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Note

Screening of pathogens from a biosecured pacific white shrimp (*Penaeus vannamei*) farm in Kattur, Tamil Nadu

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ABSTRACT

Screening for presence of various pathogens were carried out in one of the biosecurity maintained shrimp farms in Kattur Village of Tamil Nadu. Sampled shrimps exhibited pinkish discolouration of the body, pale hepatopancreas and antennal cut indicating bacterial infection. Similarly, there was also large scale size variation and reduced growth compared to the days of culture. Samples were screened for bacterial and four (including three OIE-listed) viral pathogens. The samples were also subjected to histopathological investigations. Based on the biochemical tests, the isolated bacteria was identified as *Vibrio parahaemolyticus*. Metalloprotease gene-specific PCR further confirmed the isolates to be *V. parahaemolyticus*. PCR was carried out to further investigate the early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) strain status of these isolates and were found to be negative. The samples were found to be positive for white spot syndrome virus (WSSV) by second step PCR and infectious hypodermal and haematopoietic necrosis virus (IHHNV) by direct PCR. All these were negative for monodon baculovirus, yellow head virus and gill associated virus. Infection of samples by WSSV and IHHNV were further confirmed by histopathology. The finding of the present study indicated the reduction in growth and size variation due to bacterial infection by *V. parahaemolyticus* and viral infection by IHHNV. Though bisosecurity was maintained in the farm, the pathogens are suspected to be transmitted through the seed or improper pond preparation.

Keywords: Biosecurity, Diagnosis, Pathogens, *Penaeus vannamei*

Shrimp culture is an ever growing popular food producing sector in India. *Penaeus vannamei* Boone, 1931 is one of the economically important penaeid shrimp which is extensively cultivated in many countries including India during the past few years. India was a leading exporter of black tiger shrimp until this market was virtually replaced by *P. vannamei.* The success of culturing this exotic species using imported specific pathogen free (SPF) stocks in the Indian sub-continent has led to more farmers adopting this species in the country (Regunathan and Kitto, 2011). Cultivation of penaeid shrimp in Asia has changed dramatically since 2002 because of the widespread adoption of this species.

With intensification of penaeid aquaculture industry, occurrence of diseases has increased which poses a major risk and primary constraint to the growth of shrimp culture industry in many parts of the world including India. Important shrimp viral diseases caused by white spot syndrome virus (WSSV), hepatopancreatic parvo-like virus (HPV), infectious hypodermal haematopoietic necrosis virus (IHHNV), yellow head virus (YHV) and monodon baculo virus (MBV) have been reported from India (Manivannan *et al*., 2002; Bondad-Reantaso *et al*., 2005; Flegel, 2006; Kalaimani *et al*., 2013). To date, In India WSSV remains the most important virus causing mass mortalities in shrimp farms, leading to severe economic losses (Rai *et al*., 2009). IHHNV is one of the major viral pathogens of penaeid shrimps worldwide (Lightner and Redman, 1998). Though IHHNV infection does not cause mortality in stocks of *P. vannamei* and *Penaeus monodon*, it can result in a disease condition, which is characterised by reduced growth as well as deformities of cuticle and rostrum (Bell and Lightner, 1984; Kalagayan *et al.,* 1991; Primavera and Quinitio, 2000). Recently Otta *et al.* (2014) reported WSSV and IHHNV infection from *P. vannamei* farms in Tamil Nadu. Except for a few reports, there is no information available on the diseases of *P. vannamei* from Indian shrimp culture. With this background, the present investigation was undertaken to screen for the presence of various pathogens in one of the biosecurity maintained shrimp farm at Kattur Village of Tamil Nadu.

P. vannamei samples were collected from a shrimp farm at Kattur Village in Minjur Taluk, Thiruvallur District of Tamil Nadu (Fig.1). The ponds were having bird and crab fence. Adequate reservoir ponds were there for preliminary water treatment and release into ponds. Entry to the farm was regulated through partial sanitisation. Samples were collected from two different shrimp ponds and screened for various pathogens. Samples were collected using cast net, four from each corner and one from the middle of each pond. Pooled samples from each pond were taken for pathogen screening. For PCR analysis, samples were collected in 90% ethanol. Samples for histopathological analysis were fixed with Davidson's fixative (Bell and Lightner, 1988).

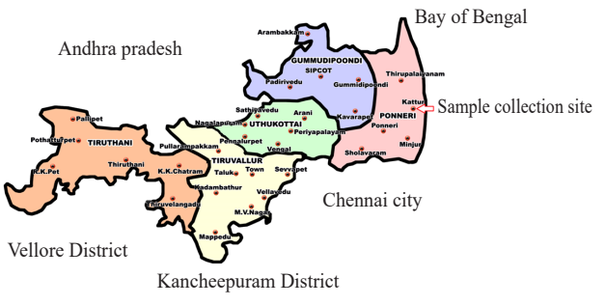

Fig. 1. Map of Thiruvallur District showing sample collection site of Kattur Village

For microbiological analysis, hepatopancreas, haemolymph and gut were directly dissected out and inoculated into Zobell marine agar and TCBS agar plates and Zobell marine broth (HiMedia, Mumbai, India). Pure culture of the predominant isolate was obtained by repeated streaking till individual colonies were obtained. Colonies from TCBS plates were subjected to biochemical tests for identification of *Vibrio* sp. as per Alsina and Blanch (1994).

For bacterial DNA extraction for PCR amplification, hepatopancreas and stomach tissues were collected and separately inoculated into the alkaline peptone water of 50 ml each and then kept at 30ºC in shaking incubator for 5 h. After incubation, 5 ml culture was taken for bacterial DNA extraction using CTAB (Bruce *et al.*, 1993). Briefly, the culture was first centrifuged at 8000 rpm for 5 min, supernatant was removed and the pellet was re-suspended in 50 µl distilled water. From this, 10 µl was taken and 567 µl of TE buffer, 30 µl of 10% SDS and 3 µl of 20 mg ml^{-1} proteinase K were added. The contents were mixed thoroughly and kept at 37ºC for 1 h. After incubation, 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl solution was added, mixed thoroughly and again incubated for 10 mins at 65ºC. Equal volume of chloroform/isoamyl alcohol was added to this, mixed by inverting and then centrifuged at 12000 rpm for 5 min. Clear aqueous phase was transferred to a fresh tube to which equal volume of phenol/chloroform/isoamyl alcohol was added and centrifuged at 12000 rpm for 5 min. Again the clear aqueous phase was transferred to a fresh tube and 0.6 volume of isopropanol was added into it and centrifuged at 12000 rpm for 5 min to precipitate the DNA. From this, supernatant was removed, pellet was washed with equal volume of 70% ethanol and air dried. This pellet was suspended in 20 µl distilled water. The extracted DNA was used for PCR reaction. To obtain crude bacterial DNA extract, 10 µl of bacterial pellet was directly resuspended into 100 µl of distilled water and then boiled for 10 mins in a water bath. From this, 1 µl of crude extract was used for PCR reaction.

For *V. parahaemolyticus* species confirmation, PCR was carried out with metalloprotease gene-specific VPM primers (Luan *et al*., 2007). To determine the EMS/AHPND status, PCR was carried out as described by Flegel and Lo (2014). The reaction volume of 25 µl containing 2x master mix (Biorad, Germany), gene specific forward and reverse primers (10 pm), 1 µl template DNA and PCR grade water was amplified in a thermocycler (Biorad, Germany). The details of primers and PCR cyclic conditions are detailed in Table 1. PCR products (5 µl each) were resolved on 1.5% gel and photographed with a gel documentation system (Biorad).

For histopathological analysis, whole animal was first injected with Davidson fixative at hepatopancreas, 3rd and 6th segments and then preserved in Davison fixative. Totally three different tissues namely gill, hepatopancreas and pleopods were taken for this study. The tissue samples were processed in automatic tissue processor (Leica, Germany), embedded in paraffin wax, sections were cut using rotary microtome (Thermofisher Scientific, Germany), mounted on glass slides and stained using hematoxylin and eosin (Bell and Lightner, 1988).

The collected shrimp samples had pinkish discolouration of the body, pale hepatopancreas and antennal cut (Fig. 2 and 3) indicating bacterial infection with large scale size variation and reduced growth compared to the days of culture.

Fig. 2. Pinkish colouration on the body (left) with normal shrimp (right)

Table 1. Details of primers used in the study

Pathogen/ Disease	PCR step	Primer sequence (5'-3')	Product size (bp)	PCR cycle conditions	Reference
EMS/ AHPND	Single step	F TCACCCGAATGCTCGCTTGTGG R CGTCGCTACTGTCTAGCTGAAG	700	94ºC 5 min, 30 cycles of 94ºC 30 s, 60ºC 30 s, 72ºC 60 s and 72ºC for 10 min	Flegel and Lo (2014)
Vibrio parahaemolyticus	Single step	VPM-1 CAGCTACCGAAACAGACGCTA VPM-2 TCCTATCGAGGACTCTCTCAAC	675	94ºC 5 min 30 cycles of 94ºC 30 s, 60ºC 30 s, 72ºC 45 s and 72ºC for 10 min	Luan *et al.* (2007)
WSSV	First step	F1 ATCATGGCTGCTTCACAGAC R1 GCTGGAGAGGACACAAGACAT	982	94ºC 4 min 39 cycles of 94ºC 1 min, 55ºC 1 min, 72ºC 2 min and 72ºC for 5 min	Kimura *et al.* (1996)
	Second step	F2 TCTTCATCAGATGCTACTGC R2 TAACGCTATCCAGTATCACG	570		
IHHNV	Single step	356F ATCGGTTCACTACTCGGA 356R TCGTACTGGCTGTTCATC	356	95ºC 5 min 35 cycles of 95ºC 30 s, 55ºC 30 s, 72ºC 1 min and 72ºC for 7 min	Nunan *et al*. (2000); Tang *et al*. (2007)
	Single step	392F GGGCGAACCAGAATCACTTA 392R ATCCGGAGGAATCTGATGTG	392		
MBV	First step	F1 CGATTCCATATCGGCCGAATA R1 TTGGCATGCACTCCCTGAGAT	533	95ºC 5 min 39 cycles of 95ºC 30s, 65ºC 30 s, 72ºC 1 min and 72ºC for 7 min	Belcher and Young (1998)
	Second step	F2 TCCAATCGCGTCTGCGATACT R2 CGCTAATGGGGCACAAGTCTC	361	95ºC 5 min 39 cycles of 95ºC 30 s, 60ºC 30 s, 72ºC 1 min and 72ºC for 7 min	
YHV-GAV	First step	GY1 GACATCACTCCAGACAACATCTG GY4 GTGAAGTCCATGTGTGTGAGACG	794	95ºC 5 min 34 cycles of 95ºC 1 min, 66ºC 1 min, 72ºC 1 min and 72ºC for 5 min	Wongteerasupaya *et al.*, (1997); Cowley *et al.* (2004)
	Second step	GY2 CATCTGTCCAGAAGGCGTCTATGA GY3 ACGCTCTGTGACAAGCATGAAGTT GY6 GTAGTAGAGACGAGTGACACCTAT	YHV - 277 GAV - 406		

Fig. 3. Shrimp sample with antennal cut

Based on the biochemical tests conducted for isolated bacteria, the species was identified as *Vibrio parahaemolyticus* (Table 2). *V. parahaemolyticus* is the dominant species in shrimp affected by red disease and tail necrosis (Jayasree *et al*., 2006). The result obtained in this study corroborates that of Alagappan *et al.* (2010) who found incidence of *V. parahaemolyticus* in shrimp ponds of Tamil Nadu and markedly higher incidence in animal surface and tissue samples. Likewise, pathogenic *V. parahaemolyticus* has been characterised from the infected shrimp samples of Tamil Nadu (Alagappan *et al*., 2013).

Table 2. Results of biochemical tests

Amino acid decarboxylase test			Salt tolerance (% NaCl)					G	I	SC	MR	VP	OG	Sugar utilisation			
Ly	Ar	Or	0	3	6	8	10							M	A	S	D
+	-	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	+
+	-	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	+
+	-	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	+
+	-	+	-	+	+	+	-	+	+	+	+	-	+	+	+	-	+

Amino acids used: Ly - Lysine, Ar - Arginine, Or - Ornithine; G - Gelatinase test; I - Indole test; SC - Simmons citrate test; MR-VP - Methyl red-Voges Proskauer test; OG - Ortho-Nitrophenyl-β-galactosidase test; Sugars used: M - Mannitol, A - Arabinose, S - Sucrose, D - D-Glucosamine

Gene-specific PCR (VPM-*V. parahaemolyticus* Metalloprotease gene) further confirmed the isolates as *V. parahaemolyticus* (Fig. 4). PCR was carried out to find out the EMS/AHPND strain status of these isolates using AHPND primers and all were found to be negative (Fig. 5). Thus, the results indicated that S/AHPND strain of *V. parahaemolyticus* (Tran *et al*., 2013), was not present in the culture system included in this study.

The samples were found to be positive for WSSV by second step PCR (Fig. 6) and IHHNV by direct PCR (Fig. 7). All these were negative for MBV and other exotic pathogens (YHV/GAV) (Fig. 8 and 9). WSSV and IHHNV infections with mortality of *P. vannamei* in culture ponds of Tamil Nadu was reported by Otta *et al.* (2014). They also found that the analysed samples were negative for taura syndrome virus, yellow head virus and infectious myonecrosis virus. This supports our result that majority of the vannamei culture is infected by the two viruses namely WSSV and IHHNV. It has also been reported that IHHNV infection on *Penaeus vannamei* results in development and growth abnormalities (Kalagayan *et al*., 1991; Lightner *et al*., 1992) and size reduction (Lightner and Redman, 1998). Similar size variation and growth reduction were also observed in the present samples. However, the typical body abnormality as observed for runt deformity syndrome (RDS) was not found in any of the shrimps during the sampling.

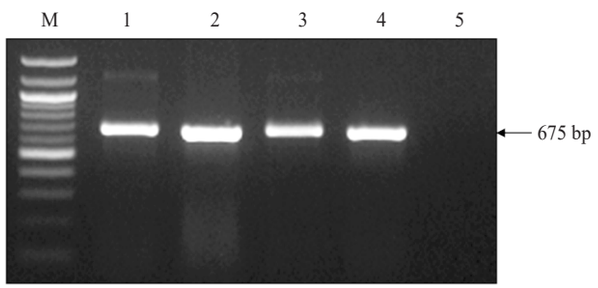

Fig. 4. PCR for the detection of *V. parahaemolyticus*
Lane M-100 bp DNA ladder, Lane-1: Bacterial DNA extract from HP, Lane-2: DNA from the HP isolated bacteria, Lane-3: Bacterial DNA extract from stomach, Lane-5: Negative control

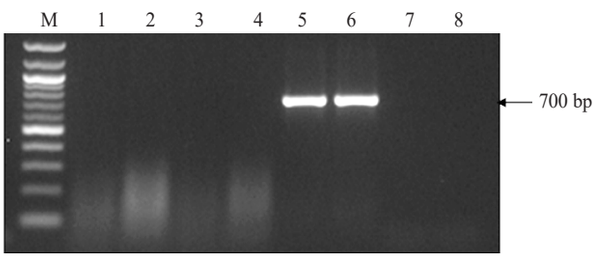

Fig. 5. PCR for the detection of shrimp Acute Hepatopancreatic necrosis disease/early mortality syndrome (AHPND/EMS)
Lane-M: 100 bp DNA ladder, Lane-1: Bacterial DNA extract from HP, Lane-2: DNA from the HP isolated bacteria, Lane-3: Bacterial DNA extract from stomach, Lane-5 and 6: Positive control, Lane-7 and 8: Negative control

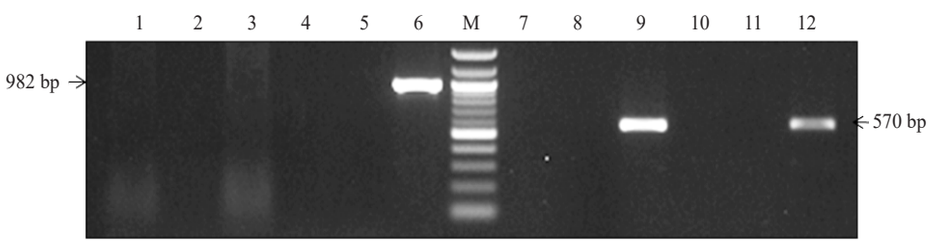

Fig. 6. PCR for the detection of white spot syndrome virus (WSSV) in shrimp
Lane-M: 100 bp DNA ladder, Lanes 1-6: First step PCR reaction (Lane-1: Undiluted sample I, Lane-2: Diluted sample I, Lane-3: Undiluted sample II, Lane-4: Diluted sample II, Lane-5: Negative control, Lane-6: Positive control). Lanes 7-12: Second step PCR (Lane-7: Undiluted sample I, Lane-8: Diluted sample I, Lane-9: Undiluted sample II, Lane-10: Diluted sample II, Lane-11: Negative control, Lane-12: Positive control)

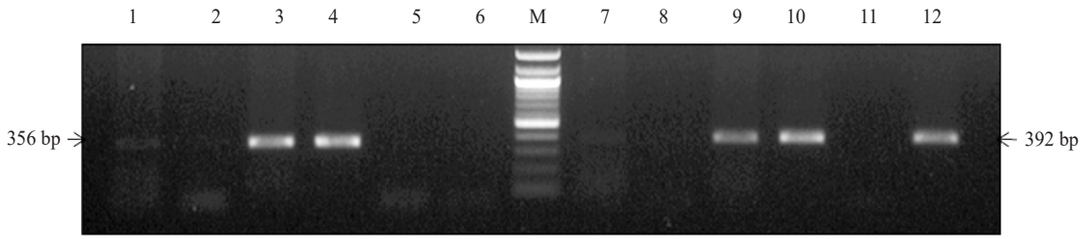

Fig. 7. PCR for the detection of infectious hypodermal and haematopoetic necrosis virus (IHHNV) in shrimp
Lane-M: 100 bp DNA ladder, Lanes 1-6: PCR for the detection of 356 bp (Lane-1: Undiluted sample I, Lane-2: Diluted sample I, Lane-3: Undiluted sample II, Lane-4: Diluted sample II, Lane-5 and 6: Negative control). Lanes 7-12: PCR for the detection of 392 bp (Lane-7: Undiluted sample I, Lane-8: Diluted sample I, Lane-9: Undiluted sample II, Lane-10: Diluted sample II, Lane-11: Negative control, Lane-12: Positive control)

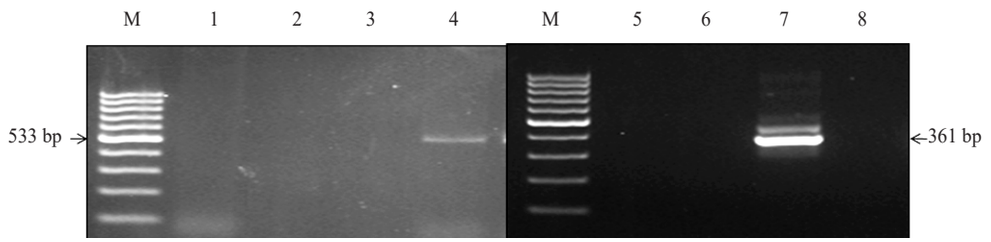

Fig. 8. PCR for the detection of monodon baculovirus (MBV) in shrimp
Lane-M: 100 bp DNA ladder, Lane-1 to 4: First step PCR (Lane-1: Sample I, Lane-2: Sample II, Lane-3: Negative control, Lane-4: Positive control). Lanes-5 to 8: Second step PCR (Lane-5: Sample I, Lane-6: Sample II, Lane-7: Positive control, Lane-8: Negative control

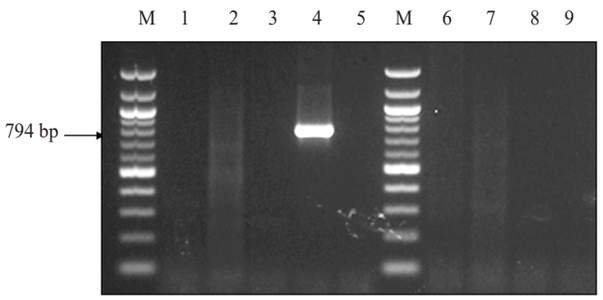

Fig. 9. PCR for the detection of yellow head virus (YHV)/Gill associated virus (GAV) in shrimp
Lane-M: 100 bp DNA ladder, Lanes-1 to 5: First step PCR (Lane-1: HP, Lane-2: Gill, Lane-3: Pleopod, Lane-4: Positive control, Lane-5: Negative control. Lanes-6 to 9: Second step PCR (Lane-6: HP, Lane-7: Gill, Lane-8: Pleopod, Lane-9: Negative control)

Infection of samples by WSSV and IHHNV was further confirmed by histopathology through the presence of intranuclear inclusion bodies. Typical WSSV inclusion bodies and Cowdry type A inclusion bodies of IHHNV infection were observed in the gills. Likewise, Otta *et al*. (2014) reported the presence of inclusion bodies for WSSV and IHHNV through histopathological analysis in *P. vannamei*.

The finding of present study indicated the reduction in growth due to *Vibrio parahaemolyticus* and size variation due to IHHNV. *V. parahaemolyticus* was isolated from the shrimp samples as a sole group indicating their pathogenic status. However, the samples were negative for specific EMS strain by PCR. This shows the strain of *V. parahaemolyticus* which cause EMS/AHPND was not present in the culture system selected for this study. Though biosecurity was maintained in the farm, the pathogens are suspected to be transmitted through the seed or improper pond preparation.

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