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Note

Prevalence of *Vibrio* spp. with special reference to *Vibrio parahaemolyticus* in farmed penaeid shrimp *Penaeus vannamei* (Boone, 1931) from selected districts of Tamil Nadu, India

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ABSTRACT

Prevalence of *Vibrio* spp. with special reference to *Vibrio parahaemolyticus* (*Vp*) infection was assessed in *Penaeus vannamei* farms in Kancheepuram and Thiruvallur districts of Tamil Nadu during the period August 2014 to February 2015. Isolation and identification of bacteria from haemolymph, stomach and hepatopancreas of *P. vannamei* was done from all the farms for the prevalence of *Vp* infection. Based on the morphological, physiological and biochemical characterisation, 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%). The PCR results showed that there were 26 (35.14%) isolates positive for *Vp* specific *toxR* and *tlh* genes and negative for human pathogenic *tdh* and *trh* genes. All isolates were also negative for acute hepatopancreatic necrosis disease (AHPND) specific AP1, AP2, AP3 ($pirA^{vp}$) and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes. The study revealed that there was no AHPND causing *Vp* strain in farmed *P. vannamei* from two important coastal districts of Tamil Nadu, India.

Keywords: Acute hepatopancreatic necrosis disease (AHPND), Early mortality syndrome (EMS), *Penaeus vannamei*, Vibriosis, *Vibrio parahaemolyticus*

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, causing the collapse of *Penaeus monodon* culture. As an alternate species, *Penaeus. vannamei* (Boone, 1931) was introduced in India during 2009. The growth in production of this exotic species in India has been stupendous. Any disease outbreak in this sector is considered to be important nationally and globally. Recently, a newly emerging disease known as early mortality syndrome (EMS) in shrimp (*P. vannamei, P. monodon* and *P. chinensis*), renamed as acute hepatopancreatic necrosis disease [AHPND] (NACA, 2012; FAO, 2013) caused by a strain of *Vibrio parahaemolyticus* (V_P) has been reported to cause unusually high mortality and morbidity within the first 30 days of culture (DOC) and led to a significant loss of one billion USD in Asia-Pacific region (FAO, 2013; Tran *et al.,* 2013). In terms of impacts on trade, several countries have suspended or banned the import of live shrimp and/or all forms of shrimp products from countries affected by AHPND (FAO, 2013). Keeping all these factors in view, the present study was carried out on prevalence of AHPND causing V_P isolates and natural occurrence of V_P infection in *P. vannamei* farms of Tamil Nadu, India.

A total of 37 shrimp farms were screened during August 2014 to February 2015. Initial bacterial isolations were made from haemolymph of infected moribund animals using thiosulphate citrate bile salts sucrose (TCBS) agar plates (Zhou *et al.,* 2012; Ananda Raja *et al.,* 2017b). The stomach and hepatopancreas from representative shrimp were enriched in tryptone soya broth (TSB) for 12 h and spread plated on TCBS plates for further isolation and identification (Antonio *et al.,* 2015; Ananda Raja *et al.,* 2017b). Thus, 74 well-separated colonies two from each pond were selected, further purified, screened and identified based on the morphological, physiological, biochemical (Baumann and Schubert, 1984; Alsina and Blanch, 1994) and molecular characterisation. For long term preservation, these isolates were stored at -20°C in TSB with 25% (v/v) glycerol.

The isolates were cultured overnight in TSB and genomic DNA of all *Vibrio* isolates were extracted by standard phenol-chloroform method. DNA was also extracted from all *Vibrio* isolates by boiling for 10 min and used as templates for PCR assays (Hossain *et al.,* 2013; Han *et al.,* 2015). The isolated DNA was quantified by spectrophotometric method and checked in 1% agarose gel electrophoresis and stored at -80°C for further use. Polymerase chain reaction (PCR) was carried out in a gradient thermal cycler (Eppendorf) for *toxR* (Kim *et al.,* 1999), *tlh* (Nordstrom *et al.,* 2007), *tdh*, *trh* (Bej *et al.,* 1999), AP1, AP2 (Flegel and Lo, 2014), AP3 (*pirA*^{vp}) and AP4 (*pirA*^{vp} *and pirB*^{vp}) (Dangtip *et al.,* 2015) genes. The amplified PCR products were analysed by agarose gel electrophoresis and visualised under UV transilluminator. PCR products were eluted using GenElute™ Gel Extraction Kit (Sigma) as per the manufacturer's instructions and used for sequencing by di-deoxy chain termination method. The nucleotide sequence obtained was assembled using Auto assembler (ABI Prism, USA) software. The isolates were identified by BLAST (Basic local alignment search tool) search in the, NCBI (National Center for Biotechnology Information) database based on the maximum percentage of homology (Ananda Raja *et al.,* 2017b) and the identified sequences were submitted to the NCBI GenBank database.

Based on the morphological, physiological and biochemical parameters, 74 isolates collected were characterised (Table 1). The isolates were identified as *V. parahaemolyticus* (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 *Pseudomonas aeruginosa* (2.7%) (Ananda Raja *et al.,* 2017b). V_P was morphologically identified based on curved, rod-shaped and Gram negative characteristics (Baumann and Schubert, 1984; Johnson *et al.,* 2012; Janakiram *et al.,* 2013). V_P isolate was found to be sensitive to heat and reduction in cells to non-detectable levels at 55°C for 5 min (Lightner *et al.,* 2013) as observed in the present study. The present study also revealed that the V_P did not grow in peptone water at 4°C after 18 h incubation and did not survive at -18°C to -20°C after 30 days of incubation. Similarly, Zorriehzahra and Banaederakhshan (2015) reported that the refrigeration at 4°C for 38 days and freezing for 27 days caused considerable reductions in cells at non-detectable levels. Furthermore, morphological, physiological and biochemical identification concurred with the results of Alsina and Blanch (1994), Jayasree *et al.* (2000) and Janakiram *et al.* (2013). The present study showed that V_P isolates were predominant (35.14%) among *P. vannamei* samples as observed by Sudha *et al.* (2014) in shellfish samples but Rosalind George (2002) identified *V. alginolyticus* (41.73%) as the most abundant species found in *P. monodon* culture pond water followed by *V. harveyi* (18.7%), *V. marinus* (6.78%), V_P (5.15%), *V. vulnificus* (5.15%), *V. logei* (4.61%), *V. mimicus* (3.52%), *V. cholera* (3.25%), *V. orientalis* (2.71%), *V. splendidus II* (2.44%), *V. fluvialis* (1.24%), *V. fischeri* (1.08%), *V. metschnikovii* (1.08%), *V. anguillarum*-like (0.54%), *V. campbellii* (0.54%), *V. splendidus I* (0.54%), *V. damsela* (0.27%) and *V. cincinnatiensis* (0.27%). Vibriosis in shrimp was usually associated with major *Vibrio* spp. such as *Vibrio 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 (Lavilla-Pitogo *et al.,* 1990; Ishimaru *et al.,* 1995; Jayasree *et al.,* 2000; Alday-Sanz *et al.,* 2002; Ananda Raja *et al.,* 2012; Zhang *et al.,* 2012; Tran *et al.,* 2013; De Schryver *et al.,* 2014; Ananda Raja *et al.,* 2017a).

Twenty six (35.14%) isolates were positive for V_P specific *toxR* and *tlh* genes (Fig. 1, 2) as observed in morphological, physiological and biochemical characterisation (Ananda Raja *et al.,* 2017b). Similarly, 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% in the Cochin Estuary and 53.3% in the shrimp farms. Letchumanan *et al.* (2015a, b) indicated that a total of 57.8 and 44.4% 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. All the 74 isolates were found to be negative for human pathogenic *tdh* and *trh* genes (Ananda Raja *et al.,* 2017b). Those isolates were also found to be non AHPND causing V_P strains using standard protocols (Kumar *et al.,* 2014; Ananda Raja *et al.,* 2017b). The PCR products of *toxR* forward and reverse showed that they had 99 and 98% homology with V_P RIMD 2210633 chromosome 1 while the PCR products of *tlh* forward and reverse confirmed that they had 96 and 99% homology with V_P RIMD 2210633 chromosome 2. Respective NCBI accession numbers obtained were KT360934 and KT360935 for *ToxR* gene and KT360936 and KT360937 for *tlh* gene (Ananda Raja *et al.,* 2017b). The PCR products of *toxR* forward and reverse showed that they had 99 and 98% homology with V_P RIMD 2210633 chromosome 1. There was deletion of T at 29th, replacement of A with T at 85th, G with A at 232nd and A with G at 265th positions in forward sequence while it was deletion of T and A at 4th and 30th position and replacement of C with T at 67th, T with A at 190th, A with T at 259th and 292nd positions in

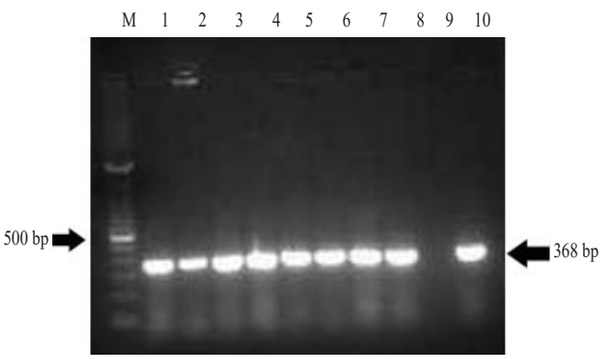
Table 1. Morphological, physiological and biochemical characterisation of bacterial isolates collected from shrimp ponds

Characteristics	V_p (26)	*V. harveyi* (16)	*V. anguillarum* (12)	*V. campbellii* (8)	*V. mimicus* (6)	*V. alginolyticus* (4)	*P. aeruginosa* (2)
Configuration	Round	Round	Round	Round	Round	Round	Round
Margin	Entire	Entire	Serrated	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Convex	Convex	Convex
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Density	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Texture	Moist	Moist	Moist	Moist	Moist	Moist	Moist
Colour on:	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
Nutrient agar							
TCBS agar	Blue-green	Yellow	Yellow	Green	Green	Yellow	Green
Cell morphology	Rod often curved	Straight rod	Curved rod	Rod	Short rod	Straight rod	Rod often curved
Size	Short	Short	Short	Short	Short	Short	Short
Arrangements	Single	Single	Single	Single	Single	Single	Single
Flagella	Single	Polar and lateral flagella	Single	Single	Single	Single	Single
Swarming	-	V	-	-	-	V	-
Motility	+	+	+	+	+	+	+
Luminescence	-	-	-	-	-	-	+
Gram staining	-	-	-	-	-	-	-
Spore forming	-	-	-	-	-	-	-
Growth in peptone with NaCl:							
0%	+	-	-	-	+	-	-
2%	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+
8%	+	+	+	+	-	+	+
10%	-	-	-	-	-	+	-
Survival at -18 to -20° C	-	-	-	-	-	-	-
Growth in peptone at:							
4°C	-	-	-	-	-	-	-
20°C	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+
40°C	+	+	-	-	+	+	+
55° C	-	-	-	-	-	-	-
Anaerobic growth	-	-	-	-	-	-	-
Decarboxylation of							
Arginine	-	-	+	-	-	-	+
Lysine	+	+	-	+	+	+	-
Ornithine	+	±	-	-	+	+	-
Production of extra-cellular enzymes							
Gelatinase	+	+	+	+	+	+	+
Urease	±	-	±	-	-	-	±
Gas production from Glucose	-	-	-	-	-	-	-
Oxidation/ Fermentation	-/+	-/+	-/+	-/+	+/-	-/+	+/-
Methyl red test	+	+	+	+	-	+	+

cont....

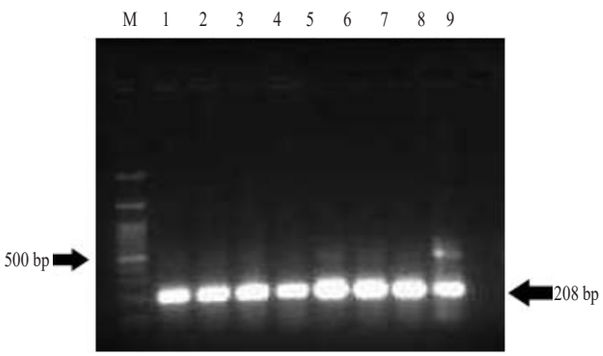
Characteristics	V_P (26)	*V. harveyi* (16)	*V. anguillarum* (12)	*V. campbellii* (8)	*V. mimicus* (6)	*V. alginolyticus* (4)	*P. aeruginosa* (2)
Voges-Proskauer	-	+	+	-	-	+	+
Nitrate reduction	+	-	+	+	-	+	+
Indole	+	±	+	+	-	-	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	-	+	+	-	+	+
Citrate utilisation	+	+	+	+	+	+	+
Ortho-Nitrophenyl-β-Galactosidase (ONPG)	-	-	+	-	+	-	-
Acid production							
Positive	Ce, Fc, Gl, Ma, Mb, Mn, Mo, Sb and Te	Fc, Gl, Ma, Mn, Mo, Su and Te	Ar, Ce, Fc, Ga, Gl, Ma, Mn, Mo, Sb, Su, Te and Xy	Ce, Fc, Ma, Mn, Mo and Te	Fc, Gl, Ma, Mb, Mn, Mo, Sb and Te	Fc, Ga, Gl, Ma, Mb, Mn, Mo, Su and Te	Ad, Ar, Ce, Fc, Ga, Gl, Ma, Mb, Mn, Mo, Sb, Te and Xy
Negative	Ad, Ar, Du, Ga, Is, La, Rf, Sa, Su and Xy	Ad, Ar, Ce, Du, Ga, Is, La, Mb, Rf, Sa, Sb and Xy	Ad, Du, Is, La, Mb, Rf and Sa	Ad, Ar, Du, Ga, Gl, Is, La, Mb, Rf, Sa, Sb, Su and Xy	Ad, Ar, Ce, Du, Ga, Is, La, Rf, Sa, Su and Xy	Ad, Ar, Ce, Du, Is, La, Rf, Sa, Sb and Xy	Du, Is, La, Rf, Sa and Su

Ad - Adonitol, Ar - Arabinose, Ce - Cellobiose, Du - Dulcitol, Fc - Fructose, Ga - Galactose, Gl - Glucose, Is - Inositol, La - Lactose, Ma - Maltose, Mn - Mannitol, Mo - Mannose, Mb - Melibiose, Rf - Raffinose, Sa - Salicin, Sb - Sorbitol, Su - Sucrose, Te - Trehalose, Xy - Xylose



Lane M – 100 bp Marker
Lane 1 to 8 – Positive samples
Lane 9 – Negative control
Lane 10 – Positive control

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



Lane M – 100 bp Marker
Lane 1 to 7 – Positive samples
Lane 8 – Negative control
Lane 9 – Positive control

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

reverse sequence, respectively. The PCR products of *tlh* forward and reverse had 96 and 99% percent homology with V_P RIMD 2210633 chromosome 2. There was deletion of A at 15th, 121st and 160th, replacement of A with C at 118th, C with T at 144th and A with T at 164th positions in forward sequence while it was replacement of T with G at 11th positions in reverse sequence, respectively (Ananda Raja *et al.,* 2017b). Natural deletion (Tinwongger *et al.,* 2014) and CAP induced mutation (Nithya Quintal *et al.,* 2009) were possible in V_P as demonstrated by earlier researchers. It needs further validation to find out the possibilities of deletion and mutation leading to the isolate becoming pathogenic to shrimp.

Differentiation of virulent bacterial strains from nonvirulent strains was difficult using traditional culture methods based on growth phenotypes (Takahashi *et al.,* 2005). So, PCR assays are becoming increasingly popular for detection of pathogenic bacterial strains targeting

virulent genes (Hossain *et al.,* 2013). Compared to the conventional bacterial identification methods, PCR based molecular techniques were found to be rapid and reliable for accomplishing a comprehensive detection of V_P. Since, the percentage of rRNA (Kita-Tsukamoto *et al.,* 1993) and *gyrB* (Venkateswaran *et al.,* 1998) 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 identified to be involved in the regulation of many other genes in *V. cholerae* (DiRita, 1992). But, the degree of homology of the *toxR* gene between V_P and *V. cholerae* was only 52%, which was much lower than that of the rRNA gene (92% identity) (Kita-Tsukamoto *et al.,* 1993; Kim *et al.,* 1999). Kim *et al.* (1999) and Anjay *et al.* (2014) found that the *toxR* gene was well conserved among V_P and therefore, they established a *toxR*-targeted PCR protocol for the specific detection of V_P. Yang *et al.* (2014) reported that the transcriptional activator *toxR* was constantly found in AHPND causing V_P strains along with zona occludens toxin, accessory cholera enterotoxin and transmembrane regulatory protein *toxS* 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 ($pirA^{vp}$) and AP4 ($pirA^{vp}$ and $pirB^{vp}$) genes (Kumar *et al.,* 2014). Hossain *et al.* (2013) developed a multiplex PCR for detection of V_P based on *groEL*, *tdh* and *trh* genes with the sensitivity of 200 pg DNA. The same level of sensitivity was observed in the present study in detecting the species-specific marker *tlh* and virulence marker *toxR* genes.

Sahilah *et al.* (2014) characterised 44 genomic DNA of V_P for the presence of *tox*R, *tdh* and *trh* gene and found 37 isolates positive towards *tox*R gene; while, none were positive to *tdh* and *trh* genes as found in the present study. Disagreement to the present findings, Letchumanan *et al.* (2015a, b) observed 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 (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 were non-pathogenic to humans. The present study concluded that prevalence of V_P in *P. vannamei* farms was high but no AHPND causing V_P isolates were found in the samples tested. Continuous surveillance studies are required to monitor and detect AHPND emergence in India.

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