



Detection of viruses in the exotic shrimp *Penaeus vannamei* Boone, 1931 cultured in India

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ABSTRACT

Pacific white shrimp *Penaeus vannamei* Boone, 1931, is a recently introduced species in India. *P. vannamei* samples, collected from various shrimp farms of Karnataka, India were subjected to polymerase chain reaction (PCR) based detection of whitespot syndrome virus (WSSV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), taura syndrome virus (TSV) and infectious myonecrosis virus (IMNV). Out of the 81 shrimp samples analysed, 41 samples (50.6%) were found positive for WSSV and four (4.9%) were positive for IHHNV. Among 41 WSSV positive samples, 20 (48.7%) samples were found positive for WSSV by 1st step PCR, while the remaining 21 (51.2%) samples were positive by nested PCR. WSSV positive samples were further confirmed by dot blot hybridisation assay. However, clinical signs/disease symptoms were not observed in any of the shrimp samples tested positive for the viruses.

Keywords: Aquaculture, Dot-blot hybridization, IHHNV, *Penaeus vannamei*, SPF shrimp, WSSV

Introduction

Aquaculture has been in practice for centuries in small rural settings but during the past few decades it has developed into a commercial activity worldwide. (Walker and Winton, 2010). Shrimp culture has become the major aquaculture activity in many developing countries to earn foreign exchange by exporting to developed countries, including United States of America, European Union and Japan (Bondad-Reantaso *et al.*, 2005). Asia dominated the world in the production of cultured shrimp, mainly through the culture of black tiger shrimp, *Penaeus monodon* for a long time. However, its dominance has been successfully replaced by the introduction of the Pacific white shrimp, *Penaeus vannamei*, in many shrimp producing countries (Flegel, 2006b). The preference for *P. vannamei* over *P. monodon* is due to its rapid growth rate, tolerance to high stocking density, tolerance to low salinity and temperature, relatively low protein requirement, high survival rate during larval rearing and good marketability (Wayban *et al.*, 1995; Briggs *et al.*, 2004). Large scale use of specific pathogen free (SPF) *P. vannamei* has led to the highest production of cultivated shrimp in Asia (Flegel, 2006a).

Shrimp production in India which was mainly dependent on *P. monodon*, declined drastically to 75,997

t in 2008, from a relatively high export production of 1,25,668 t in 2004 (MPEDA, 2016). This sharp decline was mainly attributed to exclusive culture of *P. monodon* and the associated disease problems. Lack of alternate species suitable for culture as well as the success associated with the culture of *P. vannamei* in neighboring countries prompted Indian shrimp industry to urge Government of India to permit the import of disease-resistant, SPF *P. vannamei* to India in 2009 (Remany *et al.*, 2010). Consequent to the introduction of *P. vannamei*, the area under culture in India rapidly increased from 283 ha in 2009-10 to 50,240 ha in 2014-15 (MPEDA, 2016). However, the major concern was the possibility of introduction of pathogens along with exotic species (Tu *et al.*, 1999; Phalitakul *et al.*, 2006; Senanan *et al.*, 2009). Though, *P. vannamei* is supposed to be resistant to viruses which mainly infect *P. monodon*, there are reports of viral disease problems associated with this species (Browdy *et al.*, 1993; Lightner, 1999; Motte *et al.*, 2002; Rodriguez *et al.*, 2003; Mijangos-Alquisires *et al.*, 2006; Afsharnasab *et al.*, 2009; Dos Santos-Braz *et al.*, 2009).

Since the culture of *P. vannamei* is rapidly expanding in India, it was felt necessary to investigate on the viruses associated with the species during culture operation.

The present study investigated the presence of important shrimp viruses in *P. vannamei* cultured in the state of Karnataka, India.

Materials and methods

Sample collection

A total of 81 *P. vannamei* samples were collected from shrimp farms of Uttar Kannada and Udupi districts in Karnataka, India. The samples were immediately transported to the laboratory on ice and fixed in 95% ethanol.

Detection of shrimp viruses

The samples were checked for the presence of important DNA viruses such as WSSV, HPV, MBV, IHHNV and for the RNA viruses *viz.*, TSV and IMNV which are considered pathogenic in shrimp aquaculture. The samples preserved in 95% ethanol, were rehydrated in distilled water for 1 h and viral DNA was extracted as per Otta *et al.* (2003). The shrimp DNA extracted from gills, pleopods and cuticle were used for detection of WSSV and IHHNV, while DNA from hepatopancreas was used for detection of MBV and HPV. Total RNA was extracted from gills, pleopods and muscle using 750 µl of TRI Reagent® (Invitrogen, USA) for the detection of important RNA viruses. After 5 min incubation with vigorous mixing, 200 µl of chloroform was added and incubated for 10 min

at room temperature, followed by centrifugation at 14,000 g for 10 min at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 500 µl of 100% isopropanol and was precipitated by centrifugation at 14,000 g for 10 min at 4°C. The resultant RNA pellet was washed by adding 70% (v/v) ethanol, air dried and finally suspended in 50 µl of RNase-free water. The first strand cDNA was synthesised as per manufacturer's protocol (Invitrogen, USA) using virus specific reverse primers.

PCR analysis

The presence of DNA viruses such as WSSV, MBV, HPV and IHHNV were tested by one-step PCR and nested PCR. For the detection of RNA viruses, TSV and IMNV, OIE recommended protocols were followed (Nunan *et al.*, 1998; Poulos and Lightner, 2006; OIE, 2010). The primer sequences as well as PCR protocol used for the detection of both DNA and RNA viruses and resultant amplicon sizes are detailed in Table 1.

The PCR reactions were performed in 30 µl reaction volume containing 1X PCR buffer, 5 pmol of each primer, 50 µmol of each dATP, dCTP, dGTP and dTTP, 0.9 units of Taq DNA polymerase (HiMedia, Mumbai), 2 µl of nucleic acid extract and adjusted to a final volume of 30 µl using ultrapure water. Amplification was executed in a thermalcycler (BioRad, USA). PCR products were

Table 1. Primer sequences and PCR protocols used for the detection of shrimp viruses

Virus	Primer name	Primer sequence (5'-3')	Product size (bp)	Reference
WSSV	IK1	TGGCATGACAACGGCAGGAG	486	Hossain <i>et al.</i> (2001)
	IK2	GGCTTCTGAGATGAGGACGG		
	IK3	TGTCATCGCCAGCACGTGTGC	310	
	IK4	AGAGGTTCGTCAGAGCCTAGTC		
HPV	H441F	GCATTACAAGAGCCAAGCAG	441	Phromjai <i>et al.</i> (2002)
	H441R	ACACTCAGCCTCTACCTTGT		
	HPVnF	ATAGAACGCATAGAAAACGCT	265	Umesha <i>et al.</i> (2006)
	HPVnR	CAGCGATTTCATCCAGCGCCACC		
MBV	MBV 1.4F	GCATTACAAGAGCCAAGCAG	533	Belcher and Young (1998)
	MBV 1.4R	ACACTCAGCCTCTACCTTGT		
	MBV1.4NF	ATAGAACGCATAGAAAACGCT	361	
	MBV1.4NR	CAGCGATTTCATCCAGCGCCACC		
IHHNV	IHHNV648F	CGATTCCATATCGGCCGAATA	648	Rai <i>et al.</i> (2009)
	IHHNV648R	TTGGCATGCACTCCCTGAGAT		Tang <i>et al.</i> (2007)
	IHHNV309F	TCCAATCGCGTCTGCGATACT	309	
	IHHNV309R	CGCTAATGGGGCACAAGTCTC		
IMNV	4587F	CGACGCTGCTAACCATACAA	328	Poulos and Lightner (2006)
	4914R	ACTCGGCTGTTTCGATCAAGT		
	4725 NF	GGCACATGCTCAGAGACA	139	
	4863 NR	AGCGCTGAGTCCAGTCTTG		
TSV	9992F	AAGTAGACAGCCGCGCTT	231	Nunan <i>et al.</i> (1998)
	9195R	TCAATGAGAGCTTGGTCC		

resolved by agarose gel electrophoresis containing ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) and visualised in a Geldoc system (BioRad, USA).

Dot blot hybridisation

The PCR product of 310 bp amplified from the DNA extracted from WSSV infected *P. monodon* using primer pair IK 3-4 was purified using DNA clean up kit (Qiagen, Germany) before being used for digoxigenin (DIG) labelling. WSSV probe was prepared by DIG random primed DNA labeling using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland) following manufacturer's instructions. Each of the sample DNA was denatured by boiling for 10 min and immediately chilled on ice. Two microliters of the sample was blotted on to a nylon membrane (Biodyne B Membrane, Pall Life Science, USA) and the nucleic acid was fixed to the membrane by cross linking with UV-light (UVC 500, Hoefer, USA). DIG-labelled probes were used for hybridisation to membrane blotted nucleic acids according to standard methods. Signal colour was developed using anti-DIG conjugated with alkaline phosphatase (Roche, Germany) and NBT-BCIP substrate (Roche, Germany) as directed by the manufacturer. DNA extracted from gill tissue of healthy *P. vannamei* was used as negative control.

Results and discussion

A total of 81 cultured *P. vannamei* samples, from Uttar Kannada and Udupi districts of Karnataka, India were subjected for the detection of DNA viruses like WSSV, HPV, MBV and IHNV using both one step PCR and nested PCR whereas RNA viruses like TSV and IMNV using OIE recommended protocols. Out of the 81 samples, 45 (55.6%) were found positive for selected viruses and in 36 (44.4%) samples, the tested viruses were not detected.

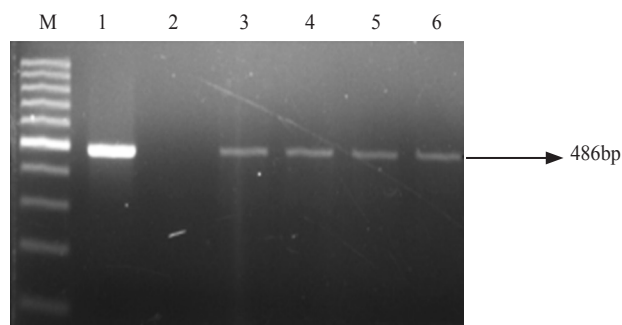


Fig. 1. PCR detection of WSSV in *P. vannamei* using WSSV specific primer sets IK1 and IK2

(1) M: 100 bp DNA ladder; (2) Lane 1: 1st step PCR amplification of positive control; (3) Lane 2: 1st step PCR amplification of negative control; (4) Lane 3 to 6: 1st step PCR amplification of DNA extracted from *P. vannamei*

Incidence of WSSV in cultured *P. vannamei* is reported from Iran (Pazir *et al.*, 2011), Taiwan (Cheng *et al.*, 2013), Ecuador (Rodriguez *et al.*, 2003) and Indonesia (Ferasyi *et al.*, 2015). WSSV infection has caused mass mortalities of farmed *P. vannamei*, bringing huge loss to shrimp farmers around the world like Ecuador (Calderon *et al.*, 1999), Brazil (Cavalli *et al.*, 2008) and Iran (Afsharnasab *et al.*, 2009). Likewise, many researchers have also reported *P. vannamei* mortality in farms due to WSSV alone or in combination with other pathogens from India (Balakrishnan *et al.*, 2011; Otta *et al.*, 2014; Sanathkumar *et al.*, 2014). In another study from India, high prevalence (72%) of WSSV in cultured *P. vannamei* samples has been reported (Moger *et al.*, 2011). In our study, out of the 81 samples tested for WSSV by PCR, 41 samples (50.6%) were found positive for the virus. Among the 41 WSSV positive samples, 20 samples were found positive for WSSV by 1st step PCR itself, suggesting high load of the virus (Fig. 1) and rest of the 21 samples were found positive by nested PCR by yielding PCR product size of 310 bp (Fig. 2). The shrimps detected positive for WSSV did not show any clinical signs of white spots. The results indicated that even though WSSV is present in the animal, this may not progress into disease unless the animal is stressed due to the deterioration of environmental parameters as observe by other workers (Tsai *et al.*, 1999; Ananda Raja *et al.*, 2012a,b). Similarly, Flegel *et al.* (2004) reported the presence of multiple viruses in non-diseased *P. monodon*.

Due to the high prevalence of WSSV in the samples, the presence of specific viral DNA was confirmed by subjecting it to dot-blot hybridisation. Dot-blot assays of DNA extracts from shrimp yielded positive results (Fig. 3) for WSSV that matched corresponding samples with positive bands for WSSV in PCR gel analysis. The

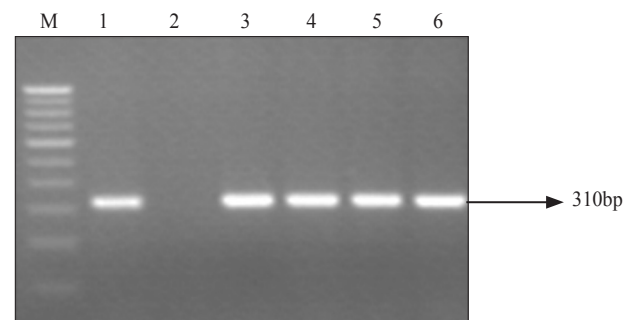


Fig. 2. Nested PCR detection of WSSV in *P. vannamei* using WSSV specific primer sets IK3 and IK4

(1) M: 100 bp DNA ladder; (2) Lane 1: Nested PCR amplification of positive control; (3) Lane 2: Nested PCR amplification of negative control; (4) Lane 3 to 6: Nested PCR amplification of DNA extracted from *P. vannamei*

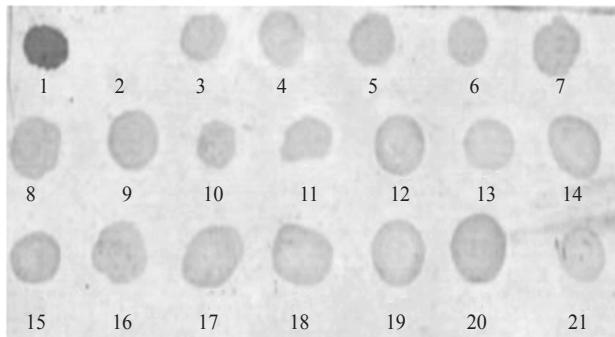


Fig. 3. Confirmation of WSSV infection of *P. vannamei* by dot-blot hybridisation

Spot 1: Positive control; Spot 2: Negative control; Spots 3-12: *P. vannamei* samples (non-nested PCR positives); Spots 13-21: *P. vannamei* samples (nested PCR positives)

positive DNA dot-blot hybridisation reactions confirmed that the positive amplification of WSSV gene in PCR is from shrimp genuinely infected with virus and ruled out the chances of amplification of DNA from mechanically present viruses or other contaminants.

IHHNV infection results in bent or deformed rostrums, wrinkled walking legs, cuticular roughness and other cuticular deformities leading to stunted growth in *P. vannamei* (Kalagayan *et al.*, 1991). Earlier researchers have reported the low (32%) and high (66%) prevalence of IHHNV infection of cultured *P. vannamei* samples in Indian farms (Moger *et al.*, 2011; Otta *et al.*, 2014). In the present study, IHHNV was detected in four shrimp samples out of 81 samples screened, giving an overall occurrence of 4.9%. This virus was detected in samples only by nested PCR, yielding a product size of 309 bp (Fig. 4), indicating lower load of the virus. Though runt deformity syndrome is the most typical sign of IHHNV infection, the infected shrimps in this study were devoid of such symptoms. This could be due to lack of optimal

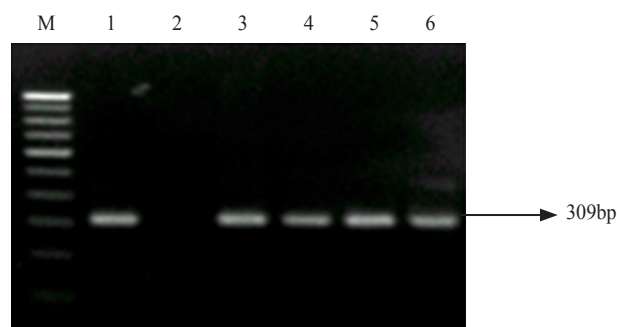


Fig. 4. Nested PCR detection of IHHNV in *P. vannamei*

(1) M: 100 bp DNA ladder; (2) Lane 1: Nested PCR amplification of positive control; (3) Lane 2: Nested PCR amplification of negative control; (4) Lane 3 to 6: Nested PCR amplification of DNA extracted from *P. vannamei*

viral concentration to cause the disease or due to chronic nature of the infection as reported by Bell and Lightner (1984) and Primavera and Qunitio (2000). The absence of clinical signs in our study could be attributed to the collection of samples during the early part of culture operation (40 days of culture). Other DNA viruses such as MBV, HPV and RNA were not detected in any of the samples during the present study.

The present study provides information regarding the prevalence of infectious viruses of *P. vannamei* cultured in Karnataka, India. *P. vannamei* was introduced to India as a hardy and disease-resistant species. Further, SPF broodstocks are being used by hatcheries in India to produce *P. Vannamei* post-larvae. In spite of this, cultured shrimp are found infected by the existing virulent viruses. Our results indicate that WSSV is highly prevalent in Indian aquaculture environment and *P. vannamei* is highly susceptible to WSSV in Indian culture conditions. The present study strongly emphasises the need to follow the best management practices (BMP), including strict biosecurity measures to get rid of viral pathogens in farms for the sustainable aquaculture of *P. vannamei* in India.

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