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Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific white shrimp, *Penaeus vannamei*

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ABSTRACT

A pathobiological study was conducted using *Vibrio parahaemolyticus* (V_P) strain isolated from vibriosis affected shrimp (*Penaeus vannamei*) farms in Kancheepuram and Thiruvallur districts of Tamil Nadu during August 2014 to February 2015. The isolate was identified based on the morphological, physiological, biochemical and molecular characters. LD_{50} value with intramuscular injection was determined as 2.6×10^4 cfu/shrimp and sequential pathology was studied giving 6.1×10^3 cfu/shrimp (LD_{25}). Total plate count (TPC) and total *Vibrio* count (TVC) in water, pond sediment, haemolymph, muscle, HP and gut were found significantly ($P < 0.01$) higher in natural cases than the experimental set up. Clinical signs and lesions observed in the natural and experimental cases were anorexia, lethargy, cuticle softening, loose shells, abdominal muscle cramp, red discoloration, opaque and whitish abdominal and tail musculature, necrosis of exoskeleton or splinter burns, reddish pleural borders of antennae, uropods and telson, swollen tail fan, ulcers, moribund shrimp sinking to bottom, and mortalities with shrunken discoloured HP with empty gut. Total haemocyte count (THC), small nongranular haemocyte (SNGH), large nongranular haemocyte (LNGH), small granular haemocyte (SGH) and large granular haemocyte (LGH) counts lowered significantly ($P < 0.01$) at 3, 6, 12, 24, 48, 96 and 192 h post injection (p.i). No LGH were found after 96 h of challenge. The post injection qPCR analyses of haemocytes showed up-regulations of penaeidin-3a, lysozyme, prophenoloxidase I, prophenoloxidase II and serine protein at 3 and 6 h of infection. There was total down-regulation of crustin from 3 to 192 h p.i. There was a remarkable elevation in the level of proPO I with concomitant depletion of proPO II. The pattern of up- and down-regulations in proPO I and SP were similar. The post infection qPCR analyses showed that these immune related genes could be used as markers for assessing the immune status of *P. vannamei*. Major histopathological manifestations observed were haemocyte infiltration/nodule in the epidermis, skeletal and cardiac muscles, atrophy of the excretory organ, and disrupted HP tubules with diffuse interstitial edema and haemocytic infiltration. Further HP showed that there was thickening of inter-tubular space, karyomegaly with prominent nucleoli, rounding and sloughing of HP tubular epithelium, many mitotic figures with bacterial colonies and apoptotic bodies, separation of shrunken tubule epithelium from myoepithelial fibers, regeneration of tubules, cystic, dilated and vacuolated appearance of HP tubules, hypoplastic changes in the tubules with no B, R and F cells, granuloma formation, concretions in tubules, calcification, necrosis, and washed out appearance with complete loss of architecture. The progression of the degenerative changes in the HP tubular epithelial cells was from proximal to distal end. In haematopoietic organ, increased mitotic activities with focal to extensive depletion and degeneration were observed. Degeneration of the stromal matrix with spheroid formation in lymphoid organ was observed among the *Vp* infected natural and experimental animals. Degeneration of glandular structures in the prehensile appendages with bacterial colonies, melanization and loss of epithelial layer in oesophagus, swelling and loss of architecture with mucinous secretion in the stomach, degeneration of

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peritrophic membrane in the lumen of intestine were observed in field cases but not in the experimental studies. Further, this study established the pathobiology of the *Vp* isolate to *P. vannamei*.

1. Introduction

Shrimp aquaculture industry accounts for 15% of the internationally traded seafood products [1]. Maintaining the health status of the animals is one of the most important criteria for a successful shrimp culture. Any disease outbreak in this sector has been one of the major challenges faced by the aquaculturists. The Asia-Pacific region, being the top producer of aquaculture products in the world, it is observed that the shrimp industry has been continuously plagued by emerging diseases leading to high mortalities and economic losses. Over the last couple of decades, several diseases such as luminous vibriosis, white spot disease (WSD), yellow head disease (YHD) and taura syndrome (TS) caused catastrophic devastation in the shrimp aquaculture of the region, causing the collapse of *Penaeus monodon* culture [2]. Being an aquatic animal, shrimps are constantly exposed to a variety of pathogens, predominantly bacteria and viruses. While most of the bacteria are harmless, probiotic but some of them can be pathogenic. Physiologically or environmentally stressed animals are easily predisposed to encounter infectious diseases. With specific reference to bacterial pathogens, bacteria belonging to the family *Vibrionaceae* are considered as one of the most important cause for mortality of cultured shrimp worldwide. Recently, a newly emerging disease known as early mortality syndrome (EMS) in shrimp (*P. vannamei, P. monodon* and *P. chinensis*) named as acute hepatopancreatic necrosis disease (AHPND) [3,4] has been reported to cause unusually high mortality nearing 100% within the first 30 days of culture (DOC) and led to a significant economic losses accounting for over one billion US$ by value [5] in Asia-Pacific region (China, Vietnam, Thailand and Malaysia [4,6]). The aetiology remained enigmatic but later it was identified to be caused by a strain of *Vibrio parahaemolyticus* (V_P). In terms of impacts on trade, several countries have suspended or banned the importation of live shrimp and/or all forms of shrimp products from countries affected by AHPND [4]. Keeping all these factors in view, the present study was carried out to find out any natural occurrences of AHPND and to study the pathobiology of V_P infection in *P. vannamei* farms in Tamil Nadu, India.

2. Materials and methods

2.1. Sample collection

A total of 37 shrimp farms were surveyed during August to December 2014. From each pond surveyed, at least 50 shrimps were collected for gross examination. Clinical signs, behavioural changes and gross lesions were recorded [3,6–11] and shrimp diseases were diagnosed as reported elsewhere [12,13]. Using a sterile container, 100 mL of water sample was collected keeping at least 2.5 cm of air space to allow adequate space for mixing the sample prior to analysis. The representative pond sediment and shrimp samples were collected on ice and the samples were within three hours after collection transported to the laboratory for microbiological analysis. Care was taken to avoid sample contamination during collection and transport. For histopathology, smaller shrimps (<1 g) were fixed directly by immersing in samples to Davidson's fixative ratio of 1:10 for 24 h and larger shrimps (>1 g) were fixed for 48 h in a wide mouth plastic bottle. For larger shrimp, the fixative was injected into iced live animals @ 10% of shrimp body weight, first into hepatopancreas (HP), then into the ventral sinus after removing haemolymph so that the fixative was equally distributed along the course of the circulatory system. The cuticle was slit open on both sides of the shrimp from the 6th abdominal segment to the rostrum using scissors before placing it in the fixative. After 24–48 h fixation in Davidson's fixative, the samples were transferred to 70% ethanol for long storage with appropriate sealing and labelling. The entire procedure was done wearing rubber gloves under a fume hood in well-ventilated room. The representative shrimp samples were also stored in 95% ethanol for further molecular based investigation [14,15].

2.2. Total bacterial load

Total plate count (TPC) and total *Vibrio* count (TVC) in water, pond sediment, haemolymph, muscle, HP and gut were estimated by serial dilution spread plate method using sterile tryptone soya agar (TSA) and thiosulphate citrate bile salts sucrose (TCBS) plates [7,16,17].

2.3. Morphological, physiological, biochemical and molecular identification of Vibrio spp.

Initial bacterial isolations were made from infected haemolymph based on the dominant five colonies observed on the TCBS plates [18]. The stomach and HP from each shrimp were removed and inoculated in to tryptone soya broth (TSB) for 12 h. The enriched media was spread plated on TCBS plates for further isolation and identification [19]. When the same isolate was present in both haemolymph and gut, it was considered as systemic infection. Thus, 74 well-separated colonies two from each pond were selected, further screened and identified based on the morphological, physiological and biochemical characters [20–22]. These isolates were stored at −20 °C in TSB medium mixed with 25% (v/v) glycerol.

For molecular characterization, the isolates were cultured overnight with TSB and genomic DNA of all *Vibrio* strains were extracted by standard phenol-chloroform precipitation method. DNA was also extracted from all *Vibrio* spp. by boiling the bacterial isolate for 10 min and used as templates for PCR assays. The isolated DNA was quantified by spectrophotometric method, checked in 1% agarose gel electrophoresis and stored at −80 °C for further use. PCR was performed with respective specific primers for different genes such as *toxR, tlh, tdh, trh, AP1, AP2, AP3 ($pirA^{vp}$),* and *AP4 ($pirA^{vp}$ and $pirB^{vp}$)* as per the reported protocols (Table 1) with slight modification wherever necessary. DNA sequencing was performed using the di-deoxy chain termination method. The nucleotide sequence obtained was assembled using Auto assembler (ABI Prism, USA) software and BLAST (Basic local alignment search tool, National Center for Biotechnology Information [NCBI], USA) to identify the isolate with the maximum percentage of homology. The bacterial sequences obtained were submitted to the NCBI for obtaining accession number.

Table 1
PCR gene specific primers for *toxR*, *tlh*, *tdh*, *trh*, multiplex PCR targeting *toxR* and *tlh*, AP1, AP2, AP3 ($pirA^{vp}$), and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes.

Method names	Primers	Target genes	Annealing	Expected amplicons size (bp)	References
toxR	toxRF - 5′-GTC-TTC-TGA-CGC-AAT-CGT-TG-3′ toxRR - 5′-ATA-CGA-GTG-GTT-GCT-GTC-ATG-3′	*toxR*	63 °C/1m	368	[23]
tlh	tlhF - 5′-ACT-CAA-CAC-AAG-AAG-AGA-TCG-ACA-A-3′ tlhR - 5′-GAT-GAG-CGG-TTG-ATG-TCC-AA-3′	*tlh*	61 °C/30s	208	[24]
tdh	tdhF - 5′-GTA-AAG-GTC-TCT-GAC-TTT-TGG-AC-3′ tdhR - 5′-TGG-AAT-AGA-ACC-TTC-ATC-TTC-ACC-3′	*tdh*	58 °C/1m	269	[25]
trh	trhF - 5′-TTG-GCT-TCG-ATA-TTT-TCA-GTA-TCT-3′ trhR - 5′-CAT-AAC-AAA-CAT-ATG-CCC-ATT-TCC-G-3′	*trh*	58 °C/1m	500	
AP1	AP1F - 5′-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3′ AP1R - 5′-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3′	AP1	60 °C/30s	700	[26]
AP2	AP2F - 5′-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3′ AP2R - 5′-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3′	AP2	60 °C/30s	700	
AP3	AP3F - 5′-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3′ AP3R - 5′-GTG-GTA-ATA-GAT-TGT-ACA-GAA-3′	$pirA^{vp}$	53°C/30s	333	[27–29]
AP4-step 1	AP4F1 - 5′-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3′ AP4R1 - 5′-ACG-ATT-TCG-ACG-TTC-CCC-AA-3′	$pirA^{vp}$ and $pirB^{vp}$	55°C/30s	1269	
AP4-step 2 (nested)	AP4F2 - 5′-TTG-AGA-ATA-CGG-GAC-GTG-GG-3′ AP4R2 - 5′-GTT-AGT-CAT-GTG-AGC-ACC-TTC-3′		55°C/20s	230	

2.4. Challenge trials

The animals in the present study were used with prior approval from Coastal Aquaculture Authority (CAA), Government of India (CAA F. No. 55-17/2011 Tech. Dated: 30.03.2015) and Institutional Animal Ethical Committee (IAEC) [O/o the Chairman, IAEC, Lr. No. 1679/DFBS/B/2015. Dated: 30.10.2015]. *P. vannamei* with average body weight (ABW) of 5.05 ± 0.23 g were collected from a local extensive shrimp farm at Kattur, Thiruvallur district of Tamil Nadu, India. They were stocked in FRP (fibre reinforced polymer) tank (500 L capacity) containing chlorinated water in the laboratory and acclimatized for five days to the laboratory condition before experimentation. Water was chlorinated @ 10 ppm followed by dechlorination and found sterile before stocking. The shrimps were fed a commercially-available shrimp feed (1.2–2.2 mm crumble, Avanti Private Limited) with minimum of 34 and 5% crude protein and crude fat, and maximum of 8–10, 5 and 17% moisture, crude fibre and ash, respectively. The shrimp were fed thrice a day at 8.00, 13.00 and 18.00 h @ 10% of the body weight. Uneaten feed was not observed in the control tanks. However, excessive and uneaten feed was removed every 24 h before the morning feeding from the tanks where shrimp health was compromised. Faecal matter was removed every 24 h before feeding. Only shrimp in the intermoult stage were used for the study. The moult stage was identified by the examination of uropoda in which partial retraction of the epidermis was distinguished. Shrimps were randomly checked for the presence of any bacteria in the haemolymph and also major viruses such as MBV and WSD by PCR as described in OIE Manual of Diagnostic Tests for Aquatic Animals 2015. The water quality parameters [30] such as temperature, salinity and pH were maintained as 31 ± 1.0 °C, 3.5 ± 1.0 g L^{-1} and 8.2 ± 0.1, respectively. Observations on TPC and TVC were made at 24 h interval.

The V_P isolate from the gut was cultured in TSB with 1.5% NaCl (HiMedia, India) at 30 °C for 24 h. The broth culture was centrifuged at 3000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in PBS by adjusting the OD of the solution to 0.8 at 620 nm, which corresponded to 6.4 × 10^8 cfu mL^{-1} [31]. This standardized bacterial suspension was centrifuged at 3000 × g for 10 min and the pellet was resuspended in 50 μL of PBS. From this, 7.81 μL was made in to 50 μL using PBS to get 1 × 10^8 cfu/50 μL. These bacterial suspensions were serially diluted using standard dilution technique with PBS to get required concentration of 10^8 to 10^4 cfu/50 μL. Preliminary challenge was done intramuscularly in the third abdominal segment with a dose of 10^3 cfu per shrimp using 0.1 mL Tuberculin syringe and found that the dose was not causing any mortality. Hence, it was decided to inject at the concentration of 10^4 to 10^8 cfu per shrimp. Uniform size (5.05 ± 0.23 g) of *P. vannamei* was selected from the stock and six animals were culled to individual tubs (30 L capacity) of five experimental (10^4 to 10^8 cfu per shrimp), one control (injected with 50 μL of PBS) and one blank control group resulting in a stocking density of 0.2 shrimp L^{-1}. All the experiments were conducted in triplicate with constant aeration system. LD_{50} value was determined at 72 h p.i. as 2.6 × 10^4 cfu per shrimp by Probit analysis method. From LD_{50} value, the standardized V_P bacterial suspension was prepared in PBS with the concentration of LD_{25} i.e., 6.1 × 10^3 cfu 50 μL^{-1}. Uniform size (ABW-5.08 ± 0.01 g) of *P. vannamei* was selected from the stock and six animals were culled to individual tubs (30 L capacity) of seven experimental (3, 6, 12, 24, 48, 96 and 192 h) and one control group resulting in a stocking density of 0.2 shrimp L^{-1}. Intramuscular injection was given in the third abdominal segment with 50 μL of V_P at the concentration of 6.1 × 10^3 cfu per shrimp. The control group was injected with 50 μL of PBS. Clinical signs and mortality pattern were observed every 15 min during the first hour p.i. followed by every 1 h until 6 h p.i. The experiment was continued for seven days and the animals were monitored at 12 h intervals [32]. Moribund/dead and surviving shrimp were sampled for histological examination. Tissue homogenate of moribund shrimp HP was used for re-isolation of bacteria using Zobell marine agar (ZMA) plates to prove the Koch's postulate.

2.5. Water quality parameters

Water quality parameters such as temperature, pH and dissolved oxygen (DO) were recorded twice daily while other parameters such as appearance, turbidity, smell, salinity using a refractometer (ATAGO, Japan), alkalinity, hardness, total dissolved solids (TDS), ammonia-nitrogen (NH3-N), chloride, sulphate, sulphide, nitrate-nitrogen (NO3-N), nitrite-nitrogen (NO2-N), phosphate-phosphorus (PO4-P), fluoride, residual chlorine, iron, copper, lead and zinc were recorded initially and at the end of the experiments following standard methods [30].

2.6. Haematology

2.6.1. Total haemocyte count

Haemolymph (0.1 mL) was withdrawn from the ventral sinus of the first abdominal segment into a syringe containing equal volume of fixative (10% formalin in 0.45 M NaCl) and transferred to a microcentrifuge tube for total haemocyte count (THC) as well as for granular haemocyte (GH) and nongranular haemocyte (NGH) counts [33,34]. After 10 min, 20 μL of the fixed haemocyte suspension was mixed with the same volume of Rose Bengal solution (1.2% Rose Bengal in 50% ethanol) and incubated at ambient temperature (27–35 °C) for 20 min. Haemocyte counts were made in a haemocytometer (improved Neubauer, Marienfeld, Germany) in 5/25 squares (vol. of one square = 0.2 × 0.2 × 0.1 mm^3). THC was calculated as:

$$\text{THC mL}^{-1}\text{ of haemolymph} = 5 \times \text{Count} \times \left(\frac{10^3 \text{mm}^3}{0.1\ \text{mm}^3}\right) \times \text{Dilution factor}$$

2.6.2. Granular and nongranular haemocyte counts

Smear from the fixed haemocyte suspension was prepared and air dried. For better differentiation of granular cells, Periodic Acid Schiff (PAS) staining McManus' method was done with slight modification. The smear was oxidized in 0.5% periodic acid solution for 5 min at room temperature (RT) and rinsed in distilled water. Then, the smear was kept in Schiff reagent for 10 min followed by washing in running distilled water for pink colour to develop. The smear was rinsed thrice in sulphurous acid for 2–3 min each followed by rinsing in distilled water and staining in Mayer's haematoxylin for 2–3 min. It was then washed in running distilled water for 10–15 min. The slide was dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX mountant for observation under light microscope. The development of rose to purplish red with granules and blue with nuclei was observed clearly to make differential count of shrimp haemocytes. Agranular haemocytes or hyalinocytes or HC were identified based on the smallest size (12.4 × 7.8 μm) and ovoid shape with no granules [35,36]. Semi-granular haemocytes or small-granular haemocytes (SGH) were identified as ovoid and slightly longer and wider (14.8 × 8.3 μm) than HC with 1–40 granules in the cytoplasm. The third cell type was large granular haemocytes (LGH) which were ovoid and spherical in shape with the same size (13.6 × 9.5 μm) as the SGH [35,36]. The proportions of GH that included both large-granular haemocyte (LGH) and small-granular/semi-granular haemocytes (SGH) in 200 total haemocytes were recorded and these proportions were used to calculate the total number of GH (*i.e.*, GH count/200 × THC). NGH or hyaline cells (HC) counts were also calculated in the same manner [33,34].

2.7. Relative quantification of immune genes by RT-PCR/qPCR

2.7.1. Haemolymph collection for gene expression analysis

At the beginning (0 h) and after 3, 6, 12, 24, 48, 96 and 192 h of exposure, shrimps were randomly sampled from each tank at the respective time interval. Haemolymph (200 μL) was withdrawn from the ventral sinus (arthrodial membrane) of each shrimp using a 2 mL sterile disposable syringe with 23 gauge needle containing an equal volume of ice-cold anticoagulant in distilled water (gL^{-1}: Glucose-20.5; sodium citrate-8; NaCl-4.2; pH-7.5). The haemolymph from each shrimp was transferred into an individual microcentrifuge tube held on ice and centrifuged at 800 g for 10 min at 4 °C. The cell pellets were resuspended in 20 times volume of Trizol (Invitrogen) and stored at −80 °C for gene expression analysis [37].

2.7.2. Total RNA extraction and cDNA synthesis

Total RNA of haemocyte was extracted using Trizol method according to the manufacturer's instructions (Invitrogen). The quality and quantity of RNA were assessed by freshly prepared 1% agarose electrophoresis and by measuring their absorbance at 260 and 280 nm using Nanophotometer [37]. First-strand complementary DNA (cDNA) with the help of reverse transcriptase enzyme and oligo dT primer was synthesized. The reaction mixture contained total extracted RNA (1 μg/μL), Oligo dT (0.5 μg/μL) and DEPC water @ 5, 1 and 5 μL, respectively. The mixture was placed in a thermal cycler and heated at 70 °C for 5 min (denaturation step) to remove all the secondary structures present in the RNA and immediately kept on ice for 5 min. Then, the following components such as 5× reaction buffer with 20 mM $MgCl_2$, RNAase inhibitor (40 U/μL), 10 mM dNTP mix, Reverse transcriptase (20 U/μL) were added @ 4, 1, 2 and 2 μL, respectively. The reaction mixture was mixed gently and incubated in the thermal cycler at 37 °C for 1 h followed by 70 °C for 10 min and then it was stored at −20 °C for further use.

2.7.3. Relative mRNA expression of immune-related genes

Relative mRNA expressions of seven immune-related genes such as penaedin-3a, crustin, lysozyme, prophenoloxidase I [proPO I], prophenoloxidase II [proPO II], serine protein [SP] and peroxinectin [PE] were measured by qPCR at 0, 3, 6, 12, 24, 48, 96 and 192 h p.i along with one internal control (β-actin) gene in *P. vannamei* following the challenge with V_P isolate. The relative expression ratio of a target gene in haemolymph was computed based on its qPCR efficiencies (E) and the crossing point difference (CPD) for challenged sample versus a control. Primer sets for each gene were selected based on the already published primer sequences as shown in Table 2. The qPCR was carried out in the LightCycler® 96 (Roche) using SYBR Green. The amplifications were performed in a 96-well plate in a 15 μL reaction volume containing 5 μL of SYBR green master mix (TaKaRa Clontech), 0.4 μM (2 μL each) gene specific forward and reverse primers, 5 μL of cDNA and 1 μL of nuclease free water. The thermal profile for SYBR Green PCR was pre-incubation of 95 °C for 10 min followed by 45 cycles of 2 step amplification of 95 °C for 15 s and annealing at 60 °C for 30 s. After PCR amplification, melt-curve analysis was conducted to confirm that there was only one amplified product. All samples were run in triplicate for each of the eight genes using shrimp β-actin gene as the internal control. No template control (NTC) was also included for every primer set. The real-time standard curve of each gene was prepared. Relative amounts of all target genes and β-actin RNA in all cDNAs were calculated from a standard curve using the LightCycler® Software 4.1/4.05. All target mRNA levels were normalized to the β-actin RNA levels and expressed as a relative level. The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts. Relative gene expression levels were evaluated using $2^{-\Delta\Delta ct}$ method [38,39] as follows.

$$\Delta\text{Ct (Normalised mean of control)} = \text{Mean of target gene} - \text{Mean of endogenous control}$$

$$\Delta\text{Ct (Normalised mean of treatment)} = \text{Mean of target gene} - \text{Mean of endogenous control}$$

Table 2
qPCR gene specific primers and product sizes with respective references.

Target gene	Primers	Amplicons size expected (bp)	References
Housekeeping gene			
β-actin	F: 5′-CCACGAGACCACCTACAAC-3′ R: 5′-AGCGAGGGCAGTGATTTC-3′	142	[40]
Antimicrobial peptides (AMPs)			
Penaedin-3a	F: 5′-CACCCTTCGTGAGACCTTTG-3′ R: 5′-AATATCCCTTTCCCACGTGAC-3′	121	[40]
Crustin	F: 5′-ACGAGGCAACCATGAAGG-3′ R: 5′-AACCACCACCAACACCTAC-3′	141	
Lysozyme	F: 5′-GGACTACGGCATCTTCCAGA-3′ R: 5′-ATCGGACATCAGATCGGAAC-3′		
Prophenoloxidase system (proPO system)			
Prophenoloxidase I (proPO I)	Liva proPO I qPCR F: 5′-ACGTCACTTCCGGCAAGCGA-3′ Liva proPO I qPCR R: 5′-CCTCCTTGTGAGCGTTGTCAGG-3′	156	[41]
Prophenoloxidase II (proPO II)	Liva proPO II qPCR F: 5′-ACCACTGGCACTGGCACCTCGTCTA-3′ Liva proPO II qPCR R: 5′-TCGCCAGTTCTCGAGCTTCTGCAC-3′	161	
Proteinases/Proteinase inhibitors			
Serine protein (SP)	SP-F: 5′-CGTCGTTAGGTTAAGTGCGTTCT-3′ SP-R: 5′-TTTCAGCGCATTAAGACGTGTT-3′	61	[39]
Oxidative stress			
Peroxinectin (PE)	Lv Pox 1890F: 5′-CTGCCAATCCAGAAATTCG-3′ Lv Pox 1989R: 5′-TCAGACTCATCAGATCCATTCC-3′	100	[42]

$$\Delta\Delta Ct = \text{Normalised mean of treatment} - \text{Normalised mean of control}$$

$$\text{Fold change in expression} = 2^{-\Delta\Delta Ct}$$

$$\text{Log 2 value} = \text{Log 2}\left(2^{-\Delta\Delta Ct}\right)$$

2.8. Histopathology

The fixed samples were processed following standard histopathological procedures and stained with haematoxylin and eosin (H&E) [43]. Histopathological screening was done based on the characteristic lesions and the presence of intranuclear, perinuclear or intracytoplasmic inclusions for monodon baculovirus (MBV), white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), yellow head virus (YHV), taura syndrome virus (TSV), infectious myonecrosis virus (IMNV), Laem-Singh virus (LSNV) and *P. vannamei* nodavirus (PvNV) [11]. The presence of common viral diseases such as MBV and WSD were tested by PCR as described in OIE Manual of Diagnostic Test for Aquatic Animals 2015. Bacterial diseases such as vibriosis and necrotising hepatopancreatitis (NHP), and parasites were also screened histopathologically [7,22,44–48]. Photographic images were captured with an Olympus multi-head U-MDOB3 microscope attached with image analyzer system (Image Proplus 5.1).

2.9. Statistical analysis

All quantitative data were expressed as means ± SE (standard error). LD_{50} value was calculated by Probit analysis. One-way analysis of variance (ANOVA) was conducted to examine significant differences among treatments using SPSS v 18.0 (SPSS, Chicago, IL, USA). Statistically significant difference was set at $p < 0.05$ [37].

3. Results

3.1. Total bacterial load

TPC and TVC in water, pond sediment, haemolymph, muscle, HP and gut were found significantly different ($P < 0.01$) among the normal ponds, vibriosis affected ponds and wet lab sequential pathology experiment (Table 3). TPC was found to be in ascending grades as haemolymph < muscle < HP < gut < water < pond sediment among the disease free pond environment but it was as muscle < haemolymph < HP < gut < water < pond sediment among the vibriosis affected ponds. In the wet lab sequential pathology experiment, it was water < muscle < haemolymph < gut < HP. TVC was found to be in ascending grades as muscle < haemolymph < HP < gut < water < pond sediment among the disease free and vibriosis affected ponds. In the wet lab sequential pathology experiment, it was water < muscle < haemolymph < gut < HP.

3.2. Morphological, physiological, biochemical and molecular identification of Vibrio spp.

Based on the morphological, physiological and biochemical parameters, 74 isolates were identified as *Vp* (35.14%), *V. harveyi* (21.62%), *V. anguillarum* (16.22%), *V. campbellii* (10.81%), *V. mimicus* (8.11%), *V. alginolyticus* (5.41%), and *Pseudomonas aeruginosa* (2.7%). On molecular identification based on V_P specific *toxR* and *tlh* genes, 26 (35.14%) isolates were found to be positive (Figs. 1 and 2). All the

Table 3
TPC and TVC in normal and vibriosis affected shrimp monoculture ponds and sequential pathology with V_P.

TPC (10^4 cfu/mL or g)**						
Details	Water	Pond sediment	Haemolymph	Muscle	HP	Gut
Normal ponds	53.76 ± 4.86[b]	813.53 ± 7.36[a]	0.88 ± 0.26[a]	0.94 ± 0.13[a]	5.82 ± 0.44[a]	18.47 ± 1.66[a]
Vibriosis affected ponds	194.20 ± 15.50[c]	2941.50 ± 23.46[b]	8.65 ± 0.71[b]	3.25 ± 0.25[b]	28.15 ± 2.25[b]	67.10 ± 5.33[b]
Wet lab sequential pathology	0.88 ± 0.48[a]	–	6.88 ± 1.48[b]	3.88 ± 0.79[b]	7.88 ± 2.13[a]	7.63 ± 2.34[a]
TVC (10^2 cfu/mL or g)**						
Normal ponds	7.82 ± 0.43[a]	120.59 ± 0.66[a]	0.29 ± 0.11[a]	0.12 ± 0.08[a]	1.06 ± 0.06[a]	2.71 ± 0.17[a]
Vibriosis affected ponds	127.35 ± 17.50[b]	1929.50 ± 26.51[b]	5.70 ± 0.80[b]	2.15 ± 0.29[b]	18.60 ± 2.55[b]	43.95 ± 6.04[b]
Wet lab sequential pathology	0.63 ± 0.32[a]	–	5.13 ± 1.41[b]	2.88 ± 0.90[b]	5.13 ± 1.20[a]	5.00 ± 1.48[a]

**P < 0.01.
[a, b, c] - Values bearing different superscripts in a column differ significantly.

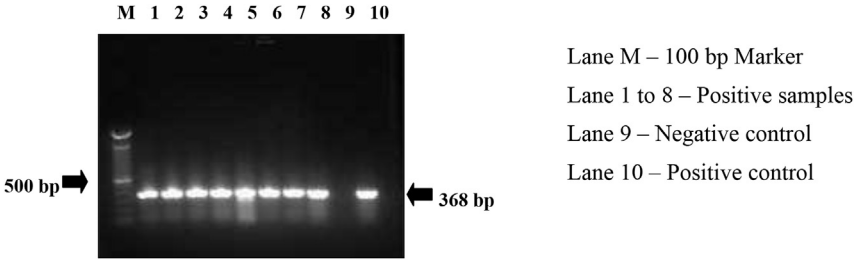


Fig. 1. V_P – *toxR* gene – Agarose gel showing the PCR product specific to *toxR* gene.

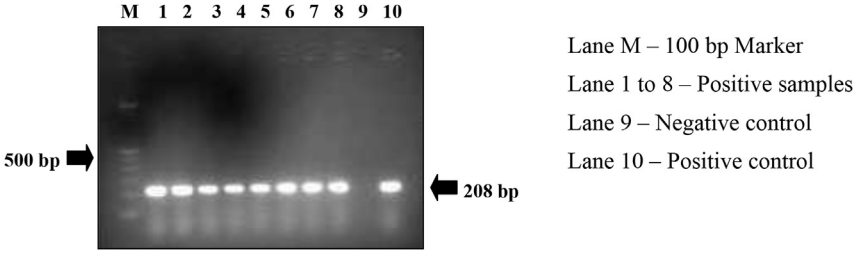


Fig. 2. V_P – *tlh* (thermolabile haemolysin) gene – Agarose gel showing the PCR product specific to *tlh* gene.

74 isolates were found to be negative for *tdh* and *trh* genes. PCR screening for the presence of AHPND causing V_P isolates among the collected farmed shrimp, *P. vannamei* samples showed that all the isolates were negative in standard protocols as well as different gradient PCR procedures.

The PCR products of *toxR* forward and reverse showed that they were 99 and 98% homologous with *V. parahaemolyticus* RIMD 2210633 chromosome 1 and the NCBI accession numbers were obtained as KT360934 and KT360936, respectively. Similarly, the PCR products of *tlh* forward and reverse showed that they were 96 and 99% homologous with *V. parahaemolyticus* RIMD 2210633 chromosome 2 and the NCBI accession number were KT360935 and KT360937, respectively.

3.3. Challenge trials

Prior to the experiment, shrimp were randomly checked and found negative for the presence of pathogenic bacteria, parasites and viruses. Clinical signs observed in sequential pathology with intramuscular injection of V_P with LD_{25} were anorexia, lethargy, inactivity, cuticle softening, loose shells, abdominal muscle cramp after 24 h p.i., red discoloration, focal to extensive opaque and whitish musculature after 48 h p.i., reddish pleural borders of antennae, uropods and telson, swelling and necrosis of tail fans, ulcers, necrosis of exoskeleton and appendages after 96 h p.i., moribund shrimp sunk to bottom and mortalities with abnormal, shrunken, small, swollen or discoloured HP after 192 h p.i.

3.4. Water quality parameters

Water quality parameters such as temperature (31.3 ± 0.12 °C), pH (morning-7.9 ± 0.02; evening-8.02 ± 0.04) and morning DO (4.22 ± 0.01 ppm), appearance, turbidity, smell, salinity (2.08 ± 0.02 g L^{-1}), alkalinity (316.67 ± 60.09 mgL^{-1}), hardness (440 ± 60 mgL^{-1}), TDS (1896 ± 60 mgL^{-1}), NH_3-N (0.6 ± 0.21 mgL^{-1}), chloride (893.33 ± 53.64 mgL^{-1}), NO_3-N (20 ± 0 mgL^{-1}), NO_2-N (0.3 ± 0.1 mgL^{-1}), residual chlorine (0.07 ± 0.07 mgL^{-1}) were found to be within the normal level as shown in the parenthesis. No PO_4-P, fluoride, sulphide, iron, copper, lead and zinc were found in the water used for the wet lab experiments.

Table 4
Time dependent haematological parameters in relation to *V. parahaemolyticus* challenging (6.1×10^3 cfu/shrimp) in juveniles of *P. vannamei*.

Sl. No.	Time (hours)	THC x 10^{6**}	SNGH x 10^{6**}	LNGH x 10^{6**}	SGH x 10^{6**}	LGH x 10^{6**}
1.	0	10.85 ± 0.15^{g}	7.41 ± 0.19^{g}	1.19 ± 0.09^{e}	1.96 ± 0.03^{e}	0.30 ± 0.09^{c}
2.	3	6.25 ± 0.05^{f}	3.63 ± 0.01^{f}	0.96 ± 0.14^{d}	1.07 ± 0.26^{d}	0.61 ± 0.07^{d}
3.	6	4.50 ± 0.00^{e}	2.57 ± 0.05^{e}	0.59 ± 0.03^{c}	1.14 ± 0.04^{d}	0.22 ± 0.02^{bc}
4.	12	3.50 ± 0.30^{d}	2.18 ± 0.21^{d}	0.37 ± 0.07^{b}	0.73 ± 0.03^{c}	0.23 ± 0.05^{bc}
5.	24	2.30 ± 0.10^{c}	1.42 ± 0.04^{c}	0.37 ± 0.01^{b}	0.32 ± 0.01^{ab}	0.19 ± 0.07^{bc}
6.	48	1.90 ± 0.00^{c}	1.20 ± 0.03^{c}	0.19 ± 0.01^{ab}	0.46 ± 0.03^{bc}	0.06 ± 0.01^{ab}
7.	96	1.05 ± 0.05^{b}	0.73 ± 0.02^{b}	0.24 ± 0.03^{ab}	0.09 ± 0.01^{a}	0.00 ± 0.00^{a}
8.	192	0.10 ± 0.00^{a}	0.07 ± 0.00^{a}	0.02 ± 0.00^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}
Sl. No.	Time (hours)		SNGH (%)**	LNGH (%)**	SGH (%)**	LGH (%)**
1.	0		68.25 ± 0.75^{c}	11.00 ± 1.00^{a}	18.00 ± 0.50^{bcd}	2.75 ± 0.75^{ab}
2.	3		58.00 ± 0.50^{a}	15.25 ± 2.25^{b}	17.00 ± 4.00^{bc}	9.75 ± 1.25^{d}
3.	6		57.00 ± 1.00^{a}	13.00 ± 0.50^{ab}	25.25 ± 0.75^{e}	4.75 ± 0.25^{bc}
4.	12		62.25 ± 0.75^{b}	10.50 ± 1.00^{a}	21.00 ± 2.50^{cde}	6.25 ± 0.75^{bcd}
5.	24		61.75 ± 0.75^{b}	16.00 ± 1.00^{b}	14.00 ± 1.00^{ab}	8.25 ± 2.75^{cd}
6.	48		63.00 ± 1.50^{b}	10.00 ± 0.50^{a}	24.25 ± 1.75^{de}	2.75 ± 0.25^{ab}
7.	96		69.50 ± 1.00^{c}	22.25 ± 1.75^{c}	8.25 ± 0.75^{a}	0.00 ± 0.00^{a}
8.	192		68.75 ± 1.25^{c}	23.25 ± 0.75^{c}	8.00 ± 0.50^{a}	0.00 ± 0.00^{a}

$^{**}P < 0.01$; $^{*}P < 0.05$ - Values bearing different superscript in a column differ significantly.

3.5. Haematology

3.5.1. Total haemocyte count

THC showed significant reduction ($P < 0.01$) at 3 h of dosing (both control and challenge) followed by significant reduction ($P < 0.01$) in treatments at different time intervals as shown in Table 4.

3.5.2. Granular and nongranular haemocyte counts

Population of small nongranular haemocyte (SNGH), large nongranular haemocyte (LNGH), and small granular haemocyte (SGH) significantly reduced ($P < 0.01$) at 3 h of dosing (both control and challenge) but large granular haemocyte (LGH) was found increased significantly ($P < 0.01$). SNGH, LNGH, SGH and LGH reduced significantly ($P < 0.01$) at different time intervals as shown in Table 4. LGH was found to be zero at and after 96 h of challenge. Percentage of differential cell count showed significant ($P < 0.01$) decrease in SNGH at 3 and 6 h followed by gradual increase up to 192 h while LNGH revealed significant ($P < 0.01$) increase in LNGH at 3 and 6 h followed by fluctuation in population between 12 and 48 h, and finally increased significantly ($P < 0.01$) at 96 and 192 h. Among the SGH population, fluctuations were observed during 0–48 h but significant ($P < 0.01$) reduction was observed at 96 and 192 h. LGH was found to be significantly ($P < 0.01$) increased at 3, 6, 12 and 24 h but found decreased significantly ($P < 0.01$) at 48 h and no cells were found at 96 and 192 h of challenge.

3.6. Relative quantification of immune genes by RT-PCR/qPCR

The post infection qPCR analyses showed that there was increase in penaeidin-3a gene at 3 and 6 h intervals followed by

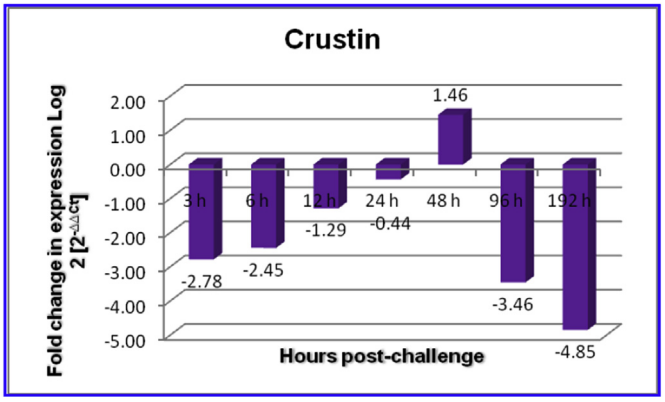


Fig. 4. qPCR relative crustin gene expression.

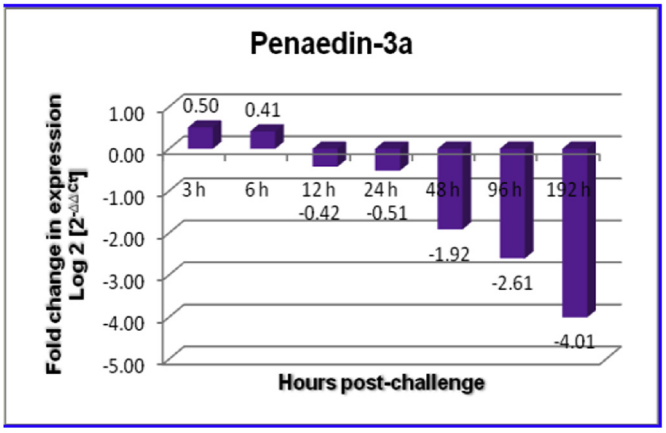


Fig. 3. qPCR relative penaeidin-3a gene expression.

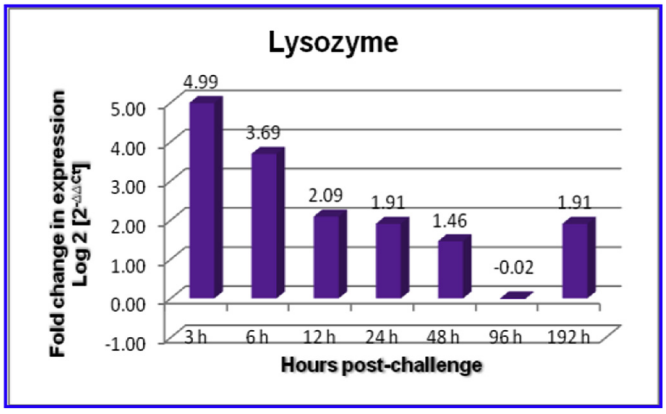


Fig. 5. qPCR relative lysozyme gene expression.

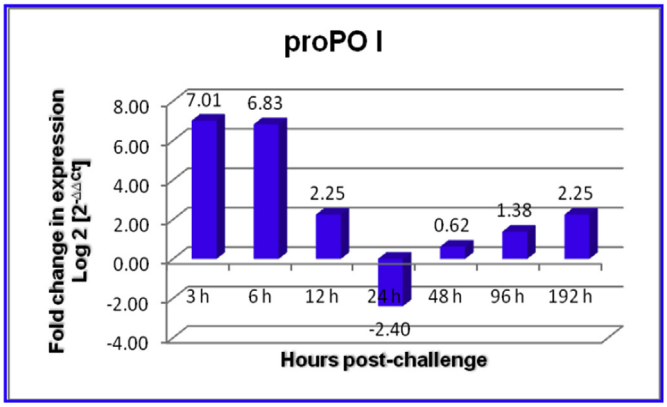


Fig. 6. qPCR relative proPO I gene expression.

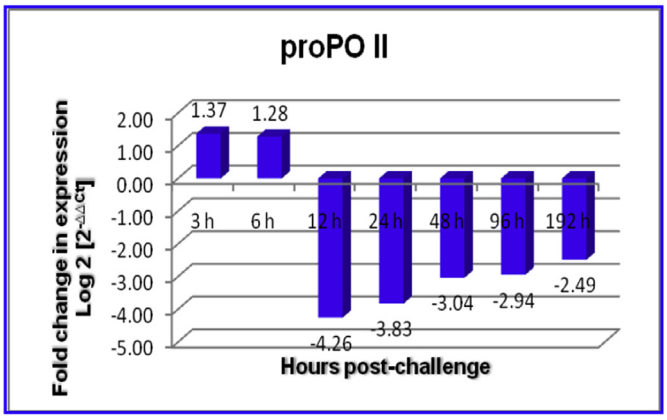


Fig. 7. qPCR relative prophenoloxidase (proPO) II gene expression.

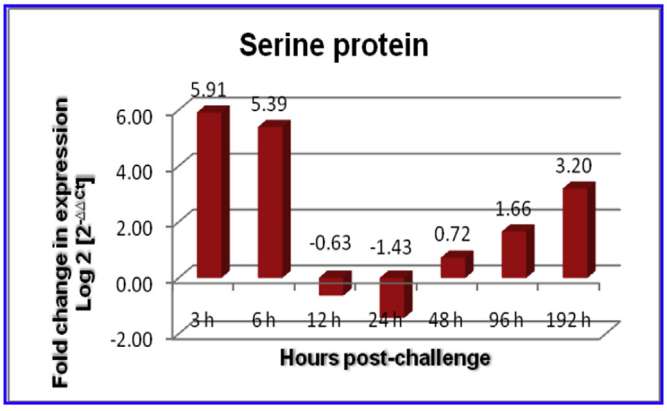


Fig. 8. qPCR relative serine protein gene expression.

decrease in expression at 12, 24, 48, 96 and 192 h intervals (Fig. 3). It was found that there was decrease in expression of crustin at different intervals except that there was increase at 48 h interval (Fig. 4). Simultaneously, there was up regulation of lysozyme at 3, 6, 12, 24, 48 and 192 h intervals but it was down regulated at 96 h interval (Fig. 5). proPO I gene was decreased at 24 h interval. But, it revealed that there was increase in expression at 3, 6, 12, 48, 96 and 192 h intervals (Fig. 6). proPO II and peroxinectin were increased initially, followed by decrease in expression up to 192 h (Figs. 7 and 9). Serine protein gene was decreased at 12 and 24 h intervals, but

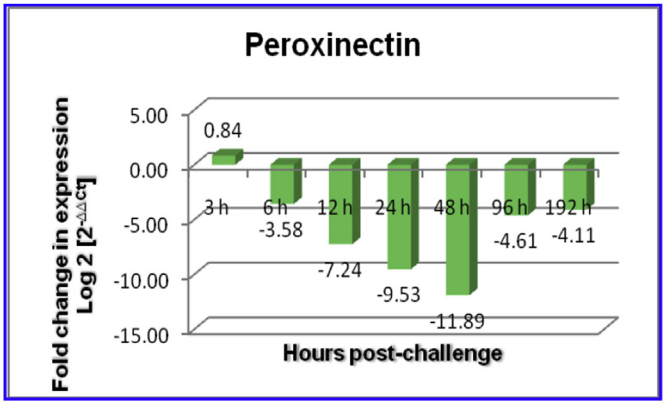


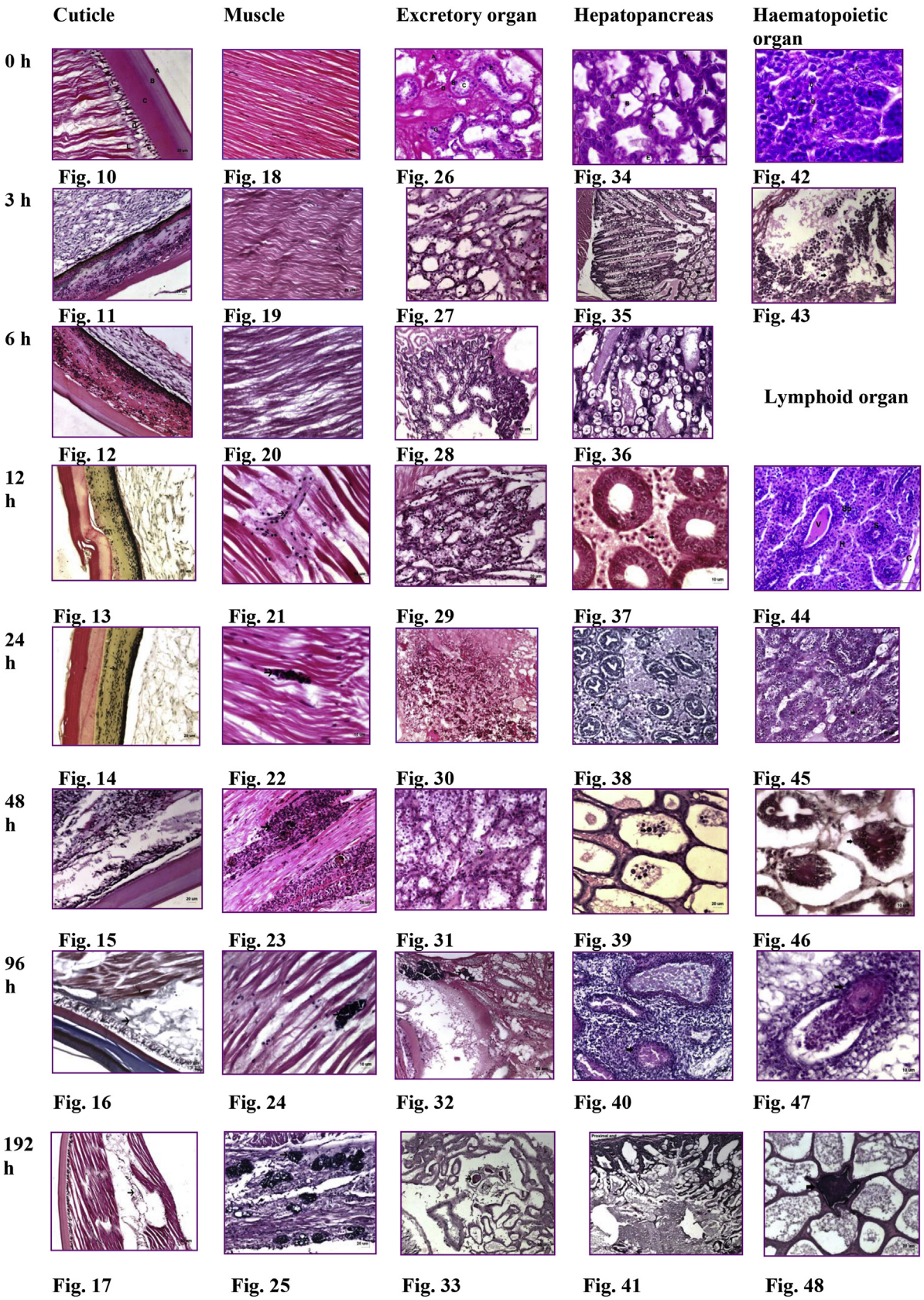
Fig. 9. qPCR relative peroxinectin gene expression.

there was increase in expression at 3, 6, 48, 96 and 192 h intervals (Fig. 8).

3.7. Histopathology

Varying degrees of cuticular damage, haemocytic infiltration and degenerative changes in the cuticle and underlying tissues (Figs. 10–17) were observed during 0, 3, 6, 12, 24, 48, 96 and 192 h p.i. Similarly, skeletal muscle showed wavy fibres (Fig. 19) at 3 h p.i., shredding of muscle fibers (Fig. 20) at 6 h p.i., muscle edema with haemocytic infiltration (Fig. 21) at 12 h p.i., and haemocytic nodule (Fig. 22) at 24 h p.i., muscle granuloma (Fig. 23) at 48 h p.i., bacterial emboli and haemocytic infiltration in muscle (Fig. 24) at 96 h p.i. and melanization with degenerative changes and muscle necrosis (Fig. 25) at 192 h p.i. Challenged animals showed loss of connective, fibrous and spongy tissue layers in the pericardium with cardiac chambers containing bacterial colonies, degenerative changes (after 48 h p.i.) in the myocardium with satellite cells and phagocytic reserve cells, and cardiac chambers containing bacterial colonies, and degenerative changes in the myocardium with haemocytic aggregation. No pathological lesions were observed in the oral region, oesophagus, stomach, intestine, anterior and posterior longitudinal intestinal blind pouch, ovary, testis, vas deferens, central nervous system (CNS) and eye of wet lab challenged animals. Excretory organ showed degenerative changes with edema (Figs. 27 and 28), loss of epithelial lining (Fig. 29), architectural loss and fibrous tissue proliferation (Fig. 30) intertubular fibrosis (Fig. 31), calcification and necrosis (Figs. 32 and 33) sequentially at 0, 3, 6, 12, 24, 48, 96 and 192 h p.i. Hepatopancreas (HP) tubules filled with eosinophilic material and reduced B and R cells (Fig. 35) at 3 h p.i., intracytoplasmic PAS positive materials in B cells (Fig. 36) at 6 h p.i., interstitial haemocytic infiltration (Figs. 37 and 38) at 12–24 h p.i., sloughing of tubular epithelium (Figs. 39 and 46) at 48 h p.i., granuloma formation (Figs. 40 and 47) at 96 h p.i., HP tubular necrosis with lesions progress from proximal to distal end (Fig. 41) and concretions in tubules (Fig. 48) at 192 h p.i. were observed as major pathological lesions of vital organ in *P. vannamei*. Haematopoietic organ (HPT) showed depletion with extensive degeneration (Fig. 43) and lymphoid organ (LO) showed degeneration with fibrous tissue proliferation and spheroid formation (Fig. 45) proportionately to the advancement of infection. Dendrobranquia gill revealed swollen and fusion of secondary gill filaments, occlusion of epithelial cells, fusion of secondary filaments with gill dilatation and loss of architecture with shrunken and fusion of secondary filaments after 48 h p.i. Tail fans among the V_P infected animals were edematous with spindle cells.

Similar lesions were observed in natural outbreaks except that



Figs. 10–48. Plate showing the histopathological changes in the *P. vannamei* juveniles at 0, 3, 6, 12, 24, 48, 96 and 192 h intervals after challenged with *V. parahaemolyticus* isolate. Normally haematoxylin and eosin (H&E) staining was used. Special stains were mentioned wherever applicable. Scale bar was depicted in the figures. Specific lesions were pointed with arrows. Normal cuticle with epicuticle (A), exocuticle (B), endocuticle (C), epidermis (D) and the underlying tissues (E) (Fig. 10); Haemocytic infiltration in the endocuticle (Figs. 11–12); Picrosirius red staining showing the red collagen in the damaged cuticle on a pale yellow background with black nuclei (Figs. 13–14); Degenerative changes in the epidermis and underlying tissues (Figs. 15); Azan trichrome staining showed brown epicuticle, blue exocuticle and dark brown endocuticle with degenerative changes in epidermis

the field cases showed degeneration of glandular structures in the prehensile appendages with bacterial colonies, melanization and loss of epithelial layer of oesophagus while stomach and intestine revealed swelling and degenerative changes with mucinous secretion.

4. Discussion

Comparison of bacterial population between normal healthy ponds, vibriosis affected ponds and wet lab experiments, the present study showed that the TPC and TVC in water, pond sediment, haemolymph, muscle, HP and gut were found to be significantly less among the normal ponds [34,49]. *Vibrio* spp. were existing as part of the normal microbiota in the marine and estuarine environments and were associated with fish and other poikilothermic animals. They acted as primary or secondary opportunistic pathogens in association with increase of *Vibrio* populations in cultured pond waters [50,51]. TPC and TVC were significantly higher in vibriosis affected ponds with salinity between 14 and 25 parts per thousand while they were significantly less among the fresh water ponds with no vibriosis, which proved that the salinity played a major role in outbreak of vibriosis [52]. The factors other than bacterial load, such as shrimp age/size, the presence of other pathogens, or environmental conditions, might influence the mortalities in the affected farms [53,54]. Sometimes, haemolymph sampled from healthy *P. vannamei* showed low numbers of *Vibrio* [55] as found in the present study ($0.88 \pm 0.26 \times 10^2$ cfu mL^{-1}). When *Vibrio* infection set in, the bacterial load significantly increased in haemolymph than muscle. The most vibrios were cleared from the haemolymph of shrimp within the early several hours after infection [56] and usually no *Vibrio* species circulate in haemolymph of live healthy shrimps [2]. The bacterial infection was found to be confined only to the digestive system on wet lab experiment with *V. harveyi* immersion and no bacteria were re-isolated from the haemolymph [57]. The present study showed that the TPC (10^4 cfu/mL or g) in haemolymph of vibriosis affected ponds and wet lab experiments were 8.65 ± 0.71 and 6.88 ± 1.48 while the TVC (10^2 cfu/mL or g) were 5.70 ± 0.80 and 5.13 ± 1.41, respectively in agreement with earlier findings [58]. The bacterial loads (TPC and TVC) in challenge experimental trials were in ascending grades as water < muscle < haemolymph < gut < HP. The literature showed that the *Vibrio* load in shrimp tissues after infection was either higher [17] or lower [59] than the present findings.

Morphological, physiological and biochemical characteristics of *Vibrio* isolates were in concurrence [21,58,60] but the *Vibrio* spp. diversity in *P. vannamei* ponds was in contrast [61,62] with the results of earlier researchers. Differentiation of virulent bacterial strains from avirulent strains became difficult using traditional culture methods based on growth phenotypes. So, the isolates were further identified based on PCR targeting V_P specific virulent genes *toxR* and *tlh* [23,24,63–65,69]. The present study revealed that the prevalence of V_P in shrimp farms were lower (35.14%) than the previous results [63–65]. Since, the percentage of rRNA [66] and *gyrB* [67] sequence homologies were more than 99 and 86.8% between V_P and *V. alginolyticus*, respectively, *toxR* gene was identified as a gene very specific to V_P. The *toxR* gene was also identified to be involved in the regulation of many other genes in *V. cholerae* [68]. But, the degree of homology of the *toxR* gene between V_P and *V. cholera* was only 52%, which was much lower than that of the rRNA gene (91–92% identity) [23,66]. The transcriptional activator *toxR* was constantly found [70] in AHPND causing V_P strains along with zona occludens toxin, accessory cholera enterotoxin, and transmembrane regulatory protein *toxS*. Even though, the V_P stains were positive to *toxR* gene in the present study, they were constantly negative to AP1, AP2, AP3 ($pirA^{vp}$), and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes which were responsible for causing AHPND in shrimp [26–29]. In the present study, all the 74 isolates were found to be negative for human pathogenic *tdh* and *trh* genes [71]. Some researchers observed in disagreement to the present findings [64,65] that 10 and 6.5% *toxR*-positive isolates exhibited the *trh* gene but none of the isolates were found positive for *tdh*. Many researchers reported that the possession of particular hemolysin genes (*tdh*, *trh*, or both) was important to cause gastroenteritis in human [25,72]. However, a small portion of clinical strains carried neither of the virulence genes, *tdh* and *trh* [73]. Therefore, the isolation and identification of the pathogenic organism in investigations of gastroenteritis due to V_P became difficult. Hence, it could not be possible to ascertain the zoonotic importance (based on PCR) of the V_P isolates obtained in the present study.

Clinical signs observed in sequential pathology with LD_{25} were anorexia, lethargy, inactivity, opaque and whitish abdominal and tail musculature, reddish pleural borders of antennae [75], uropods and telson, swollen tail fan and mortalities as observed among the field cases but the same clinical signs and lesions were also observed with the pathogenic strain of *V. alginolyticus* [7] and *V. harveyi* [74]. Three forms of bacterial infection in shrimps were localised pits in the cuticle (cuticle softening, loose shells), localised infections of the gut or HP and generalized septicaemia, which were observed in the present challenged trials with V_P [12,76]. No gross gill lesions were observed with V_P infection as reported in *P. monodon* exhibiting tea brown gill syndrome (TBGS) in Thailand [77] caused by *V. harveyi* and a bacteriophage. Opaque and whitish abdominal and tail musculature was observed with V_P infection in the present study and the same was reported as bacterial white tail disease (BWTD) in *P. vannamei* caused by a nonluminescent and

and underlying tissue (Fig. 16); Extensive degenerative changes in the cuticle underlying tissues (Fig. 17); Normal striated skeletal muscle in longitudinal section (Fig. 18); Wavy skeletal striated muscle fibres (Fig. 19); Shredding of muscle fibers (Fig. 20); Muscle edema with haemocytic infiltration (Fig. 21) and haemocytic nodule (Fig. 22); Muscle granuloma (Fig. 23); Bacterial emboli and haemocytic infiltration in muscle (Fig. 24); Melanization with degenerative changes and muscle necrosis (Fig. 25); Normal excretory organ with heamocytes in the sinus (A), tubular epithelium (B), lumen (C), blood sinus (D), antennal gland (E), collagen and reticular fibres (F) and podocytes or coelomosac epithelium (G) (Fig. 26); Degenerative changes with edema (Figs. 27–28); Loss of epithelial lining (Fig. 29); Architectural loss and fibrous tissue proliferation (Fig. 30); Intertubular fibrosis (Fig. 31); Calcification and necrosis (Figs. 32–33); Healthy hepatopancreas (HP) with tubules (A), lumen with eosinophilic materials (B), brush border (C), tubular epithelium (D), large distinctive secretory (Blasenzellen) B-cells with large vacuole encompassed by a thin layer of cytoplasm and the nucleus placed in the basal region (E), absorptive storage (Restzellen) cylindrical R-cells with small sub apical vacuoles and the nucleus placed in the basal region (F), medial region with fibrous (Fibrillenzellen) F-cells (G), tubule apex with undifferentiated embryonic (Embryonalzellen) E-cells (H), myoepithelial fibres (J) and sinus intertubular space (L) (Fig. 34); HP tubules filled with eosinophilic material and reduced B and R cells in the distal portion (Fig. 35); Intracytoplasmic PAS positive materials in B cells (Fig. 36); Interstitial haemocytic infiltration (Figs. 37–38); Sloughing of tubular epithelium (Figs. 39 and 46); Granuloma formation (Figs. 40 and 47); Tubular necrosis with lesions progress from proximal to distal end (Fig. 41); Concretions in tubules (Fig. 48); Normal haematopoietic organ (HPT) organized into distinct lobes (A) surrounded by fibrous connective tissue (B) with haematopoietic tissue and haemocyte precursor cells in different developmental stages (C), mitotic figures (D), A-type cells deeply stained with bigger nuclei (E), B type cells comparatively light stained with binucleated cells (F), C-type ells round shape with light stained binucleated cells (G) and D-type cells pear shaped light stained cells (H) (Fig. 42); Depletion with extensive degeneration (Fig. 43); Normal lymphoid organ (LO) showed intact tubular structures (LO cords) with multiple layers of sheath of lymphoid cells in the stromal matrix (S), central haemolymph vessel (V), hemal sinus with haemocytes (H), spheroids (Sp) with lack of central vessel and more basophilic cytoplasmic cells, and connective tissue fibre (C) (Fig. 44); Degeneration with fibrous tissue proliferation and spheroid formation (Fig. 45). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

highly virulent strain of *V. harveyi* [18]. On the contrary, some researchers reported white patch disease (WPD) in *P. vannamei* caused by *Bacillus cereus* with major symptoms of white opaque patches in the carapace, necrosis, whitish blue coloration, loss of appetite and pale white muscles, and high mortality (>70%) [78]. In the present study, signs and lesions close to AHPND [3,6,8–10,53,54,79] were observed with mass mortality between 20 and 80 DOC but specifically corkscrew or spiral swimming as observed in AHPND was not observed in both field and wet lab experiments.

All the water quality parameters in wet lab experiments were maintained within the normal level which was conducive for healthy shrimp. Increased concentrations of sulfide in water increased the susceptibility of *P. vannamei* to *V. alginolyticus* infection by a depression in THC and HC population [80]. Since no sulphide was found in the water used for wet lab experiments in the present study, it might not have played role in decreasing haemocyte population. Since the wet lab experiments were conducted with treated water under the controlled environment, the influence of temperature on the density of V_P [79] in water was nullified. pH itself did not seem to have an effect on AHPND causing V_P strain. Those isolates were susceptible to common disinfectants and found no cultural cells after exposure to pH 5 for 15 min [81]. But, pH of the water used for challenge trials were optimum 7.9 ± 0.03 and 8.03 ± 0.04 during morning and evening, respectively.

The present study showed that there was significant reduction in THC at 3 h of dosing (both PBS control and challenge) [82]. Previous workers showed that the THC in shrimp varied according to the moult stage due to change in HC population [83] but the present trials were conducted using the intermoult stage. Hence, influence of moult stage on haemocytic population was ruled out. Haemocytopenia was observed gradually and highly significantly at different time intervals (0, 3, 6, 12, 24, 48, 96 and 192 h). Similarly, hypoxic stress and/or intramuscular injection of *V. alginolyticus* in *P. stylirostris*, *P. vannamei* and *Fenneropenaeus indicus* could cause a significant decrease in the THC due to significant decrease in HC and SGC [84–86]. Haematopoiesis is an important process in generating newborn haemocytes to refill circulating haemocytes, which took place in the HPT. The lifespan of white shrimp *P. vannamei* haemocytes was short; between 129 and 156 h [87] and the reduction in the percentage of large cells might be due to degranulation of GC. HPT was severely affected during vibriosis which led to decrease in THC [41]. Sequential pathology with V_P showed the differential cell count percentages of SNGH, LNGH, SGH and LGH between 57 ± 1 and 69.5 ± 1, 10 ± 0.5 and 23.25 ± 0.75, 8 ± 0.5 and 25.25 ± 0.75, and zero and 9.75 ± 1.25, respectively in contrary to the previous findings in healthy animals [88]. The differential haemocyte population (x 10^6) in the present study among the control *P. vannamei* were 7.41 ± 0.19, 1.19 ± 0.09, 1.96 ± 0.03 and 0.30 ± 0.09 in SNGH, LNGH, SGH and LGH, respectively [33]. THC, SNGH, LNGH, SGH and LGH reduced (haemocytopenia) gradually and highly significantly at different time intervals. LGH were found to be zero at and after 96 h of challenge. But, some researchers concluded that an injection of *V. alginolyticus* to *P. vannamei* rapidly reduced the shrimp's immunity by decreasing HC (SNGH, LNGH), GC (SGH and LGH) and PO activity within 3–24 h, followed by a slow recovery during 72–168 h p.i. [89]. Hypoxic stress and/or intramuscular injection of *V. alginolyticus* in *P. stylirostris* and *P. vannamei* could cause a significant decrease in HC and SGC [84–86].

Haemocytes played an important role in the cellular immune response [90]. Hence, they were selected as target cells for relative quantification of gene expression in the present study. Significant reduction in differential haemocyte count between 3 and 192 h p.i. of V_P was directly proportionate to the down regulation of all the seven immune genes expressions. When injected with the highly pathogenic *Vibrio* spp., PLs normally died within two days. In contrast, juvenile shrimp were able to survive even at a higher dose of bacteria. Hence, juvenile shrimp were used as a model to study effects of infections on immune gene expression. AMPs such as penaeidin-3a, crustin and lysozyme [90] showed significant up and down regulations in *P. vannamei* challenged with V_P strain. It was observed that there was swift up-regulation of penaeidin during 0–6 h, followed by down-regulation but earlier it was reported that the penaeidin-3 (PEN-3) was swiftly down-regulated (0–24 h) in haemocytes and HP of *P. vannamei* in response to dietary inclusion of β-1,3-glucan [40]. There was 2.09 fold raises in penaeidin against *V. harveyi* in *P. monodon* PLs in agreement to the up-regulation of penaeidin during 0–6 h [91,92]. It was found that there was 2.78, 2.45, 1.29, 0.44, 3.46 and 4.85 fold decrease in expression at 3, 6, 12, 24, 96 and 192 h, respectively except that there was 1.46 fold rise in crustin gene at 48 h. Similar to the present results, LPS injection and β-1,3-glucan in feed caused a rapid decrease in mRNA levels of crustin [40,93]. Oral administration of peptidoglycan and sodium alginate induced a marked increase in crustin mRNA levels of *M. japonicus* [94] and *P. monodon* [95], respectively. Lysozyme could be one of the marker genes used for assessing the immune status of the *P. vannamei* since there was typical pattern of up-regulation on V_P challenge trials. Supporting the present findings, there were reports that the lysozyme was swiftly up-regulated in haemocytes and HP of *P. vannamei* in response to dietary inclusion of β-1,3-glucan [40] and the post infection with *V. harveyi* in *P. monodon* [91]. Lysozyme was expressed and stored mainly in all types of hemocytes, although the intensity of expression was higher in granular than hyaline hemocytes [90,96,97]. Present study showed that there was significant reduction in THC at different time intervals but increase in LNGH up to 192 h, SGH up to 48 h and LGH up to 24 h might have played a role in up regulation of lysozyme. The expression of c-type lysozyme in shrimp against *V. alginolyticus*, V_P and *V. anguillarum* were more active than against *V. cholerae* [96,98] as observed against V_P in the present study. Up-regulation of proPO in *P. vannamei* fed with *B. subtilis* against *V. harveyi* infection was observed by previous researchers [99] as observed in the present study against V_P infection. The proPO gene was specifically expressed in the hemocytes; therefore, the proPO transcript was used as a hemocyte marker to monitor hemocyte infiltration into different tissues [100]. Concurrent to the present findings, up-regulation of SP in *P. vannamei* challenged with *V. harveyi* after eight weeks of feeding with probiotic *B. subtilis* diets were reported [39,99] but transient strong down-regulation of *Pm*ClipSP2, a clip-domain SP mRNA after infection with *V. harveyi* was also reported elsewhere [101]. Up-regulation of SP gene in shrimp was directly proportional to up-regulation of proPO gene [102]. Almost similar pattern was observed between proPO and SP in the present study. Hence, SP and proPO genes could also be used as marker genes for assessing the immune status of the *P. vannamei* against V_P infection. PE can also be one of the marker genes used for assessing the immune status of the *P. vannamei* since there was typical pattern of down-regulation in V_P challenge trials.

Cuticle necrosis was observed among the field and challenged wet lab experimental cases suggested that this was due to the chitinoclastic and chitinolytic effect of V_P [103]. Muscle granuloma, melanization, necrosis and mineralization were noticed after 48 h.p.i. [104] and 168 h p.i. [74] in *P. vannamei* injected with *V. harveyi*. It indicated that the bacterial strain used in the present study was more virulent. The muscle lesions were localised to the site of injection in the present study [86]. Colonization of bacteria along with circumscribed haemocytic nodules formation was also observed [22,74]. Cardiac haemocytic aggregation and nodule formation was reported at 48 h p.i. but the same lesions were reported

after 168 h p.i. in *P. vannamei* injected with *V. harveyi* (Vh CAIM 1792) [74]. Excretory organ involved in removal of pathogenic organisms. Hence, it showed atrophy, edema, degeneration with architectural loss, fibrous tissue proliferation, calcification and necrosis.

Field natural outbreaks showed degeneration of glandular structures in the prehensile appendages with bacterial colonies [44]. Since the challenge was done by intramuscular injection, neither lesions nor bacterial colonization were found in the oral region, oesophagus, stomach and intestine of wet lab challenged animals. But, oesophagus revealed melanization [104] and loss of epithelial layer while stomach and intestine revealed swelling and degenerative changes with mucinous secretion in natural outbreaks. Midgut was not lined by cuticle and therefore it provided a favourable site for invasion of pathogens unlike foregut and hindgut [105]. Typically, no lesions were observed in V_P challenged animals. No lesions were found in ovary, testis, vas deferens, CNS and eye.

HP from V_P challenged animals showed hemocyte infiltration [57], concretions, development of granulomas [58], calcifications and necrosis after 96 h.p.i. Nodule formation with coagulated protein (eosinophilic coccoid bodies) in the interstitial space, haemocytic infiltration, granuloma formation, necrosis of hepatopancreatic cells, thickened basal lamina and granulomatous encapsulation were observed in severe epizootic of bacterial diseases in *P. monodon* [59] as observed in the present study. Severity of the pathological changes was directly proportional to the dose and duration of infection. Disrupted tubules with only the thin connective tissue capsule was observed in HP [106] in *P. monodon* fed with different carbohydrates which supported the fact that the HP was the prime organ in shrimps for digestion, absorption, metabolism and detoxification. Lesions like atrophy and necrosis of the hepatopancreatic tubules were also observed [47] on experimental *V. harveyi* infections in *P. vannamei* larvae. Copper had the capacity to cause sloughing of epithelial cells (<75%), infiltration of hemocytes (<75%) and reduction in R and B cells (100%) after 10 days exposure at salinities of >5, 5 and 10 practical salinity unit (psu) [107]. The severity of the lesions was more in the lowest salinities. But in the present study, similar lesions were observed with V_P infection wherein the copper concentration in water was absolutely zero. As observed in the present study, granulomatous reaction with a bacteria-filled center, dilated tubules, devoid of epithelial cells lining the tubule, varying degrees of sloughed, necrotic epithelial cells and cellular debris were observed in *P. vannamei* exposed to 35 ppm nitrate-N at 11 ppt [108]. Since the nitrate-N and salinity in the present study were 20 ± 0 mgL^{-1} and 2.08 to 2.12 ± 0.02 $g\ L^{-1}$, respectively which was considerably very less and within the accepted level for shrimp culture, it could be concluded that the lesions were not pathognomonic to V_P infection. Similar lesions were also observed by *V. alginolyticus* infections in *P. monodon* [28]. The lesions observed in the present study were comparable with the lesions observed in AHPND caused by a strain of V_P having the AP1, AP2, AP3 ($pirA^{vp}$), and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes [3,4,6,8–10,28,31,48,109–111]. The histopathology revealed that the lesions in the present study were in consistent with AHPND but did not meet the case definition for AHPND as provided in the draft OIE Aquatic Code.

Haematopoiesis was completely affected among V_P challenged animals as evidenced by drastic reduction in THC, SNGH, LNGH, SGH and LGH due to extensive depletion and degeneration of HPT [104]. Since HPT was severely depleted during vibriosis, both haemocytes death and haematopoiesis kinetically affected the number of haemocytes [41]. As observed in the present study, nodule formation in the HPT was reported elsewhere [59] in severe epizootic of bacterial diseases in *P. monodon*.

Gills were more susceptible to bacterial penetration because of their thin exoskeleton but gill fouling was not observed in the present study [108]. Lesions such as multiple melanised nodules formation [104] and spheroid formation, degeneration of stromal matrix, generalized necrosis were observed in lymphoid organ in *P. japonicus* [45] and *P. vannamei* [74] with *Vibrio* sp. PJ and *V. harveyi* (Vh CAIM 1792), respectively as observed in the present study.

From the present study, it was found that there was no AHPND causing isolate found among the shrimp ponds surveyed but the V_P isolated from the field was proved to cause severe pathology with considerable mortalities in juvenile *P. vannamei*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.06.020.

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