels of antimicrobial peptides (AMPs) genes *viz.,* crustin, penaeidin, anti-lipopolysaccharides factor (ALF) and stylicin, were quantified in the haemocytes samples using quantitative real-time PCR (qRT-PCR). Gene-specific oligonucleotide primers were designed using NCBI Primer Blast tool, and beta-actin was used as the reference gene for normalization and as an internal control (Panigrahi *et al.,* 2021). The sequences of all primers used in this study are provided in Table 2. Real-time amplification was performed on an Applied Biosystems' Step One Plus Real-Time PCR system using SYBR Green chemistry. The expression of each target AMP gene was analysed using its respective gene-specific primer pair, including thiose of crustin, penaeidin, ALF and stylicin.

Briefly, quantitative real-time PCR (qRT-PCR) reactions were performed in triplicate using the following thermal cycling conditions: an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Each 20 µl reaction mixture contained 10 µl of 2× SYBR® Green qPCR Master Mix (Bio-Rad, USA), 1 µl each of forward and reverse primers (10 pmol), 1 µl of template DNA (30–60 ng), and 7 µl of nuclease-free PCR-grade water. The relative expression levels of the target genes were calculated using the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). To see the significance of the expression of the gene at different time points. The $2^{-\Delta\Delta CT}$ method was used to determine the relative quantification of AMP gene expression and to compare expression levels across the different post-infection time points.

Statistical analysis

All data were statistically analyzed using IBM SPSS Statistics version 26.0. Significant differences between samples were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for *post hoc* comparisons. Data are presented as mean±standard error (SE). Differences were considered statistically significant at $p<0.05$. Graphs were generated using GraphPad Prism (Version 8.4.2).

Results

Molecular identification and phylogenetic analysis

The partial 16S rRNA gene sequence analysis confirmed the isolate as *V. parahaemolyticus* and rhe and the sequence was deposited in GenBank under the Accession No. PQ821090. The isolate showed 98.24% sequence similarity with *V. parahaemolyticus* strain vp-201911 (GenBank Accession No. CP150862). A phylogenetic tree was constructed based on partial 16S rRNA gene sequences using the maximum likelihood method. The analysis included the obtained *V. parahaemolyticus* sequence (PQ821090) along with closely related species such as *V. harveyi*, *V. campbellii*, and *V. alginolyticus*, with *Pseudomonas aeruginosa* used as the outgroup (Fig. 1).

Virulence gene identification

The virulence genes, including *toxR*, haemolysin-related genes, and biofilm-associated genes, of *V. parahaemolyticus* were characterised.

Table 2. Primers used for quantitative RT-PCR analysis of antimicrobial peptide genes expression

Target	Gene	Sequence 5'-3'	Size (bp)	Reference/ NCBI accession no.
Housekeeping gene	β-actin	F-CAACCGCGAGAAGATGACAC R-TCGGTCAGGATCTTCATCAGG	243	Panigrahi *et al*. (2021)
Antimicrobial peptides genes	Crustin	F- CAGGCATCAGGAACAGACCC R- TTCAAGCAGGTGTCGTAGCA	85	Antony *et al*. (2010) (GQ469987.1)
	Penaeidin	F- CTGGCACTCGACCATTCAGT R- TCGTTGTCTTCTCCATCGGC	146	PP981516
	ALF	F- GGCTGTGGAGGAACGAGAAA R- GGCTGTGGAGGAACGAGAAA	244	PP982467
	Stylicin	F- TCATGTGAATGCGGCTACCA R- AACTCGGCGATGGGACTTTC	77	PQ114162

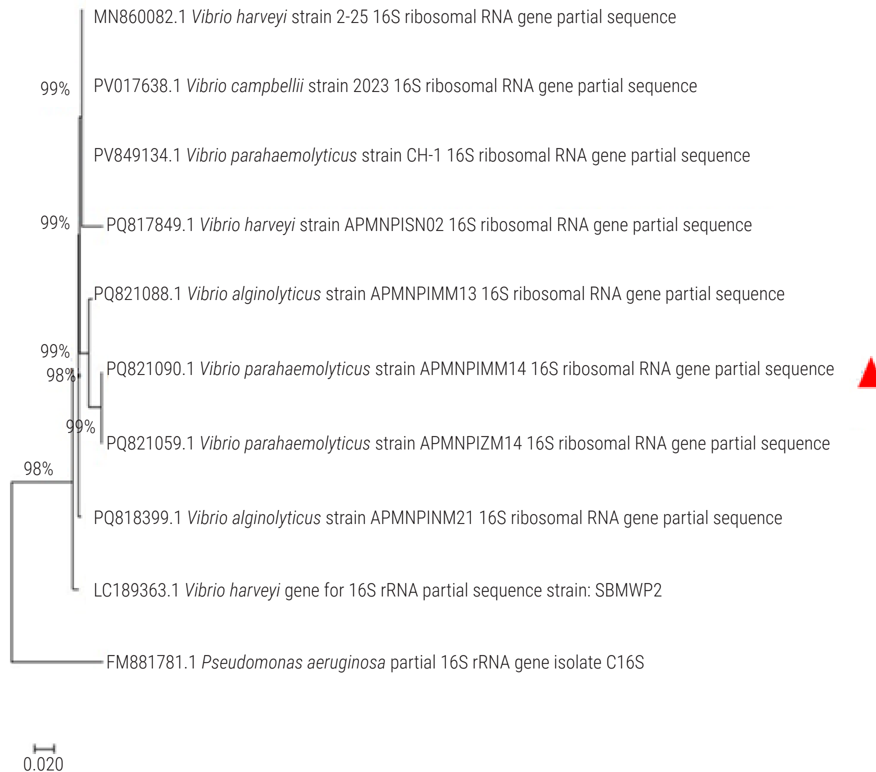

Fig. 1. Phylogenetic analysis of *V. parahaemolyticus* based on partial 16S rRNA sequences (PQ821090). The phylogenetic tree was constructed using the maximum likelihood method with with 1000 bootstrap replicates to assess branch support. *Pseudomonas aeruginosa* was included as an outgroup

PCR amplification showed positive results for *toxR*, *trh*, *aphA*, *moxT*, *fliG*, *mcp*, *opaR*, and *pvuA*, while negative results were observed for *tdh*, *tlh*, *luxO*, and *pomA* (Fig. 2).

Biofilm quantification and gene expression analysis

Biofilm formation by *V. parahaemolyticus* was evaluated at 27°C and 32°C over incubation periods of 24 and 48 h. The highest biofilm formation (71.8%) was observed at 32°C after 48 h, followed by 68.6% at 27°C for the same duration, with differences being statistically significant ($p<0.05$). At 24 h, biofilm formation was 66.1% and 60.5% at 27°C and 32°C, respectively. Overall, the results indicated significantly enhanced biofilm formation at 32°C after 48 h of incubation ($p<0.05$) (Fig. 3).

The relative expression levels of biofilm-related genes (*toxR, aphA, motX, fliG, opaR, pomA,* and *mcp*) were analysed at 24 and 48 h. At 48 h and 32°C, *toxR* (6.8-fold) and *aphA* (7.6-fold) were significantly upregulated ($p<0.05$), followed by *motX* (1.09-fold), *pomA* (1.88-fold), and *mcp* (1.99-fold). In contrast, lower expression levels were observed

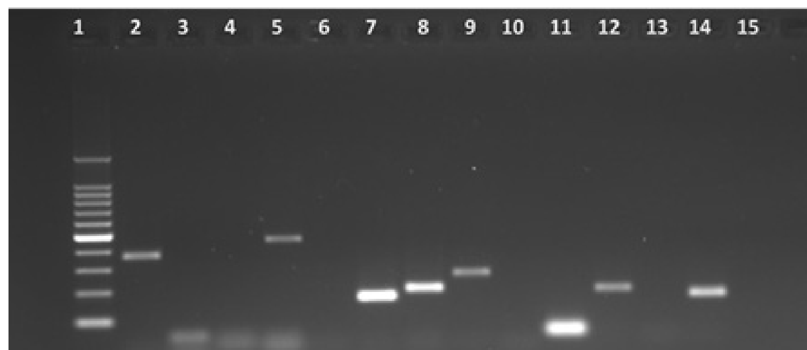

Fig. 2. Agarose gel electrophoresis showing PCR amplification of virulence genes from the genomic DNA of *V. parahaemolyticus* (Genbank No: PP883791) isolate. (Lane 1:100bp plus DNA ladder; Lane 2: tox-R (+); Lane 3: tdh (-); Lane 4: tlh (-); Lane 5: trh (+); Lane 6: Negative control; Lane 7: aphA (+); Lane 8: Mot-X (+); Lane 9: Flig (+); Lane10: Luxo (-); Lane 11: mcp (+); Lane 12: OpaR (+); Lane 13: Poma (-);Lane 14-puva (+); Lane 15: Non-template control (NTC)

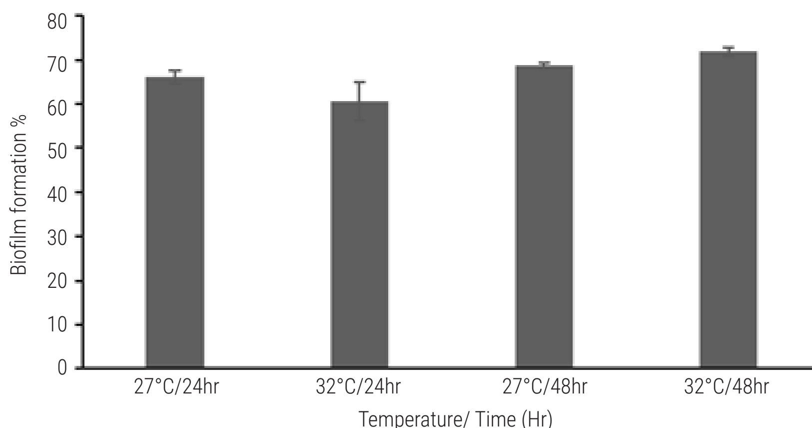

Fig. 3. *In vitro* biofilm formation by *V. parahaemolyticus* isolate at different temperatures and incubation periods

for *fliG* (0.77-fold) and *opaR* (0.67-fold). However, at 24 h, *fliG* (1.17-fold) and *opaR* (1.2-fold) showed slight upregulation, indicating time-dependent changes in gene expression ($p<0.05$) (Fig. 4).

Cumulative mortality and clinical signs of P. indicus during V. parahaemolyticus challenge

The cumulative mortality of juvenile *P. indicus* was recorded following an immersion challenge with *V. parahaemolyticus* (1×10^8 CFU ml^{-1}). Mortality increased progressively in the challenged group, reaching 23%, 45%, 65%, and 73% at 24, 48, 72, and up to 96 h hpi, respectively. Mortality was observed from 12 to 96 hpi.

During the experimental challenge, shrimps infected shrimps exhibited distinct external pathological signs characteristic of bacterial infection. Affected individuals showed body discolouration and necrotic lesions around the rostrum and the bases of antennae, along with melanised dark patches on the cephalothorax and abdominal segments. These clinical signs are indicative of cuticular damage, possible bacterial colonisation, and tissue necrosis (Fig. 5 and Fig. 6).

Expression profile of AMPs-related genes by qRT-PCR

The relative expression levels of AMP genes, including crustin, penaeidin, ALF and stylicin, were analysed using quantitative real-time PCR (qRT-PCR) to elucidate their transcriptional responses during *V. parahaemolyticus* infection. A time-course analysis was conducted to assess the relative expression levels of each AMP gene in shrimp following immersion challenge with *V. parahaemolyticus*, with unchallenged shrimp serving as the control.

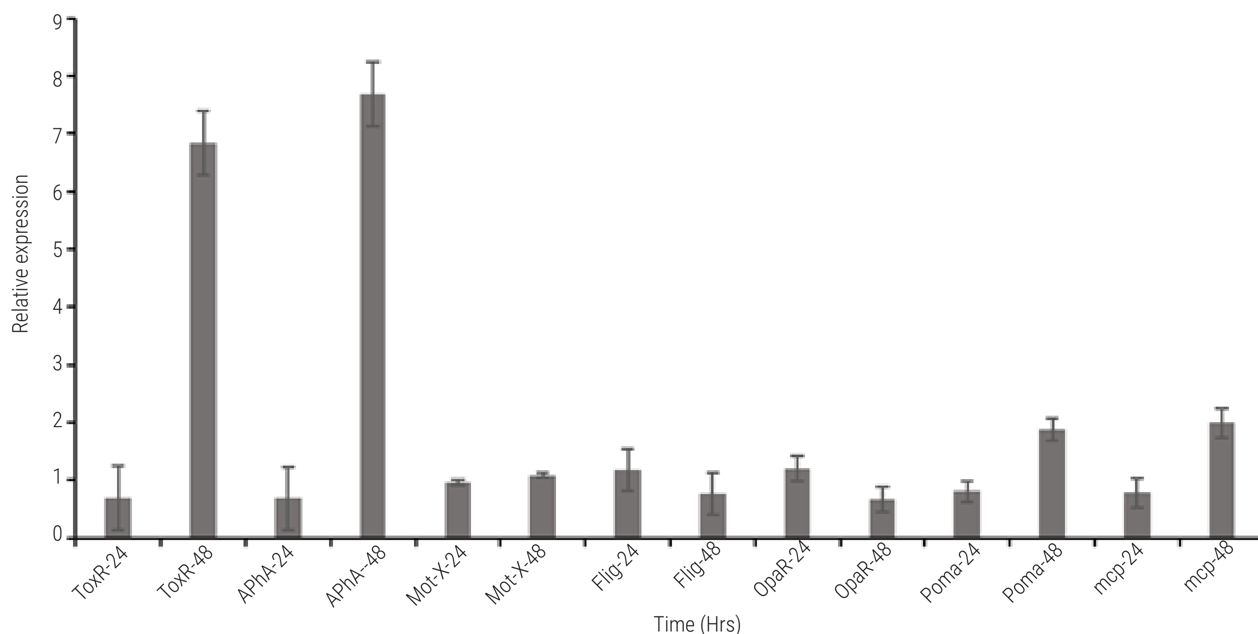

Fig. 4. Relative expression level of biofilm-related genes of *V. parahaemolyticus* at different time points

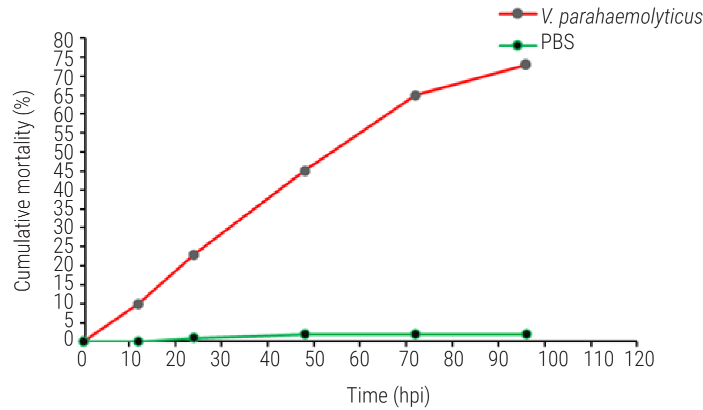

Fig. 5. Cumulative mortality of shrimps in each group following *V. parahaemolyticus* immersion challenge

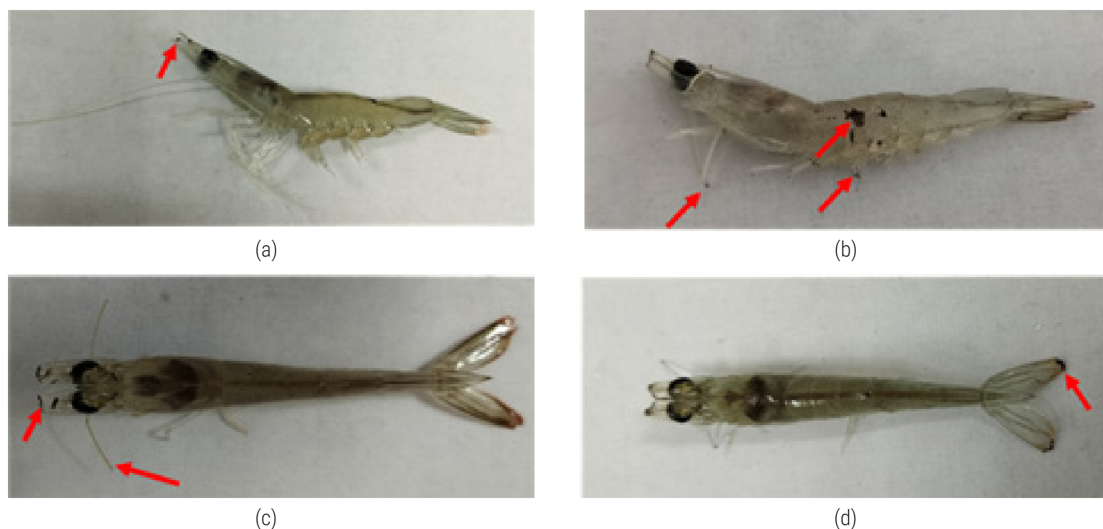

Fig. 6. The clinical signs observed in *P.indicus* following experimental infection with *V. parahaemolyticus*. Infected shrimps exhibited necrosis of antennules, antennular scale, antenna cut, uropods, and exoskeleton (a, b, c, d)

As shown in Fig. 7, transcripts of crustin, penaeidin, ALF and stylicin were detected in haemocytes of *P. indicus* post-infection, indicating their involvement in the immune response against *V. parahaemolyticus*. Crustin transcript levels initially decreased up to 48 hpi, but subsequently increased, reaching 1.5 fold at 72 hpi and 2 fold at 96 hpi ($p<0.05$). Similarly, penaeidin expression decreased up to 48 hpi, followed by a significant increase to 3.85 fold at 72 hpi and 1.75 fold at 96 hpi ($p<0.05$).

The expression of ALF was downregulated at 12, 24, and 72 hpi, but showed a significant upregulation at 96 hpi (3.95-fold; $p<0.05$). In contrast, stylicin exhibited downregulation up to 72 hpi, followed by the highest expression at 96 hpi ($p<0.05$). Among the four AMPs analysed in the haemocytes of *P. indicus*, stylicin showed the highest expression at 96 hpi compared to crustin, penaeidin, and ALF following *V. parahaemolyticus* challenge.

Discussion

Previous investigations have reported the presence of *V. parahaemolyticus* in shrimp aquaculture regions along the Indian coastline (Kumar *et al.*, 2014; Navaneeth *et al.*, 2020). Moreover, pathobiological studies have demonstrated that *V. parahaemolyticus* significantly affects the immune response of haemocytes in penaeid shrimp (Raja *et al.*, 2017). This bacterium possesses three major haemolysin genes: thermostable direct haemolysin (*tdh*), *tdh*-related haemolysin (*trh*), and thermolabile haemolysin (*tlh*). Among these, *tdh* and *trh* are considered the primary determinants of pathogenicity (Raja *et al.*, 2017; Li *et al.*, 2019a). Molecular characterisation of *V. parahaemolyticus* through the amplification of species-specific genes such as *toxR*, *tdh*, *tlh*, and *trh* facilitates the identification of virulence-associated factors (Bej *et al.*, 1999; Kim *et al.*, 1999; Zulkifli *et al.*, 2009).

Bacterial biofilms play a crucial role in survival within host environments (Han *et al.*, 2016). *Vibrio* spp. exhibit two types of flagellar motility systems *viz*., polar and lateral flagella enabling movement in liquid environments (swimming) and across surfaces (swarming on viscous media) (Stewart *et al.*, 2003). The *motX* and *motY* genes are essential for sodium-driven polar flagellar motility (Thompson *et al.*, 2006). Biofilm formation and exopolysaccharide (EPS) matrix production are regulated by several transcriptional regulators, including *aphA, motX, fliG, opaR, mcp,* and *toxR* in *V. parahaemolyticus* (Zhang *et al.*, 2012; Wang *et al.*, 2013; Lu *et al.*, 2021; Chakrapani *et al.*, 2024).

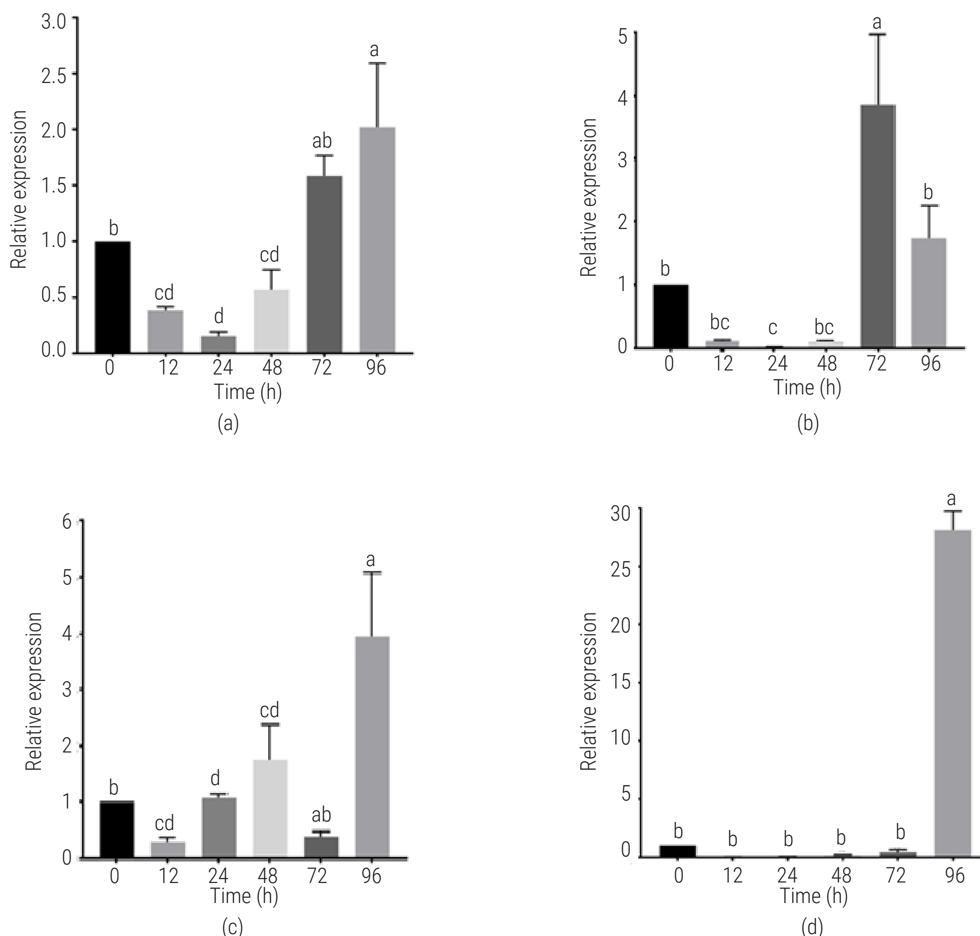

Fig. 7. Relative expression of AMP genes (a) Crustin, (b) Penaeidin, (c) Anti-lipolysaccharide factor (ALF) and (d) Stylicin genes in haemocytes of *P. indicus* following *V. parahaemolyticus* challenge at 0, 12, 24, 48, 72, and 96 hpi. Non-infected shrimp group (0 h) served as the control

Earlier studies have reported that pathogenic *V. parahaemolyticus* isolates from shrimp farms along the Indian coast were positive for *toxR, tdh,* and *trh* virulence genes, while some isolates also carried the *tlh* gene (Silvester *et al.,* 2015; Paria *et al.,* 2019). In contrast, another study reported the absence of *tdh* and *trh* genes in isolates obtained from shrimp, water, and sediment samples (Ayyappan *et al.,* 2018). However, a high prevalence of *tdh*-positive *V. parahaemolyticus* strains has also been observed in shrimp farming environments (Narayanan *et al.,* 2020), indicating variability in virulence profiles. The *aphA* gene encodes a key quorum-sensing regulator that functions under low cell density conditions (Sun *et al.,* 2012) and plays a critical role in regulating swimming and swarming motility, biofilm formation, and virulence in *V. parahaemolyticus* (Wang *et al.,* 2013). A biofilm is a structured microbial community embedded in an EPS matrix and adherent to biotic or abiotic surfaces (Wang *et al.,* 2013). Previous studies have shown that *V. parahaemolyticus* exhibits strong biofilm-forming capacity at temperatures ranging from 25°C to 37°C (Ahmed *et al.,* 2018). Additionally, biofilm development has been reported to increase across a temperature range of 15 to 37°C under varying environmental conditions (Han *et al.,* 2016), with gradual enhancement observed at 25°C compared to 15°C and 37°C (Song *et al.,* 2017). In the present study, biofilm formation by *V. parahaemolyticus* was higher at 32°C compared to 27°C. These findings are consistent with previous reports indicating that environmental temperature and stress conditions significantly influence bacterial growth and biofilm-forming ability (Mizan *et al.,* 2016).

The present study investigates the expression profiles of antimicrobial peptide (AMP) genes, including crustin, penaeidin, ALF, and stylicin, in the haemocytes of *P. indicus* following *V. parahaemolyticus* challenge *via* an immersion method that mimics natural infection routes. Shrimp haemocytes play a crucial role in immune responses against pathogenic infections. Following bacterial challenge, changes in AMP gene expression have been reported in circulating haemocytes as well as in haematopoietic tissues (Soonthornchai *et al.,* 2010). In general, AMPs exert antibacterial activity through multiple mechanisms, including disruption of bacterial membrane integrity, interaction with lipopolysaccharides, promotion of bacterial agglutination, and inhibition of bacterial proliferation (Tassanakajon *et al.,* 2011; An *et al.,* 2016; Benfield and Henriques, 2020; Mookherjee *et al.,* 2020).

Crustin is a cationic antimicrobial peptide containing a functional whey acidic protein (WAP) domain at the C-terminal region, which confers both antibacterial and antiprotease properties, primarily through disruption of bacterial cell membranes. Crustin has been identified in the haemocytes of *Penaeus indicus* (Antony *et al.,* 2010;

Sruthy *et al.,* 2017). A recombinant crustin peptide (rFi-crustin2) has demonstrated antibacterial activity against *Vibrio parahaemolyticus, V. alginolyticus, V. vulnificus, V. proteolyticus,* and *V. cholerae* (Sruthy *et al.,* 2017). In *P. vannamei*, crustin expression in haemocytes decreased at 12 h post-infection following *Vibrio* challenge (Vargas-Albores *et al.,* 2004). Conversely, the crustin transcript (*LvCrustinB*) in haemocytes of *P. vannamei* showed upregulation at 24 h post-infection in response to *V. parahaemolyticus* infection (Li *et al.,* 2019b). Similarly, previous studies have reported upregulation of crustin gene expression in the haemocytes of *Metapenaeus japonicus* at 12 and 24 h post-infection with *V. alginolyticus* (Sun *et al.,* 2017). In the present study, a significant increase in crustin gene expression was observed in the haemocytes of *P. indicus* at 72 h post-infection following *V. parahaemolyticus* challenge.

Penaeidins are antimicrobial peptides with strong antibacterial activity and are predominantly distributed in shrimp haemocytes (Asfal *et al.,* 2016). A penaeidin member, LvBigPEN, identified in *P. vannamei*, plays a crucial role in inhibiting *V. parahaemolyticus* infection (Xiao *et al.,* 2021). Structurally, penaeidins contain several cationic amino acid residues that facilitate binding to negatively charged bacterial cell membranes and interaction with bacterial DNA (Xiao *et al.,* 2021). Previous studies have reported decreased mRNA expression levels of PEN2, PEN3, PEN4, and crustin in the haemocytes of *P. vannamei* following lipopolysaccharide (LPS) injection (Okumura *et al.,* 2007). Specifically, LPS exposure significantly reduced the expression of PEN2 and PEN3 at 4 and 24 h hpi, and PEN4 and crustin at 4 hpi; these levels subsequently returned to baseline at 72 hpi for PEN2 and PEN3, and at 24 hpi for PEN4 and crustin (Okumura *et al.,* 2007). Similarly, *Vibrio* exposure has been shown to reduce penaeidin expression within 1-6 h post-injection, followed by recovery to baseline levels at 24-48 h and a subsequent threefold increase at 72 hpi (Muñoz *et al.,* 2003). In the present study, juvenile *P. indicus* challenged with *V. parahaemolyticus* exhibited a significant upregulation of penaeidin transcripts in haemocytes at 72 hpi. This pattern is consistent with earlier findings indicating an initial suppression followed by a delayed induction of AMP expression as part of the shrimp innate immune response (Munoz *et al.,* 2003; Vargas-Albores *et al.,* 2004).

ALFs represent another important group of AMPs, characterised by variable amino acid lengths and sequences that confer diverse biological functions. Many ALFs possess predominantly cationic properties, enabling them to bind to bacterial cell walls and neutralise bacterial toxins (Tassanakajon *et al.,* 2011; Rosa *et al.,* 2013; Zhou et al., 2019). Recombinant ALF peptides have demonstrated antibacterial activity by interacting with bacterial membranes, leading to cytoplasmic leakage and effective inhibition of pathogens such as *Vibrio anguillarum* (Jiang *et al*., 2015) and *V. parahaemolyticus* (Zhang *et al.,* 2020). In penaeid shrimps, ALFs are predominantly expressed in haemocytes (Ponprateep *et al.,* 2012; Jiang *et al.,* 2015; Zhou *et al.,* 2019). For instance, ALF-D expression in the haemocytes of *P. stylirostris* was significantly upregulated between 12 and 24 h following *V. penaeicida* infection (de Lorgeril *et al.,* 2008). However, some studies have reported relatively lower expression levels of mjALF-D in haemocytes compared to other tissues (Jiang *et al.,* 2015), indicating tissue-specific variation in ALF expression. In this study, ALF expression was significantly upregulated in the haemocytes of *P. indicus* at 96 h hpi in response to *V. parahaemolyticus*. Stylicins represent a novel class of antimicrobial peptides (AMPs) first identified in the penaeid shrimp *Litopenaeus stylirostris*. A representative peptide, Ls-stylicin, is characterised by a proline-rich region at the N-terminal and a cysteine-rich region at the C-terminal (Rolland *et al.,* 2010). Recombinant Ls-stylicin-1 has been reported to exhibit relatively low antibacterial activity and limited LPS-binding agglutination activity against *V. penaeicida* (Rolland *et al.,* 2010). Previous studies have also reported the expression of the *Mj-Sty* gene in the gills of kuruma shrimp (*Marsupenaeus japonicus*), with levels comparable to those observed in haemocytes (Liu *et al.,* 2015). This suggests that, under normal physiological conditions, stylicin plays an important role in gill tissues, which serve as the first line of defence against pathogens, in addition to its function in haemocytes. In *P. vannamei*, elevated expression of stylicin (*Lv-stylicin-2*) has been reported in circulating haemocytes in response to *Vibrio* spp. infection (Farias *et al.,* 2019). In contrast, the present study observed a relatively lower expression level of stylicin transcripts in the haemocytes of *P. indicus* following *V. parahaemolyticus* challenge. However, there is currently limited evidence regarding stylicin expression in the haemocytes of *P. indicus*, highlighting the need for further investigation.

In the present study, *V. parahaemolyticus* exhibited notable virulence characteristics along with a strong biofilm-forming ability, which may facilitate immune evasion and enhance pathogenicity in the host. The expression profiles of AMP genes, including crustin, penaeidin, ALF, and stylicin, were analysed in the haemocytes of *P. indicus*. The results demonstrated that AMP expression varied across different time points following *V. parahaemolyticus* challenge, indicating a dynamic and time-dependent immune response. These findings provide valuable insights into the host–pathogen interaction and highlight the potential of AMPs as biomarkers for health assessment in shrimp aquaculture. Furthermore, future studies focusing on the molecular mechanisms underlying AMP-mediated defence during bacterial infection may contribute to the development of effective therapeutic and disease management strategies in shrimp farming.

Acknowledgements

The authors are grateful to the Director, ICAR-CIFE, Mumbai and ICAR-CIBA, Chennai for the scientific research support for the work. This research was funded by the PMMSY-GIPPI SD (4001/543) project, ICAR-CIBA. The authors also thank ICAR-CIFE-AEHMD, ICAR-CIBA-CCD and AAH&ED for providing the Lab facilities.

References

Afsal, V. V., Antony, S. P., Philip, R. and Bright Singh, I. S. 2016. Molecular characterization of a newly identified subfamily member of penaeidin from two penaeid shrimps, *Fenneropenaeus indicus* and *Metapenaeus monoceros*. *Probiotics Antimicrob Proteins*, 8(1): 46-52. https://doi.org/10.1007/s12602-015-9203-9.

Ahmed, H. A., El Bayomi, R. M., Hussein, M. A., Khedr, M. H., Remela, E. M. A. and El-Ashram, A. M. 2018. Molecular characterization, antibiotic resistance pattern and biofilm formation of *Vibrio parahaemolyticus* and *V. cholerae* isolated from crustaceans and humans. *Int. J. Food Microbiol.*, 274: 31-37. https://doi.org/ 10.1016/j.ijfoodmicro.2018.03.013.

An, M. Y., Gao, J., Zhao, X. F. and Wang, J. X. 2016. A new subfamily of penaeidin with an additional serine-rich region from kuruma shrimp (*Marsupenaeus japonicus*) contributes to antimicrobial and phagocytic activities. *Dev. Comp. Immunol.*, 59: 186-198. https://doi.org/10.1016/j.dci.2016.02.001.

© 2026 Indian Council of Agricultural Research

Antony, S. P., Bright Singh, I. S. and Philip, R. 2010. Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian white shrimp, *Fenneropenaeus indicus*. *Fish Shellfish Immunol.*, 28(1): 216. https://doi.org/ 10.1016/j.fsi.2009.10.013.

Ayyappan, M. V., Balange, A. K., Nayak, B. B. and Kumar, S. 2018. Distribution of potentially pathogenic *Vibrio parahaemolyticus* in seafood and the aquatic environment of Mumbai, India. *Fish Technol*, 55(3): 205-211.

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C., Jones, D. D. and Kaysner, C. A. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tl, tdh and trh. *J. Microbiol. Methods*, 36(3): 215-225. https://doi.org/10.1016/ S0167-7012(99)00037-8.

Benfield, A. H. and Henriques, S. T. 2020. Mode-of-action of antimicrobial peptides: Membrane disruption *vs.* intracellular mechanisms. *Front. Med. Technol.*, 2: 610997. https://doi.org/10.3389/fmedt.2020.610997.

Chakrapani, S., Panigrahi, A., Palanichamy, E., Thangaraj, S. K., Radhakrishnan, N., Panigrahi, P. and Nagarathnam, R. 2024. Evaluation of therapeutic efficiency of stylicin against *Vibrio parahaemolyticus* infection in shrimp *Penaeus vannamei* through comparative proteomic approach. *Probiotics Antimicrob Proteins*, 16(1): 76-92. https://doi.org/ 10.1007/s12602-022-10006-w.

Das, R. R., Sarkar, S., Saranya, C., Esakkiraj, P., Aravind, R., Saraswathy, R., Rekha, P. N., Muralidhar, M. and Panigrahi, A. 2022. Co-culture of Indian white shrimp, *Penaeus indicus* and seaweed, *Gracilaria tenuistipitata* in amended biofloc and recirculating aquaculture system (RAS). *Aquaculture*, 548(1): 737432. https://doi.org/ 10.1016/j.aquaculture.2021.737432.

De Lorgeril, J., Gueguen, Y., Goarant, C., Goyard, E., Mugnier, C., Fievet, J., Piquemal, D. and Bachère, E. 2008. A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a vibrio infection. *Mol. Immunol.*, 45(12): 3438-3445. https://doi.org/10.1016/j.molimm.2008.04.002.

De Souza Valente, C. and Wan, A. H. 2021. Vibrio and major commercially important vibriosis diseases in decapod crustaceans. *J. Invertebr. Pathol.*, 181: 107527. https://doi.org/ 10.1016/j.jip.2020.107527.

Essig, A., Hofmann, D., Münch, D., Gayathri, S., Künzler, M., Kallio, P. T., Sahl, H. G., Wider, G., Schneider, T. and Aebi, M. 2014. Copsin, a novel peptide-based fungal antibiotic interfering with the peptidoglycan synthesis. *J. Biol. Chem.*, 289(50): 34953-34964. https://doi.org/10.1074/jbc.M114.599878.

Farias, N. D., Falchetti, M., Matos, G. M., Schmitt, P., Barreto, C., Argenta, N., Rolland, J. L., Bachère, E., Perazzolo, L. M. and Rosa, R. D. 2019. *Litopenaeus vannamei* stylicins are constitutively produced by hemocytes and intestinal cells and are differentially modulated upon infections. *Fish Shellfish Immunol.*, 86: 82-92.

Gross, P. S., Bartlett, T. C., Browdy, C. L., Chapman, R. W. and Warr, G. W. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Dev. Comp. Immunol.*, 25(7): 565-577. https://doi.org/10.1016/S0145-305X(01)00018-0.

Guo, D., Yang, Z., Zheng, X., Kang, S., Yang, Z., Xu, Y., Shi, C., Tian, H. and Xia, X. 2019. Thymoquinone inhibits biofilm formation and attachment-invasion in host cells of *Vibrio parahaemolyticus*. *Foodborne Pathog Dis.*, 16(10): 671-678. .

Han, J. E., Tang, K. F., Tran, L. H. and Lightner, D. V. 2015. Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Org.*, 113(1): 33-40. https://doi.org/10.3354/dao02830.

Han, N., Mizan, M. F. R., Jahid, I. K. and Ha, S. D. 2016. Biofilm formation by *Vibrio parahaemolyticus* on food and food contact surfaces increases with rise in temperature. *Food Control*., 70: 161-166. https://doi.org/ 10.1016/j.foodcont.2016. 05.054.

Jayasree, L., Janakiram, P. and Madhavi, R. 2006. Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). *J. World Aquac. Soc.*, 37(4): 523-532. https://doi.org/ 10.1111/j.1749-7345.2006.00066.x

Jiang, H. S., Lv, L. X. and Wang, J. X. 2022. Anti-lipopolysaccharide factor D from kuruma shrimp exhibits antiviral activity. *Mar. Life Sci. Technol.*, 1-10. https://doi.org/ 10.1007/s42995-021-00113-y.

Jiang, H. S., Zhang, Q., Zhao, Y. R., Jia, W. M., Zhao, X. F. and Wang, J. X. 2015. A new group of anti-lipopolysaccharide factors from *Marsupenaeus japonicus* functions in antibacterial response. *Dev. Comp. Immunol*, 48(1): 33-42. https://doi.org/ 10.1016/j.dci.2014.09.001.

Joshi, J., Srisala, J., Truong, V. H., Chen, I. T., Nuangsaeng, B., Suthienkul, O., Lo, C. F., Flegel, T. W., Sritunyalucksana, K. and Thitamadee, S. 2014. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, 428: 297-302. https://doi.org/10.1016/j.aquaculture.2014.03.030.

Kim, Y. B., Okuda, J. U. N., Matsumoto, C., Takahashi, N., Hashimoto, S. and Nishibuchi, M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. *J. Clin. Microbiol.*, 37(4): 1173-1177. .

Kulkarni, A., Krishnan, S., Anand, D., Kokkattunivarthil Uthaman, S., Otta, S. K., Karunasagar, I. and Rajendran, K. V.. 2021. Immune responses and immunoprotection in crustaceans with special reference to shrimp. *Rev. Aquac*, 13(1): 431-459. https://doi.org/10.1111/raq.12482.

Kumar, B. K., Deekshit, V. K., Raj, J. R. M., Rai, P., Shivanagowda, B. M., Karunasagar, I. and Karunasagar, I. 2014. Diversity of *Vibrio parahaemolyticus* associated with disease outbreak among cultured *Litopenaeus vannamei* (Pacific white shrimp) in India. *Aquaculture*, 433: 247-251. https://doi.org/10.1016 /j.aquaculture.2014. 06.016.

Kumar, S., Kumar, C. B., Rajendran, V., Abishaw, N., Anand, P. S., Kannapan, S., Nagaleekar, V. K., Vijayan, K. K. and Alavandi, S. V. 2021. Delineating virulence of V*ibrio campbellii*: A predominant luminescent bacterial pathogen in Indian shrimp hatcheries. *Sci. Rep.*, 11(1): 15831. https://doi.org/10.1038/s41598-021-94961-4.

Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128(3-4): 203-209. https://doi.org/10.1016/0044-8486(94) 90309-3

Li, L., Meng, H., Gu, D., Li, Y. and Jia, M. 2019a. Molecular mechanisms of *Vibrio parahaemolyticus* pathogenesis. *Microbiol. Res.*, 222: 43-51. https://doi.org/ 10.1016/j.micres.2019.03.003.

Li, M., Ma, C., Zhu, P., Yang, Y., Lei, A., Chen, X., Liang, W., Chen, M., Xiong, J. and Li, C. 2019b. A new crustin is involved in the innate immune response of shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.*, 94:398-406. https://doi.org/ 10.1016/j.fsi.2019.09.028.

Liu, H. T., Wang, J., Mao, Y., Liu, M., Niu, S. F., Qiao, Y., Su, Y. Q., Wang, C. Z. and Zheng, Z. P. 2015. Identification and expression analysis of a novel stylicin antimicrobial peptide from Kuruma shrimp (*Marsupenaeus japonicus*). *Fish Shellfish Immunol.*, 47(2): 817-823. https://doi.org/10.1016/j.fsi.2015.09.044.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*, 25(4): 402-408. https://doi.org/10.1006/meth.2001.1262.

Lu, R., Sun, J., Qiu, Y., Zhang, M., Xue, X., Li, X., Yang, W., Zhou, D., Hu, L. and Zhang, Y. 2021. The quorum sensing regulator OpaR is a repressor of polar flagellum genes in *Vibrio parahaemolyticus*. *J. Microbiol.*, 59(7): 651-657. https://doi.org/10. 1007/s12275-021-0629-3.

Matos, G. M., Schmitt, P., Barreto, C., Farias, N. D., Toledo-Silva, G., Guzmán, F., Destoumieux-Garzón, D., Perazzolo, L. M. and Rosa, R. D. 2018. Massive gene expansion and sequence diversification is associated with diverse

tissue distribution, regulation and antimicrobial properties of anti-lipopolysaccharide factors in shrimp. *Mar. Drugs*, 16(10): 381. https://doi.org/10.3390/md16100381.

Mizan, M. F. R., Jahid, I. K., Kim, M., Lee, K. H., Kim, T. J. and Ha, S. D. 2016. Variability in biofilm formation correlates with hydrophobicity and quorum sensing among *Vibrio parahaemolyticus* isolates from food contact surfaces and the distribution of the genes involved in biofilm formation. *Biofouling*, 32(4): 497-509. https://doi.org/ 10.1080/08927014.2016.1149571.

Mookherjee, N., Anderson, M. A., Haagsman, H. P. and Davidson, D. J. 2020. Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug Discov.*, 19(5): 311-332. https://doi.org/10.1038/s41573-019-0058-8.

Muñoz, M., Vandenbulcke, F., Gueguen, Y. and Bachère, E. 2003. Expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *Penaeus vannamei*. *Dev. Comp. Immunol.*, 27(4): 283-289. https://doi.org/10.1016/S0145-305X(02)00102-7.

Muñoz, M., Vandenbulcke, F., Saulnier, D. and Bachère, E. 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. *Eur. J Biochem.*, 269(11): 2678-2689. https://doi.org/ 10.1046/j. 1432-1033.2002.02934.x.

Narayanan, S. V., Joseph, T. C., Peeralil, S., Koombankallil, R., Vaiyapuri, M., Mothadaka, M. P. and Lalitha, K. V. 2020. Tropical shrimp aquaculture farms harbour pathogenic *Vibrio parahaemolyticus* with high genetic diversity and Carbapenam resistance. *Mar. Pollut. Bull.*, 160: 111551. https://doi.org/10.1016/j.marpolbul.2020.111551.

Navaneeth, K. A., Bhuvaneswari, T., Rajan, J. J. S., Alavandi, S. V., Vijayan, K. K. and Otta, S. K. 2020. Characterization of *Vibrio parahaemolyticus* isolates from shrimp farms of Southeast coast of India with special reference to acute hepatopancreatic necrosis disease (AHPND) status. *Aquaculture*, 518: 734813. https://doi.org/10.1016/j.aquaculture.2019.734813.

Okumura, T. 2007. Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol*., 22(1-2): 68-76. https://doi.org/10.1016/j.fsi.2006.03.013.

Panigrahi, A., Das, R. R., Sundaram, M., Sivakumar, M. R., Jannathulla, R., Lalramchhani, C., Antony, J., Anand, P. S., Kumar, K. V., Jayanthi, M. and Dayal, J. S. 2021. Cellular and molecular immune response and production performance of Indian white shrimp *Penaeus indicus* (H. Milne-Edwards, 1837), reared in a biofloc-based system with different protein levels of feed. *Fish Shellfish Immunol*., 119: 31-41.

Panigrahi, A., Esakkiraj, P., Saranya, C., Das, R. R., Sundaram, M., Sudheer, N. S., Biju, I. F. and Jayanthi, M. 2022. A biofloc-based aquaculture system bio-augmented with probiotic bacteria *Bacillus tequilensis* AP BFT3 improves culture environment, production performances, and proteomic changes in *Penaeus vannamei*. *Probiotics Antimicrob. Proteins,* 14(2): 277-287. https://doi.org/10.1007/s12602-022-09926-4.

Paria, P., Kunal, S. P., Behera, B. K., Mohapatra, P. K. D., Das, A., Parida, P. K. and Das, B. K. 2019. Molecular characterization and genetic diversity study of *Vibrio parahaemolyticus* isolated from aquaculture farms in India. *Aquaculture*, 509: 104-111. https://doi.org/10.1016/j.aquaculture.2019.04.076.

Ponprateep, S., Tharntada, S., Somboonwiwat, K. and Tassanakajon, A. 2012. Gene silencing reveals a crucial role for anti-lipopolysaccharide factors from *Penaeus monodon* in the protection against microbial infections. *Fish Shellfish Immunol.*, 32(1): 26-34. https://doi.org/10.1016/j.fsi.2011.10.010.

Radhakrishnan, N., Panigrahi, A., Rajeshwar, B. N. and Bedekar, M. K. 2026. Shrimp immune responses and antimicrobial peptides: An overview with emphasis on *Vibrio* infection. *Aquac. Int.*, 34(3): 78. https://doi.org/10.1007/s10499-026-02444-y.

Raheem, N. and Straus, S. K. 2019. Mechanisms of action for antimicrobial peptides with antibacterial and antibiofilm functions. *Front. Microbiol.*, 10: 2866.

Raja, R. A., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S. and Nagarajan, K. 2017. Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific white shrimp, *Penaeus vannamei*. *Fish Shellfish Immunol.*, 67: 368-381. https://doi.org/ 10.1016/j.fsi.2017.06.020.

Rolland, J. L., Abdelouahab, M., Dupont, J., Lefevre, F., Bachere, E. and Romestand, B. 2010. Stylicins, a new family of antimicrobial peptides from the Pacific blue shrimp *Litopenaeus stylirostris*. *Mol. Immunol.*, 47(6): 1269-1277.

Rosa, R. D., Vergnes, A., de Lorgeril, J., Goncalves, P., Perazzolo, L. M., Saune, L., Romestand, B., Fievet, J., Gueguen, Y., Bachere, E. and Destoumieux-Garzon, D. 2013. Functional divergence in shrimp anti-lipopolysaccharide factors (ALFs): From recognition of cell wall components to antimicrobial activity. *PLoS ONE*, 8(7): e67937. https://doi.org/ 10.1371/journal.pone.0067937.

Sato, K. and Homma, M. 2000. Multimeric structure of PomA, a component of the Na+-driven polar flagellar motor of *Vibrio alginolyticus*. *J. Biol. Chem.*, 275(26): 20223-20228. https://doi.org/10.1074/jbc.M002236200.

Saucedo-Vázquez, J. P., Gushque, F., Vispo, N. S., Rodriguez, J., Gudiño-Gomezjurado, M. E., Albericio, F., Tellkamp, M. P. and Alexis, F. 2022. Marine arthropods as a source of antimicrobial peptides. *Mar. Drugs*, 20(8): 501. https://doi.org/10.3390/md20080501.

Saulnier, D., Haffner, P., Goarant, C., Levy, P. and Ansquer, D. 2000. Experimental infection models for shrimp vibriosis studies: A review. *Aquaculture*, 191(1-3): 133-144. https://doi.org/10.1016/S0044-8486(00)00423-3.

Seyfi, R., Kahaki, F. A., Ebrahimi, T., Montazersaheb, S., Eyvazi, S., Babaeipour, V. and Tarhriz, V. 2020. Antimicrobial peptides (AMPs): Roles, functions and mechanism of action. *Int. J. Pept. Res. Ther.*, 26: 1451-1463. https://doi.org/10.1007/s10989-019-09946-9.

Silvester, R., Alexander, D. and Ammanamveetil, M. H. A. 2015. Prevalence, antibiotic resistance, virulence and plasmid profiles of *Vibrio parahaemolyticus* from a tropical estuary and adjoining traditional prawn farm along the southwest coast of India. *Ann. Microbiol.*, 65(4): 2141-2149. https://doi.org/10.1007/s13213-015-1053-x.

Song, X., Ma, Y., Fu, J., Zhao, A., Guo, Z., Malakar, P. K., Pan, Y. and Zhao, Y. 2017. Effect of temperature on pathogenic and non-pathogenic *Vibrio parahaemolyticus* biofilm formation. *Food Control*, 73: 485-491. https://doi.org/10.1016 /j.foodcont. 2016.08.041.

Soonthornchai, W., Rungrassamee, W., Karoonuthaisiri, N., Jarayabhand, P., Klinbunga, S., Soderhall, K. and Jiravanichpaisal, P. 2010. Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev. Comp. Immunol.*, 34(1): 19-28. https://doi.org/10.1016/j.dci. 2009.07. 007.

Sruthy, K. S., Nair, A., Puthumana, J., Antony, S. P., Singh, I. B. and Philip, R. 2017. Molecular cloning, recombinant expression and functional characterization of an antimicrobial peptide, Crustin from the Indian white shrimp, *Fenneropenaeus indicus*. *Fish Shellfish Immunol.*, 71: 83-94. https://doi.org/10.1016/j.fsi.2017.09.071.

Stewart, B. J. and McCarter, L. L. 2003. Lateral flagellar gene system of *Vibrio parahaemolyticus*. *J. Bacteriol*, 185(15): 4508-4518. https://doi.org/10.1128/jb.185. 15.4508-4518.2003.

Sun, F., Zhang, Y., Wang, L., Yan, X., Tan, Y., Guo, Z., Qiu, J., Yang, R., Xia, P. and Zhou, D. 2012. Molecular characterization of direct target genes and cis-acting consensus recognized by quorum-sensing regulator AphA in *Vibrio parahaemolyticus*. *PLoS ONE,* 7(9): e44210. https://doi.org/10.1371/journal.pone.0044210.

Sun, B., Wang, Z. and Zhu, F. 2017. The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection. *Fish Shellfish Immunol.*, 66: 487-496. https://doi.org/10.1016 /j.fsi. 2017.05.055.

Supungul, P., Klinbunga, S., Pichyangkura, R., Jitrapakdee, S., Hirono, I., Aoki, T. and Tassanakajon, A. 2002. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). *Mar. Biotechnol*, 4: 487-494. https://doi.org/ 10.1007/s10126-002-0043-8.

Talapko, J., Mestrovic, T., Juzbasic, M., Tomas, M., Eric, S., Horvat Aleksijevic, L., Bekic, S., Schwarz, D., Matic, S., Neuberg, M. and Skrlec, I. 2022. Antimicrobial peptides: Mechanisms of action, antimicrobial effects and clinical applications. *Antibiotics*, 11(10): 1417. https://doi.org/10.3390/ antibiotics11101417.

Tamura, K., Stecher, G. and Kumar, S. 2021. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.*, 38(7): 3022-3027.

Tassanakajon, A., Amparyup, P., Somboonwiwat, K. and Supungul, P. 2011. Cationic antimicrobial peptides in penaeid shrimp. *Mar. Biotechnol*, 13: 639-657.

Tassanakajon, A., Somboonwiwat, K., Supungul, P. and Tang, S. 2013. Discovery of immune molecules and their crucial functions in shrimp immunity. *Fish Shellfish Immunol.*, 34(4) :954-967. https://doi.org/10. 1016/j.fsi.2012.09.021.

Tharntada, S., Somboonwiwat, K., Rimphanitchayakit, V. and Tassanakajon, A. 2008. Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. *Fish Shellfish Immunol.,* 24(1): 46-54. https://doi.org/10.1016/j.fsi.2007.07.010.

Thompson, F. L., Brian Austin and Jean Swings 2006. *The biology of vibrios,* ASM Press, Washington, D. C., USA, 455 p. https://doi.org/10. 1128/9781555815714.ch3.

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K. and Lightner, D. V. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Organ.*, 105(1): 45-55. https://doi.org/10.3354/dao02621.

Vargas-Albores, F., Yepiz-Plascencia, G., Jiménez-Vega, F. and Ávila-Villa, A. 2004. Structural and functional differences of *Litopenaeus vannamei* crustins. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, 138(4): 415-422. https://doi.org/10. 1016/j.cbpc. 2004.05.007.

Wang, L., Ling, Y., Jiang, H., Qiu, Y., Qiu, J., Chen, H., Yang, R. and Zhou, D. 2013. AphA is required for biofilm formation, motility, and virulence in pandemic *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.*, 160(3): 245-251. https:// doi.org/ 10.1016/ j.ijfoodmicro.2012.11.004.

Xiao, B., Liao, X., Wang, H., He, J. and Li, C. 2021. BigPEN, an antimicrobial peptide of penaeidin family from shrimp *Litopenaeus vannamei* with membrane permeable and DNA binding activity. *Fish Shellfish Immunol. Rep.*, 2: 100034.

Zhang, Y., Qiu, Y., Tan, Y., Guo, Z., Yang, R. and Zhou, D. 2012. Transcriptional regulation of opaR, qrr2–4 and aphA by the master quorum-sensing regulator OpaR in *Vibrio parahaemolyticus*. *PLoS ONE*, 7(4): e34622. https://doi.org/10.1371/ journal.pone. 0034622.

Zhou, L., Li, G., Li, A., Jiao, Y., Li, S., Huang, J., Yang, L. and Wang, C. 2019. Characterization of a group D anti-lipopolysaccharide factor (ALF) involved in anti-*Vibrio* response in *Penaeus monodon. Fish Shellfish Immunol.,* 89: 384-392. https://doi.org/10.1016 /j.fsi.2019.03.049.

Zulkifli, Y., Alitheen, N. B., Son, R., Yeap, S. K., Lesley, M. B. and Raha, A. R. 2009. Identification of *Vibrio parahaemolyticus* isolates by PCR targeted to the toxR gene and detection of virulence genes. *Int. Food Res. J.*, 16(3): 291-298.