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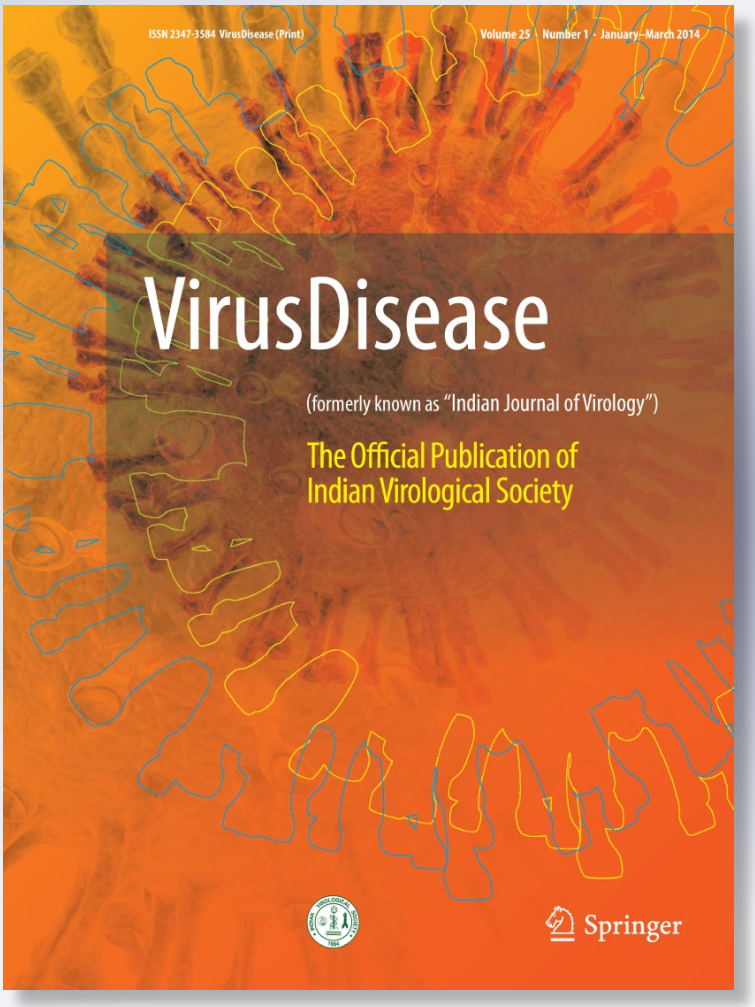
Indian Journal of Virology

ISSN 0970-2822
Volume 25
Number 1

Indian J. Virol. (2014) 25:63-68
DOI 10.1007/s13337-013-0180-x



Springer

VirusDis. (January–March 2014) 25(1):63–68
DOI 10.1007/s13337-013-0180-x

ORIGINAL ARTICLE

Association of dual viral infection with mortality of Pacific white shrimp (*Litopenaeus vannamei*) in culture ponds in India

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Received: 17 November 2012 / Accepted: 11 November 2013 / Published online: 30 November 2013

Abstract Pacific white shrimp, *Litopenaeus vannamei* has been introduced recently for culture practice in India. Though SPF stocks are imported for larval production and thereafter culture practice, these are prone to infection with the existing viruses in the environment. Here we report mortality of *L.vannamei* in several farms in India with minimum biosecurity. The shrimp were harvested early within 50–72 days of culture due to the onset of disease and consequent mortality. As per the analysis carried out, the shrimp were infected with two virus, white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV). About 80 % of the samples collected had either or both of the viruses. A majority of these samples (60 %) had dual infection with WSSV and IHHNV. Infection of shrimp with WSSV and IHHNV could be detected both by PCR and histopathology. Some of the samples had either exclusively WSSV infection or IHHNV infection and were also harvested before the completion of the required culture period. All the samples analyzed were negative for taura syndrome virus, yellow head virus and infectious myonecrosis virus. While it is difficult to point out the exact etiological agent as the cause of mortality, strict biosecurity measures are advisable for the continuity of *L. vannamei* culture in India.

Keywords *Litopenaeus vannamei* · Dual viral infection · Shrimp mortality · India

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Introduction

For quite a long time, viral diseases have been one of the major constraints in the progress of shrimp aquaculture industry throughout the world. Although several viruses have been reported to infect shrimp [13], two of the major viruses, white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) have been found to be the most prevalent and widespread ones. After its first appearance in eastern Asia during 1992 to 1993 [7], WSSV has spread rapidly to different parts of the world. During the process of detection and identification, this virus has been named differently by several investigators [5]. WSSV is a double-stranded rod-shaped DNA virus and belongs to the family Nimaviridae [17]. This virus is well known for its high virulence and thereby bringing severe mortality within a short span of time [19, 34]. IHHNV was discovered as early as 1981 when it was highly virulent to *Penaeus stylirostris* resulting in high mortality [14]. This is a single stranded, small and non-enveloped DNA virus [16]. Except *P. stylirostris*, this virus has not been reported to be responsible for severe mortality in other penaeid shrimps. However, poor growth, slow mortality and external deformity caused by IHHNV in different shrimps have been reported [2, 8].

Culture of tiger shrimp, *Penaeus monodon*, was in steady progress globally, until 1992-1993, when it was struck by WSSV [6]. Within a short span of time, the disease became pandemic and the farmers suffered severe loss as most of the farms were virtually wiped out because of this. Shrimp aquaculture in India too suffered significantly due to WSSV infection [9, 22]. Subsequently, several attempts made by the farmers faced repeated failures and they continued to bear the loss. Difficulties in captive breeding of tiger shrimp could not make it possible for the

development of specific pathogen free (SPF) and genetically improved strains with disease resistance. Therefore, the entrepreneurs started looking for the alternatives. In this context, Pacific white leg shrimp (*Litopenaeus vannamei*) was the most preferred one for its several qualities and possibility of developing SPF strain was the most important one. Special attempts were also made to screen wild stock for the prevalence of WSSV and IHHNV, to acquire SPF *L. vannamei* for inclusion into the US Marine Shrimp Farming Programme's genetic breeding program [20]. Taking all these into consideration, there was an increased interest for the farmers of non-native region to adopt this species for farming practice. With the creation of regulatory process by the Ministry of Agriculture through Coastal Aquaculture Authority (CAA), farmers were allowed to undertake hatchery production and culture practice of this species in several coastal regions of India.

Unfortunately, the SPF status of this shrimp has not made it possible to avoid the existing diseases in the areas where it was introduced. The threat of shrimp viruses, particularly that of WSSV and IHHNV continued to remain. WSSV infection brought huge loss to *L. vannamei* farmers in Mexico [15] and Brazil [4]. Similarly, mortality in *L. vannamei* farms due to WSSV infection has also been reported from India [1]. Though not reported from culture ponds, it has been shown on experimental basis that IHHNV can severely affect the growth and survival of *L. vannamei* [27]. Presence of this virus in cultured *L. vannamei* as a single agent [10, 33] or co-infection of this virus either with WSSV [23, 35] and other viruses [28, 32] have been reported.

Here we report the co-infection of WSSV and IHHNV and subsequent mortality of *L. vannamei* from several shrimp farms located in Tamil Nadu state of India.

Materials and methods

Sampling

Shrimps, *L. vannamei,* were collected from different culture ponds of Tamil Nadu, India. Wherever possible, moribund samples were collected directly from the ponds and processed at the pond site for different analysis. Samples for histopathology were immediately fixed in Davidson's fixative as described by Bell and Lightner [3]. For PCR analysis, organs such as pleopod, telson and gill were collected in 90 % ethyl alcohol and RNAlater. All the samples were transported to lab for further analysis.

PCR analysis

DNA and RNA from the respective samples were extracted using the IQ 2000 kit (Gene Reach Biotechnology, Taichung, Taiwan) and following manufacturer's instruction. PCR amplification of WSSV was carried out as per Kimura et al. [11]. A pair of outer primers (5′-ATC ATG GCT GCT TCA CAG AC-3′ and 5′-GGC TGG AGA GGA CAA GAC AT-3′) amplifying a product of 982 bp were used for 1st step amplification and a pair of inner primers (5′-TCT TCA TCA GAT GCT ACT GC-3′ and 5′-TAA CGC TAT CCA GTA TCA CG-3′) designed to amplify a product of 570 bp were used for nested PCR for WSSV. For the amplification of IHHNV, two sets of primers described in OIE protocol [21] were used in this study. The 1st set of primers (5′-GGG CGA ACC AGA ATC ACT TA-3′ and 5′-ATC CGG AGG AAT CTG ATG TG-3′) amplifies a product of 392 bp and the 2nd set (5′-ATC GGT GCA CTA CTC GGA-3′ and 5′-TCG TAC TGG CTG TTC ATC-3′) gives a product of 356 bp size. For final reaction, 1 × Taq DNA polymerase master mix red (BIO-RAD, CA, USA), 10 pm of each primers and 2 µl of extracted DNA was taken in a total volume of 25 µl. The mixture was run in a gradient thermocyler (Eppendorf, Hamburg, Germany) using the specific programmes reported. Detection of IHHNV was also verified by IQ 2000 kit (Gene Reach Biotechnology, Taichung, Taiwan) following the protocol described in the kit. For the amplification of 3 RNA viruses, yellow head virus (YHV), taura syndrome virus (TSV) and infectious myconecrosis virus (IMNV), IQ 2000 kits were used. All the IQ 2000 kits used for this study were designed for the detection of the respective virus by nested PCR.

The PCR-amplified products were separated in ethidium bromide-incorporated 1 % agarose gel using 0.5x Tris Boric acid EDTA buffer. The products were either loaded directly (in case of master mix red) or mixed with 6x dye buffer (for IQ 2000 kit) and then loaded into separate wells. The gels were observed and photographed with a gel documentation system (BIO-RAD, CA, USA).

Histopathology

Samples injected with Davidson's fixative were kept at room temperature for 48 h to facilitate the penetration of the fixative after which it was transferred to 70 % alcohol. Individual organs were then dissected out and arranged in embedding cassette (Leica, Nussloch, Germany). The tissues were processed with different grades of alcohol and other reagents as described by Bell and Lightner [3]. The paraffin-embedded tissues were cut into sections of 5 µm thickness using a rotary microtome (Leica, Nussloch, Germany) and were stained using hematoxylin and eosin [12]. Finally, the sections were mounted with DPX (Sigma, MO, USA). Observation of the slides and photomicrography were done using a microscope (Olympus CX41) with digital camera (Olympus, Japan).

Results

General observation of samples

Shrimp were found to be moribund and white spots on the carapace, typical clinical symptoms of WSSV infection, were also observed. Other than this, the shrimp did not have any other external deformities.

PCR analysis

Samples from all the farms that were included in this study were found to have different degrees of WSSV infection and were detected by PCR (Fig. 1). Overall, 73.33 % samples analyzed were positive for WSSV and 53.33 % were positive in the 1st step indicating high load of the virus. Of the 15 samplings carried out from this region, only 4 (26.66 %) were negative for WSSV. This could be due to the timing of samplings and status of the shrimp (healthy or moribund). Eventually all the farms were harvested with a low DOC and it was not possible to collect samples from all the farms during harvesting to verify the actual status.

Similar to WSSV, high prevalence of IHHNV was also observed in different samples analyzed for this study. This is as evidenced by the PCR analysis (Figs. 1, 2). Presence of IHHNV was confirmed by different primer pairs and by IQ 2000 kit. This was carried out to avoid the possibility of false positive due to genomic integration of this virus [29, 31]. All the samples from different farms described here were negative for IMNV, TSV and YHV as detected by IQ 2000 kit (Fig. 2).

Histopathology

Further confirmation for the presence of WSSV and IHHNV infection were verified by histopathology. Typical WSSV inclusion bodies were observed in the gills (Fig. 3). Similarly, Cowdry type A inclusion bodies in gill sections of affected shrimps, suggestive of IHHNV infection were also observed (Fig. 4). Histopathologically, there was no evidence of infection by YHV, TSV or IMNV as analyzed in different organs of the shrimp. From this and PCR analysis, it was clear that the mortality of the shrimp was caused by either WSSV alone or it was a combined effect of both WSSV and IHHNV.

Discussion

Usually, shrimp infected with IHHNV show slow growth rate and rostrum deformity, clinically called as runt-deformity syndrome [8]. Any of such external symptoms of IHHNV infection were not observed in the present study. Even though higher load of IHHNV compared to WSSV was observed in some of the samples (Table 1), clinical signs specific to IHHNV were not observed. Emergency harvesting of shrimp at an early culture period (maximum

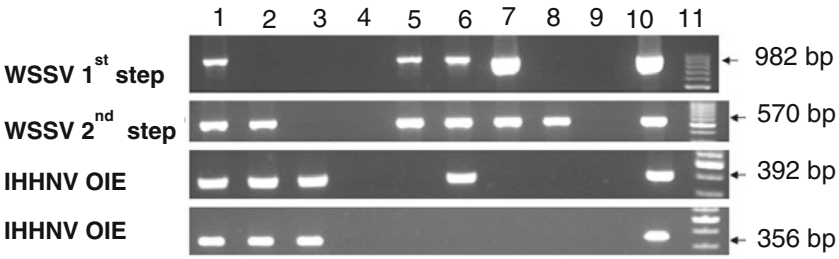

Fig. 1 PCR detection of WSSV and IHHNV by different sets of primers in representative samples from different farms of Tamil Nadu, India. *Lanes 1–8 L. vannamei* samples from different farms. *Lane 9* negative control, *Lane 10* positive control, *Lane 11* 100 bp molecular weight marker

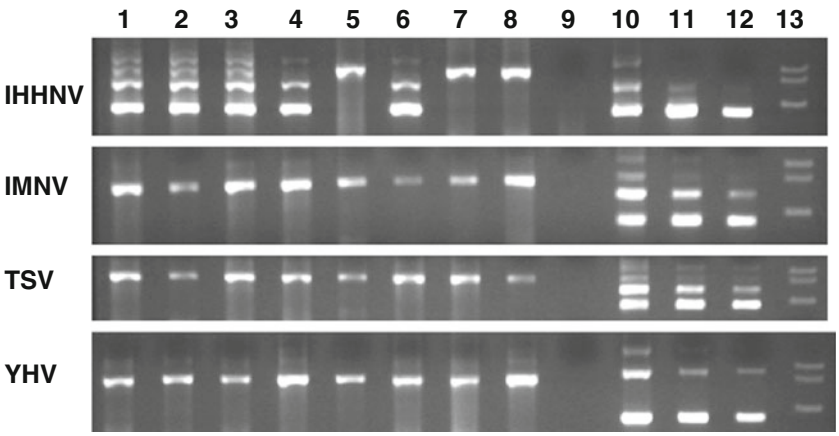

Fig. 2 PCR detection of shrimp viruses by IQ 2000 kit in representative samples from different farms of Tamil Nadu, India. *Lanes 1–8*: L. vannamei samples from different farms *Lane 9*: Negative control, *Lane 10–12*: Positive controls with different copy numbers to show the degree of infection (highest degree with 3 bands, moderate with 2 and lowest with 1 band), *Lane 13*: IQ 2000 Molecular weight Marker

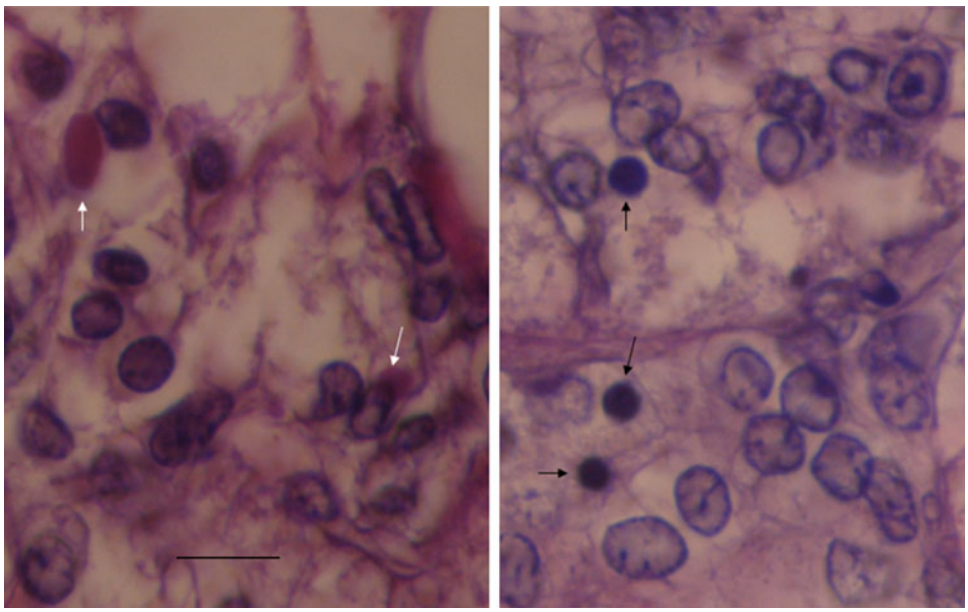

Fig. 3 Sections of gill tissue from infected *L. vannamei* showing eosinophilic (*white arrow*) and basophilic (*black arrow*) inclusion bodies suggestive of WSSV infection (Magnification ×100). *Bar* 15 μm

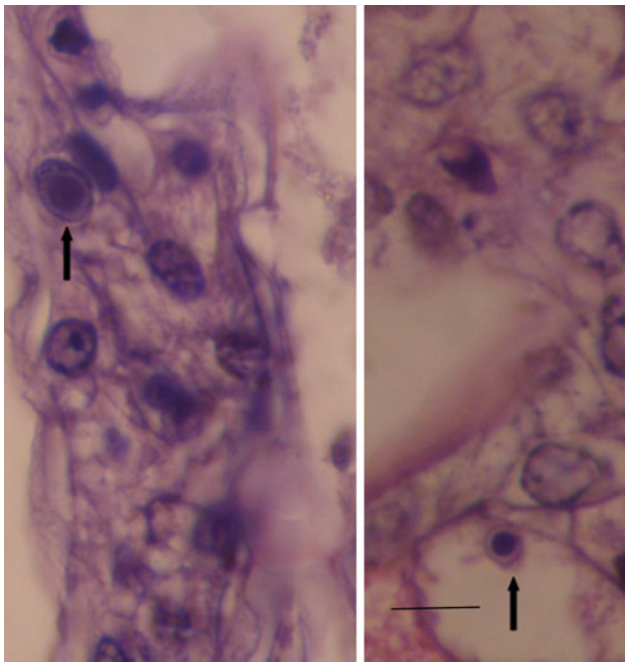

Fig. 4 Sections of gill tissue of infected *L.vannamei* showing Cowdry type A inclusion bodies (*blocked arrow*) suggestive of IHHNV infection (Magnification ×100). *Bar* 15 μm

72 days, Table 1) to avoid crop loss could have been a reason for this. In most of the studies, the effect of IHHNV has been described to be chronic [2, 24] and probably the appearance of symptoms would have been possible, had the shrimp were cultured for a little longer period.

Litopenaeus vannamei has been reported to be sensitive to WSSV infection and severe mortality due to WSSV infection has been reported from different parts of world [4, 15]. Even *L. vannamei* mortality in India due to WSSV infection has also been reported recently [1]. In an investigation, Moger et al. [18] observed high prevalence (72 %) of WSSV in cultured *L. vannamei* samples from India. This clearly indicates the WSSV risk associated even with the SPF stocks.

Large number of samples (66 %) analysed were found to be positive for IHHNV (Table 1) of which 53 % were first-step positive indicating heavy viral load. Low prevalence of IHHNV infection (32 %) in cultured *L. vannamei* samples from India has been reported earlier [18] where the shrimp were apparently healthy without any signs of disease. Mortality of shrimp due to IHHNV infection alone has been reported only in *P. stylirostris* and not in any other penaeid shrimps [14]. However, sporadic reports indicating IHHNV to be the sole agent causing mortality are also available. Heavy mortality of *P. monodon* due to IHHNV infection has been reported from India [26]. Similarly, a recent report indicates severe mortality of *L. vannamei* in Vietnam where IHHNV was detected from the dead shrimp (http://talkvietnam.com/2012/05/virus-decimates-shrimp-population). More information on genetic variation associated with the virulence or any other factors responsible to bring such kinds of mass mortality are however necessary to confirm such incidences.

Co-infection of IHHNV with other viruses has been found to be common in both *P. monodon* [25] and *L. vannamei* [23, 28, 32, 35]. In the present study, about 60 % of the samples were found to have dual infection of IHHNV and WSSV. Similar high prevalence of co-infection has been reported by Yeh et al. [36] where 75 % of the samples were infected with both WSSV and IHHNV. However, the exact outcome of such co-infection is not yet known. Many times the mortality that has occurred due to such co-infection is attributed to the

Table 1 Sample details and PCR analysis of shrimp samples from different farms of Tamil Nadu, India

Farm number	Pond number	Sampling number[a]	DOC at Sampling	WSSV		IHHNV		IMNV		YHV		TSV		DOC at harvest
				I Step	II Step	I Step	II Step	I Step	II Step	I Step	II Step	I Step	II Step	
Farm 1	Pond 1	1	27	−	+	+	+	−	−	−	−	−	−	50
		2	50	−	−	+	+	−	−	−	−	−	−	
	Pond 2	1	27	−	+	+	+	−	−	−	−	−	−	
Farm 2	Pond 2	1	64	+	+	−	−	−	−	−	−	−	−	64
	Pond 4	1	55	−	−	−	−	−	−	−	−	−	−	68
		2	65	−	+	+	+	−	−	−	−	−	−	
	Pond 5	1	57	−	+	+	+	−	−	−	−	−	−	60
	Pond 6	1	39	+	+	−	+	−	−	−	−	−	−	57
		2	57	+	+	+	+	−	−	−	−	−	−	
Farm 3	Pond 1	1	56	+	+	+	+	−	−	−	−	−	−	56
Farm 4	Pond 1	1	64	−	+	−	−	−	−	−	−	−	−	70
Farm 5	Pond 1	1	36	−	−	−	−	−	−	−	−	−	−	72
		2	62	+	+	−	+	−	−	−	−	−	−	
	Pond 5	1	62	−	−	−	−	−	−	−	−	−	−	
	Pond 6	1	72	−	+	+	+	−	−	−	−	−	−	

DOC days of culture

[a] Each sampling included 3 samples from a particular pond

presence of already known high virulent viruses of either WSSV [23, 28, 32, 35, 36] or TSV [28]. Moreover, such type of co-infection has also found to provide resistance to WSSV by experimental studies [30]. In other studies, co-infection was found to regulate the virus load in the host. An inverse relationship between the two viruses, when they are present together has been observed [32]. In this report, only the presence of different viruses has been studied and the exact virus load with respect to each other has not been attempted.

The present study provides the information regarding the sustainability of *L. vannamei* culture in India. Even though genetically improved SPF stocks are used for culture practice, these are still susceptible to the existing virulent viruses that can bring mortality and loss to shrimp farmers. Therefore, strict biosecurity measures are necessary to prevent the shrimp from such kinds of infection. As white shrimp culture practice is just picking up, it is essential to adopt stringent measures to make shrimp farming sustainable.

Acknowledgments Funding for this research was granted from National Fisheries Development Board, Hyderabad (India). The authors are thankful to the Director, Central Institute of Brackishwater Aquaculture, Chennai for facilities provided to carry out this research.

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