Full Length Article

SUSCEPTIBILITY STUDIES OF FARMED PACIFIC WHITE SHRIMP, *Penaeus vannamei* TO NON-AHPND STRAIN OF *Vibrio parahaemolyticus*

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ABSTRACT

Isolation and identification of bacteria from haemolymph, stomach and hepatopancreas (HP) were carried out from 37 farms in Tamil Nadu, India to find out the prevalence of Vibrio parahaemolyticus (Vp) infections with special reference to acute hepatopancreatic necrosis disease (AHPND). Bacterial identifications were done based on the morphological, physiological and biochemical characteristics. Vp isolates were identified by polymerase chain reaction (PCR) targeting Vp specific toxR and tlh genes, human pathogenic tdh and trh genes, and AHPND causing AP1, AP2, AP3 ($pirA^{vp}$) and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes. Histopathological and immunohistochemical (IHC) techniques were employed to identify the pathology due to Vp in HP tissues. Based on the morphological, physiological, biochemical and molecular characterization, 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 V. aeruginosa (2.7%). All isolates were negative for tdh, trh, AP1, AP2, AP3 and AP4 genes. Histopathology and IHC revealed that the HP was the prime organ affected in Vp infection. It further proved that the IHC could be used as an important tool to identify the establishment of infection in hepatopancreatic tissues.

Keywords: Acute hepatopancreatic necrosis disease (AHPND) - early mortality syndrome (EMS) - immunohistochemistry - polyclonal antibodies - *Penaeus vannamei* - *Vibrio parahaemolyticus*

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INTRODUCTION

The Asia-Pacific region, the top producer of aquaculture products in the world, is continuously besotted by emerging aquatic animal diseases that could lead to high mortalities and economic losses.

The disease, known as early mortality syndrome (EMS) also called as acute hepatopancreatic necrosis disease (AHPND), has caused large-scale die-offs of cultivated

shrimp in several Asian countries since 2012 (FAO, 2013). Shrimp farmers sensed a déjà vu with AHPND as they recalled the last decade hardship with white spot disease (WSD). The causative agent, a strain of *Vibrio parahaemolyticus* (V_P) with potential phage involvement, was later identified (Tran *et al*., 2013). Since V_P is a common inhabitant of coastal and estuarine environments all over the world, it is often found positive by polymerase chain reaction (PCR). But Immunohistochemistry (IHC) has an advantage of revealing the establishment of V_P infection in the tissues. Once the organism is established in shrimp body, disease outbreak occurs based on certain environmental conditions such as temperature, salinity, zooplankton, tidal flushing and dissolved oxygen (DO). The present study was carried out to find out any natural occurrence of AHPND. Since there was no polyclonal antibody based immunohistochemical (IHC) techniques available for diagnosis of V_P infection in shrimp, a new technique was developed and the same is discussed in this paper.

MATERIALS AND METHODS

Isolation and identification of *Vibrio* spp.

A total of 37 shrimp farms were screened from two important brackish water aquaculture coastal districts of Tamil Nadu, India. Initial bacterial isolations were taken from haemolymph, stomach and hepatopancreas (HP) of moribund and weak infected animals based on the dominant five colonies observed on the thiosulphate citrate bile salts sucrose (TCBS) plates (Zhou *et al*., 2012; Raja *et al*., 2017a; Raja *et al*., 2017b). Seventy four well-separated colonies two from each pond were selected, further screened and identified based on the morphological, physiological and biochemical characterization (Raja *et al*., 2017b; Alsina and Blanch, 1994; Raja *et al*., 2017c; Baumann and Shubert, 1984). PCR was carried out in a gradient thermal cycler (eppendorf, HiMedia) for *toxR* (Raja *et al*., 2017a; Kim *et al*., 1999), *tlh* (Raja *et al*., 2017a; Nordstrom *et al*., 2007), *tdh*, *trh* (Raja *et al*., 2017a; Bej *et al*., 1999), AP1, AP2 (Raja *et al*., 2017a; Flegel and Lo, 1999) AP3 ($pirA^{vp}$) and AP4 ($pirA^{vp}$ *and* $pirB^{vp}$) (Raja *et al*., 2017a; Dangtip *et al*., 2015) genes as per the protocols cited in the references. PCR products were eluted using GenElute™ Gel Extraction Kit (Sigma) as per the manufacturer's instruction and sequenced by 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. These isolates were stored at -20°C in tryptose soya broth (TSB) mixed with 25 per cent (v/v) glycerol.

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 Ethics Committee (IAEC) [O/o the Chairman, IAEC, Lr. No. 1679/DFBS/B/2015. Dated: 30.10.2015]. Healthy *P. vannamei* juveniles

with average body weight (ABW) of 5.05±0.23 g were collected from a shrimp farm. The experiment was conducted in triplicate with constant aeration system following the standard protocol. Six animals were culled to individual tubs (30 L capacity) of five experimental (10^4 to 10^8 cfu shrimp^{-1}), one control (injected with 50 µL of PBS) and one blank control group with a stocking density of 0.2 shrimp L^{-1}. The V_P isolate was prepared in PBS to get required concentration of 10^4 to 10^8 cfu 50 µL^{-1} and the shrimp were challenged intramuscularly in the third abdominal segment using 0.1 mL tuberculin syringe. Clinical signs and mortality pattern were observed every 15 min during the first hour post-injection (p.i.) followed by every 1 h until 6 h p.i. The experiment was continued for seven days and the animals were monitored every 12 h (Raja *et al*., 2017a; Selvin and Lipton, 2003). LD_{50} value was determined at 72 h p.i. by probit analysis method. Moribund/ dead and surviving shrimp were sampled for histopathological and IHC 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.

Raising polyclonal antibody against V_P

Immunisation program for raising anti sera in New Zealand White rabbits against V_P

Clinically two healthy New Zealand White rabbits (five months old) weighing approximately 1.5 kg, procured from Post Graduate Research Institute in Animal Sciences (PGRIAS), Rabbit Unit, Kattuppakkam, TANUVAS, India were used for raising antiserum (Jayaprakash, 2005). They were housed in stainless steel cages and kept in a well-ventilated animal room. They were provided with pelleted feed, fodder and drinking water *ad libitum*. The rabbits were acclimatised for two weeks before immunisation. Prior to immunisation, the rabbits were bled through marginal ear vein and the serum was tested for the presence of anti *Vibrio* antibodies by slide agglutination technique. This serum was used as control. Fur was removed using fine scissors at injection sites a day before injection. The V_P antigen was prepared from 18 h TSB young culture (10^9 cfu mL^{-1}) by centrifuging (6000 rpm for 10 min) and killed with 0.5 per cent formalin in normal saline. After 48 h incubation at 28±1°C, cells were washed thrice in PBS and adjusted to optical density (OD) 1.0 at A_{600nm} using phosphate buffer saline (PBS) to get ~10^9 cfu mL^{-1}. Formalin inactivation of V_P was confirmed for no growth on tryptose soya agar (TSA) plates. A 1:1 mixture of cell suspension and complete Freund's adjuvant (CFA) [Sigma] was mixed thoroughly and the rabbits were injected subcutaneously (0 day) 1 mL each. Incomplete Freund's adjuvant (IFA) was used for subsequent antigen boosts and injected on 7th, 14th and 21st day.

Antiserum collection and determination of antiserum titre

One week after the last injection of immunisation schedule (28th day), blood (~10 mL) was collected in a single bleed in clean sterile screw cap tubes and maintained in slanting position for 2 h at room temperature (RT). Serum was collected in microcentrifuge tubes, centrifuged at 3000 rpm for 10 min at 4°C to remove RBCs and other particulates.

The supernatant was collected in fresh sterile microcentrifuge tubes and the antiserum was maintained at -20°C until further use. The agglutination titre of antiserum was determined by slide agglutination method. The overnight grown culture of V_P from TSB with 1.5 per cent NaCl was harvested using PBS and adjusted to OD 1.0 at A_{600nm} to get a cell count of ~10^9 cfu mL^{-1} (Raja *et al*., 2017a; Raja *et al*., 2017b). Two separate drops (20 µL each) of above culture suspension were placed on the clean sterile glass slides. To one of the suspensions, a drop (20 µL) of control serum was added and mixed. To the other suspensions, one drop (20 µL) of antiserum diluted to the concentrations of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096 and 1:8192 was added and mixed. The slides were gently rocked for one to two minutes and observed visually and then confirmed under light microscope for agglutination. The titre of the antiserum was expressed as the reciprocal of the last dilution, which showed agglutination (Jayaprakash, 2005). A series of cross-reaction tests were done to verify the specificity of the antiserum by slide agglutination method. *Vibrio* spp. used in the tests were V_P (26 isolates), *V. harveyi* (16), *V. anguillarum* (13), *V. campbellii* (8), *V. mimicus* (5), *V. alginolyticus* (4), and *V. aeruginosa* (4).

Histopathology and Immunohistochemical (IHC) staining

The fixed samples were processed following standard histopathological procedures and stained with haematoxylin and eosin (H&E) (Bell and Lightner, 1988). Histopathological screening was carried out 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) (Raja and Jithendran, 2015). The presence of common viral diseases was also tested by PCR as described in OIE Manual of Diagnostic Test for Aquatic Animals 2015.

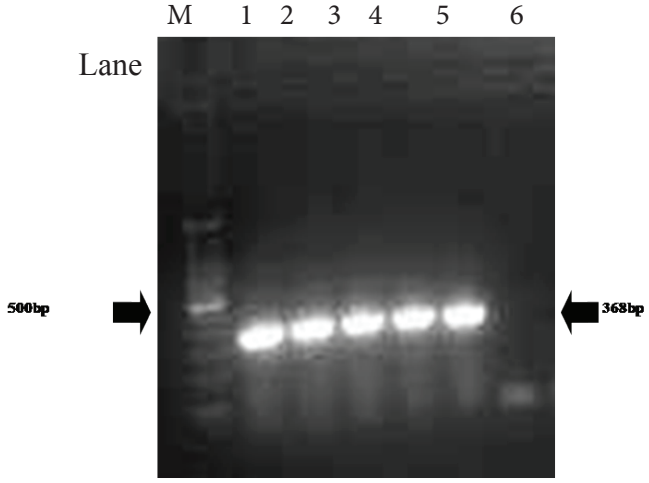
Standardised IHC staining for V_P antigen was carried out by polymeric methods (BioGenex, USA) as per the manufacturer's protocol with slight modification wherever needed. Tissue sections (3-4 µm thickness) were mounted on 3-Aminopropyl Triethoxy-Silane coated slides and incubated at 60°C for 1 h. The slides were deparaffinised in xylene for 3 min, in xylene and alcohol (1:1) for 3 min and then dexylenized in graded alcohols followed by rehydration in running tap water for 10 min. Antigen retrieval was done in 1M Tris EDTA (pH 9.0) at 120°C for 7 min followed by cooling to RT for 20 min and rinsing with PBS (pH 7.4) twice 5 min each. The slides were incubated with peroxidase block (3% hydrogen peroxide in water) for 10 min and rinsed with PBS (pH 7.4) twice 5 min each. Power block solution was applied for 10 min and the slides were incubated with primary antibody (raised in rabbit) against V_P (1:30 dilution) for 60 min at RT. After washing in PBS (pH 7.4) twice 5 min each, treated with Super Enhancer™ for 25 min followed by washing in PBS (pH 7.4) twice 5 min

each. The slides were incubated with mouse anti-rabbit IgG HRPO conjugate (secondary antibody) for 30 min at RT and washed in PBS (pH 7.4) twice 5 min each. Finally the slides were incubated with DAB or AEC chromogen kit + substrate buffer for 10 min and rinsed in PBS (pH 7.4) twice 5 min each. Counterstaining was done with haematoxylin for 30s and washed in running water for 5 min. Air dried slides were cleared with xylene and mounted with DPX, and examined under Olympus BX-51 microscope attached with image analyzer system (Image Proplus 5.1) for the development of reddish brown colour in the V_P positive tissue sample.

RESULTS

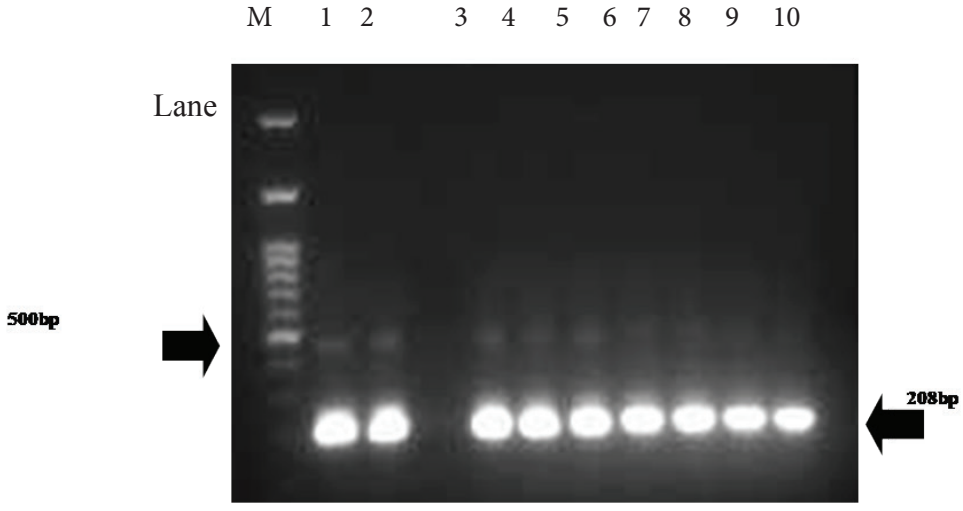
Isolation and identification of *Vibrio* spp.

Vibriosis due to V_P infection was observed among 48.6 per cent of the *P. vannamei* farms with 20 to 100 per cent mass mortality between 20 and 80 days of culture (DOC). Based on the morphological, physiological and biochemical parameters, 74 isolates collected were characterized as V_P (35.14%), *V. harveyi* (21.62%), *V. anguillarum* (16.22%), *V. campbellii* (10.81%), *V. mimicus* (8.11%), *V. alginolyticus* (5.41%) and *V. aeruginosa* (2.7%). Twenty six (35.14%) isolates were positive for V_P specific *toxR* and *tlh* genes (Fig. 1 and 2) as observed in morphological, physiological and biochemical characterization. NCBI accession numbers were obtained as KT360934 and KT360936 for *ToxR* gene and KT360935 and KT360937 for *tlh* gene forward and reverse, respectively. All the 74 isolates were found to be negative for human pathogenic and AHPND causing V_P genes.



M – 100 bp Marker
Lane 1 to 4 – Positive samples
Lane 5 – Positive control
Lane 6 – Negative control

Fig. 1: VP – toxR gene – Agarose gel showing the PCR product specific to toxR gene



M – 100 bp Marker
Lane 3 – Negative control
Lane 2 & 4 to 10 – Positive samples
Lane 1 – Positive control

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

Challenge trials

LD_{50} value was found to be 2.6 x 10^4 cfu $shrimp^{-1}$ at 72 h p.i. Clinical signs observed in LD_{50} trial were anorexia, lethargy, inactivity, opaque and whitish abdominal and tail musculature, abdominal muscle cramp, red discoloration, reddish pleural borders of antennae, uropods and telson, swollen tail fan, necrosis of exoskeleton and mortalities. No gross gill lesions were observed. Tissue homogenate of moribund shrimp HP proved the Koch's postulate.

Determination of antiserum titre and cross-reactions

The agglutination titre of the antiserum raised against V_P antigen was found to be 2048 by the slide agglutination method. The undiluted antiserum was found to react with all the isolates of V_P and *V. alginolyticus* whereas the PBS @ 1:64 diluted antiserum did not react with *V. alginolyticus*. Cross-reactions of antiserum with other *Vibrio* spp. were not observed.

Histopathology and IHC staining

HP from V_P infected field and LD_{50} trial animals showed disrupted tubules with undifferentiated cells containing abundant basophilic F cells replacing R and B cells (Fig. 3), hepatopancreatitis with increased intertubular space and haemocytic infiltration (Fig. 4), cystic HP tubules (Fig. 5), karyomegaly with prominent nucleoli

and sloughing of tubular epithelium (Fig. 6), regeneration of HP tubules with many mitotic figures (Fig.7), hyperplastic tubule epithelium with haemocytic infiltration (Fig. 8), rounding and sloughing of tubular epithelial cells into the lumen (Fig. 9), separation of tubule epithelium from myoepithelial fibers (Fig. 10), bacterial emboli occlusion in the tubular lumen (Fig. 11), and concretion in tubules with complete loss of tubular cells (Fig. 12). Severity of the pathological changes was directly proportional to the dose and duration of infection. IHC staining revealed that there was intense expression of V_P antigen in HP compared to normal (Fig. 13 and 14).

DISCUSSION

The present study showed 48.6 per cent of vibriosis outbreaks with 20 to 100 per cent mass mortality between 20 and 80 DOC in *P. vannamei* farms. Similar mortalities were observed within 40–50 DOC in the coast of Andhra Pradesh, India (Kumar *et al*., 2014). Vibriosis in shrimp was usually associated with major *Vibrio* spp. such as *V. harveyi*, *V. splendidus*, *V. cholerae*, *V. penaeicida, V. alginolyticus, V. anguillarum*, V_P, *V. vulnificus*, *V. campbelli*, *V. mimicus* and *V. nigripulchritudo*. Many researchers reported that the opportunistically pathogenic *Vibrio* species were the major cause of disease problems in shrimp aquaculture in several parts of the world (Tran *et al*., 2013; Raja *et al*., 2017a; Raja *et al*., 2017b; Alsina and Blanch, 1994; Raja *et al*., 2017c; Jayasree *et al*., 2000; Lavilla-Pitogo *et al*., 1990; Raja *et al*., 2015). The morphological, physiological and biochemical identification in the present study concurred with the results of previous researchers (Alsina and Blanch, 1994; Baumann and Schubert, 1984; Jayasree *et al*., 2000). The present study showed that the V_P isolates were predominant (35.14%) among *P. vannamei* samples as observed by Sudha *et al.* (2014) in shellfish samples but George (2002) identified *V. alginolyticus* (41.73%) as the most abundant species in *P. monodon* culture pond water. Silvester *et al.* (2015) confirmed 75 isolates as V_P using a PCR assay targeting the species-specific *tlh* gene and found that the prevalence of V_P was 71.6 per cent in the Cochin estuary and 53.3 per cent in the shrimp farms. Letchumanan *et al.* (2015) indicated that a total of 57.8 per cent isolates were positive for V_P with *toxR*-based PCR assay. The present study revealed that the prevalence of V_P in shrimp farms were lower (35.14%) than the previous results. Many researchers reported that the possession of particular haemolysin genes (*tdh*, *trh*, or both) was important to cause gastroenteritis in human (Bej *et al*., 1999; DePaola *et al*., 2003). However, a small portion of clinical strains carried neither of the genes, *tdh* and *trh* (Banerjee *et al*., 2014). So, it could not be possible to ascertain that the V_P isolates obtained in the present study was not pathogenic to human. Yang *et al.* (2014) reported that the transcriptional activator *toxR* was constantly found in AHPND causing V_P strains but were not in other strains. Even though, the V_P stains in the present study were positive to *toxR* gene, they were constantly negative to AHPND causing AP1, AP2, AP3 and AP4 genes (Liu *et al*., 2004).

The clinical signs and lesions observed in the present study were observed with the pathogenic strain of *V. alginolyticus* (Liu *et al*., 2004) and *V. harveyi* (Soto-Rodriguez

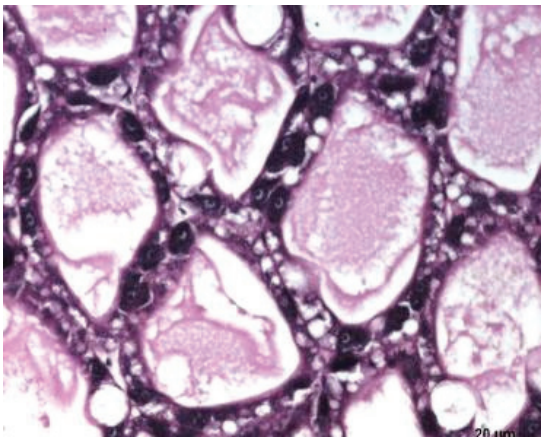


Fig. 3. HP – Tubules containing abundant basophilic F cells (arrow) replacing R and B cells H&E Scale bar : 20 µm

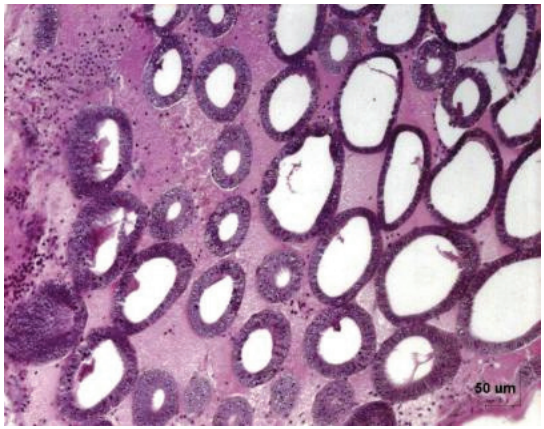


Fig. 4. HP – Hepatopancreatitis – Increased intertubular space with haemocytic infiltration and undifferentiated cells H&E Scale bar : 50 µm

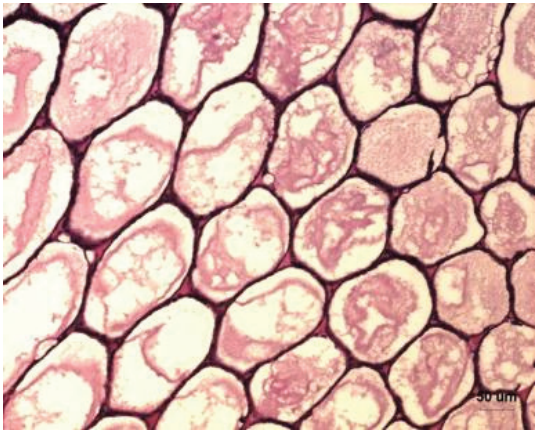


Fig. 5. HP – Cystic hepatopancreatic tubules H&E Scale bar : 50 µm

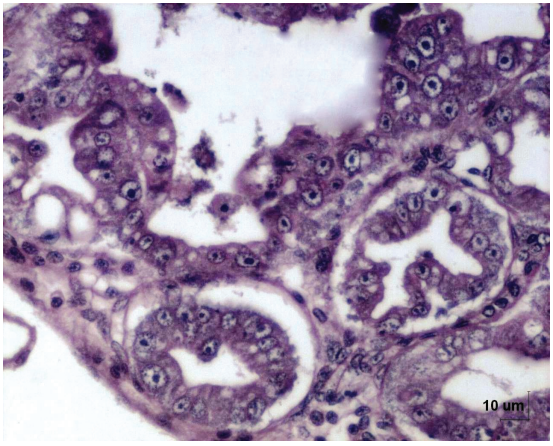


Fig. 6. HP – Karyomegaly with prominent nucleoli and sloughing of tubular epithelium H&E Scale bar : 10 µm

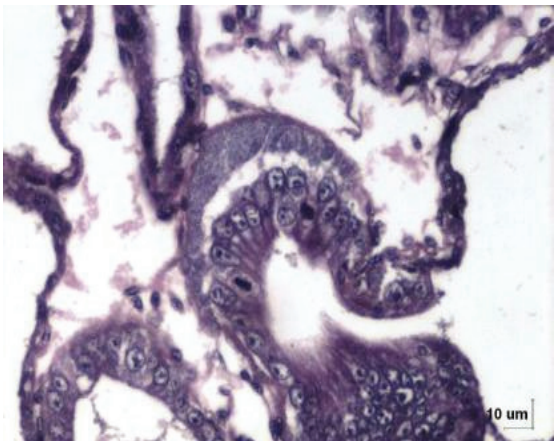


Fig. 7. HP – Regeneration of HP tubules with many mitotic figures

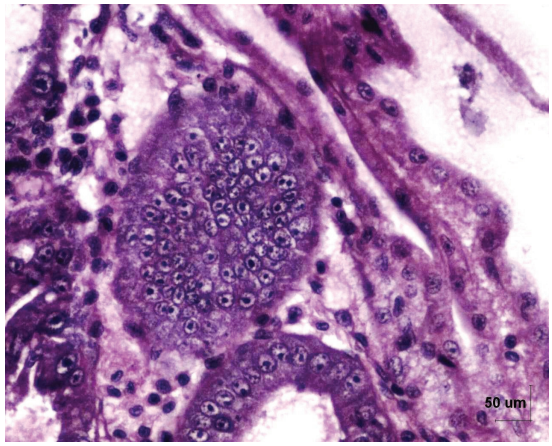


Fig. 8. HP – Hyperplastic epithelium - Haemocytic infiltration H&E Scale

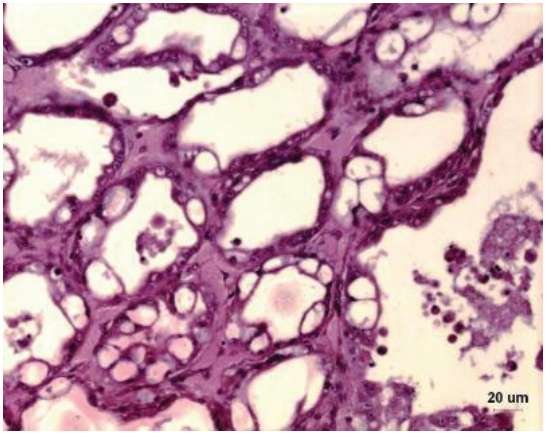


Fig. 9. HP – Rounding and sloughing of tubular epithelial cells into the lumen H&E Scale bar : 20 µm

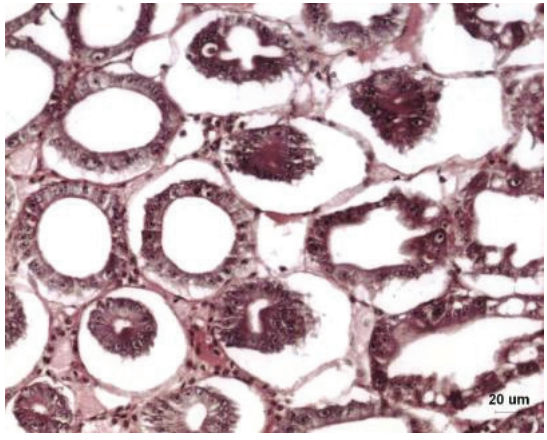


Fig. 10. HP – Separation of tubule epithelium from myoepithelial fibres H&E Scale bar : 20 µm

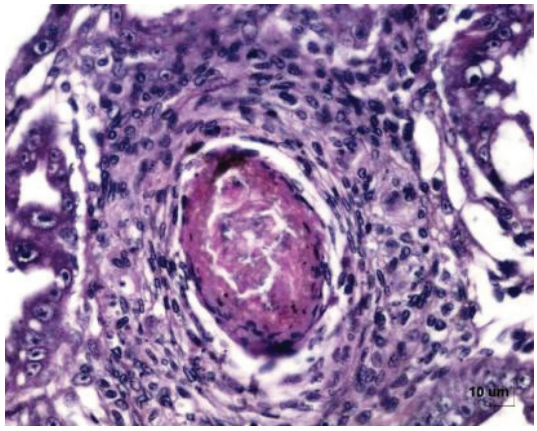


Fig. 11. HP – Bacterial emboli H&E Scale bar : 1 0 µm

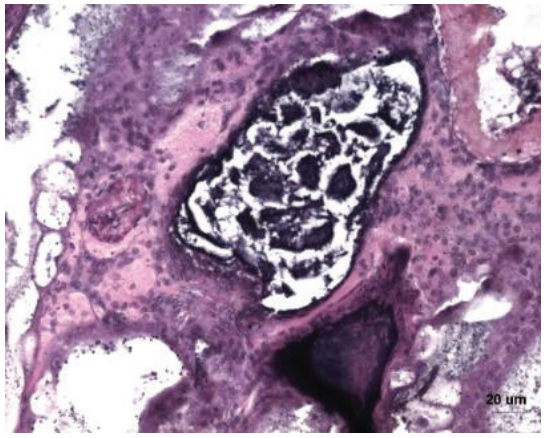


Fig. 12. HP – Concretion in tubules and complete loss of tubular cells H&E Scale bar : 20 µm

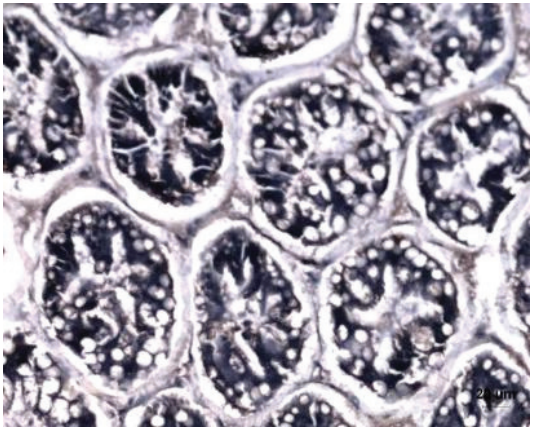


Fig. 13. HP – Control - IHC – No expression of VP antigen DAB brown Scale bar : 20 µm

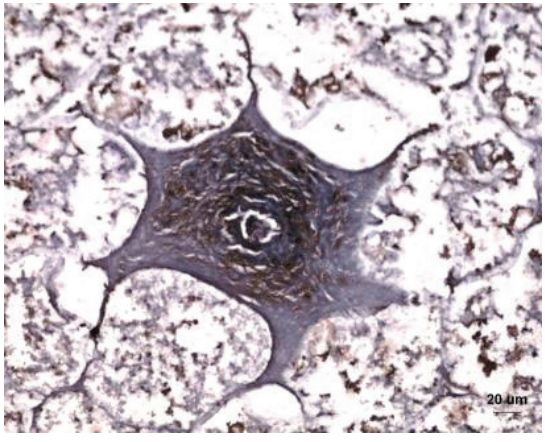


Fig. 14..HP - IHC – Concretion with intense expression of VP antigen DAB brown Scale bar : 20 µm

et al., 2012). 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 generalised septicaemia, which were observed in the present challenged trials with V_P (Lightner, 1993; Ruangpan and Kitao, 1991). 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 *V. harveyi* (Zhou *et al*., 2012). 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 and pale white muscles with >70 per cent mortality (Velmurugan *et al*., 2015). In the present study, signs and lesions close to AHPND (Tran *et al*., 2013) 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.

Polyclonal antibodies based rapid detection method was very much less expensive and less time consuming to produce when compared to the monoclonal antibody (mAb). In addition, mAb could only react with one specific epitope. Polyclonal antibodies could recognize multiple epitopes, making them more suitable to identify even small changes in the nature of the antigen. Polyc1onal antibodies were used for detection of denatured proteins and could also be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken and others, giving the users many options in experimental design (Greenfield, 1988). In the present study, rabbit was used for raising hyper immune serum. The homologous agglutination titre of the antiserum raised against V_P antigen was found to be 2048 by the slide agglutination method. But, Jayaprakash (2005) reported that there was agglutination of *V. alginolyticus* @ 1:4096. The undiluted antiserum was found to react with all the isolates of V_P and *V. alginolyticus* whereas the diluted antiserum with PBS @ 1:64 did not react with *V. alginolyticus*. Contrarily, Jayaprakash (2005) reported that there was cross-reaction of *V. alginolyticus* and V_P with undiluted un-purified antiserum whereas no cross-reaction was observed with the diluted antiserum (1:128). Cross-reactions of antiserum with other *Vibrio* spp. were not observed (Jayaprakash, 2005). The observed cross-reaction with *V. alginolyticus* might arise from the fact that, they share common antigens (Kita-Tsukamoto *et al*., 1993; Ruimy *et al*., 1994; Venkateswaran *et al*., 1998), and could be avoided by diluting the antiserum. No cross-reactions of antiserum to other genera explained its antigenic specificity unique to the bacterial species in question. Similar result was observed by Jayaprakash (2005) against both *V. alginolyticus* and V_P.

Severity of the pathological changes was directly proportional to the dose and duration of infection. Haemocyte infiltration, HP concretions, development of granulomas (Jayasree *et al*., 2012), calcifications and necrosis were observed after 96 h p.i. Similarly, Soonthornchai *et al.* (2010) also reported numerous haemocytic infiltrations and detachment of epithelial cells from the basement membrane of affected HP tubules in infected *P. monodon* after 48 h *V. harveyi* immersion. Nodule formation with coagulated

protein (eosinophilic coccoid bodies) in the interstitial space, haemocytic infiltration, granuloma formation, necrosis of HP cells, thickened basal lamina and granulomatous encapsulation were observed by Abraham *et al.* (2013) in severe epizootic of bacterial diseases in *P. monodon* as observed in the present study. Disrupted tubules with only the thin connective tissue capsule was observed in HP by Piedad-Pascual *et al.*(1983) in *P. monodon* fed with different carbohydrates which supported the fact that the HP was the prime organ in shrimp for digestion, absorption, metabolism and detoxification (Franceschini-Vicentini *et al*., 2009). Lesions like atrophy and necrosis of the HP tubules were also observed by Robertson *et al.* (1998) on experimental *V. harveyi* infections in *P. vannamei* larvae. IHC revealed that there was intense expression of V_P antigen in the HP. *In situ* hybridization (ISH) showed similar finding in HP of *Vibrio* spp. infected shrimp (Lightner *et al*., 2013). No other reports were available on IHC against V_P. But only reports were available on IHC against yellow-head virus (YHV) (Sithigorngul *et al*., 2002) and infectious myonecrosis virus (IMNV) (Kunanopparata *et al*., 2011). The present study concluded that the prevalence of V_P in the *P. vannamei* farms was predominant but there was no AHPND causing V_P isolates among the samples collected. Continuous monitoring and screening is required to ascertain non-emerging of AHPND in India in future.

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