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Classical Runt Deformity Syndrome Cases in Farmed *Penaeus vannamei* Along the East Coast of India

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ABSTRACT

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Infectious hypodermal and haematopoietic necrotic viral disease caused by the infectious hypodermal and hematopoietic necrosis virus (IHHNV), a member of the Family *Parvoviridae*, Genus *Penstyldensovirus*, is the smallest known shrimp penaeid viruses. This viral disease poses a threat to shrimp farming as it causes runt deformity syndrome in *Penaeus vannamei* and thereby causing economic loss to the farmers. An analysis was carried out in various *P. vannamei* farms (n=350) along the East Coast of India from 2013-2018, and it was found that 30 farm samples positive for IHHNV. The shrimps in these farms exhibited classical IHHNV clinical signs like deformed sixth abdominal segment, deformed rostrum, cuticular roughness and wrinkled antennae. There was a wide size variation in growth among the affected farms. These samples on histological analysis showed prominent intranuclear, Cowdry type A inclusion bodies characteristic of IHHNV. The inclusion bodies observed were in the tissues of the ectodermal hypodermal epithelium of fore- and hindgut, mesodermal origins like haematopoietic organs, antennal gland and lymphoid organ. All the samples were positive for IHHNV by PCR using OIE primers. An experiment was conducted in *P. vannamei* (n=100) to study the disease transmission wherein, the animals were fed orally by the infected IHHNV tissue, and it was found that the animals got the infection by day five. The experimentally infected animals did not exhibit the classical IHHNV symptoms as that was seen in animals in the farming conditions.

ADDITIONAL INDEX WORDS: *Disease transmission, IHHNV,* Penaeus vannamei, *Runt deformity syndrome.*

INTRODUCTION

Aquaculture also referred to as aquafarming, reported as early as 6000 BC is climbing steeply due to the intensification of farming by modern and scientific practices. Shrimp farming, an important sector of aquafarming, contributing significantly to the economy of a country like India. In spite of the steady progress in this sector, the diseases remain as a major stumbling block for high production to meet the global demands. Notably, this sector is highly susceptible to especially viral pathogens like White spot syndrome virus (WSSV), Infectious hypodermal hematopoietic virus (IHHNV), Infectious myo necrosis virus (IMNV), Yellow head virus (YHV), Taura syndrome virus (TSV) and brings severe mortality in shrimps. Infectious Hypodermal Hematopoietic Necrosis Virus, is one such important virus, also termed as *Penaeus stylirostris densovirus* (*Pst*DV), smallest known virus of the shrimp that infected *Penaeus stylirostris* and was reported first in Hawaii in the 1980s with mass mortalities in *P. stylirostris* and later found infecting several other penaeid shrimps (OIE, 2018). The current study was aimed to know the prevalence of IHHNV infections in the cultured *P. vannamei* farms practised along the east coast of India and to find out the existence of this disease in the culturing systems with its clinical signs as well as its relation to the degree of infection.

METHODS

Shrimp samples collected from different *P. vannamei* shrimp farms (n=350) located along the east coast states of India, *viz*., Tamil Nadu, Andhra Pradesh, Odisha, and West Bengal were used for the screening of IHHNV infection. Clinical observation based on the gross changes as well as clinical signs displayed by the infected shrimps from various *P. vannamei* farms were categorized into Type A, Type B and Type C farms (Table 1).

Molecular diagnosis was made to confirm the IHHNV infection in the samples. Gills and pleopods were excised and preserved in 90% ethyl alcohol for total DNA extraction. Around 30-50 mg of shrimp tissues were taken for the DNA extraction (Laird *et al*., 1991) by using alkaline lysis buffer (10 mM Tris HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 100 mM NaCl), and DNA pellet was resuspended in double distilled water and stored at -20°C until further use. The quality of the DNA was estimated by using the Nanophotometer (Implen, Germany), at 260 nm. The PCR screening for IHHNV performed as described by OIE (2018), targeting a 309 bp amplicon and using the primers 309-F-5' GGG CGA ACC AGA ATC ACT TA 3' and

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309-R-5'ATC CGG AGG AAT CTG ATG TG 3' (Tang, Navarro, and Lightner, 2007). The 25 µl PCR reaction mixture constituted 200 ng of DNA template, ten picomoles of forward and reverse primers and 12.5 µl of 2X master mix (Gene Technologies, Denmark). It was performed in a thermal cycler (Applied Biosystems, Singapore) using the specified programme (OIE, 2018). The amplified PCR products were analyzed in 1.5% agarose gels and stained with 0.5 µg ml^{-1} ethidium bromide using 1x Tris Acetate EDTA buffer. The gels were visualised using UV transilluminator (BIORAD, CA, USA).

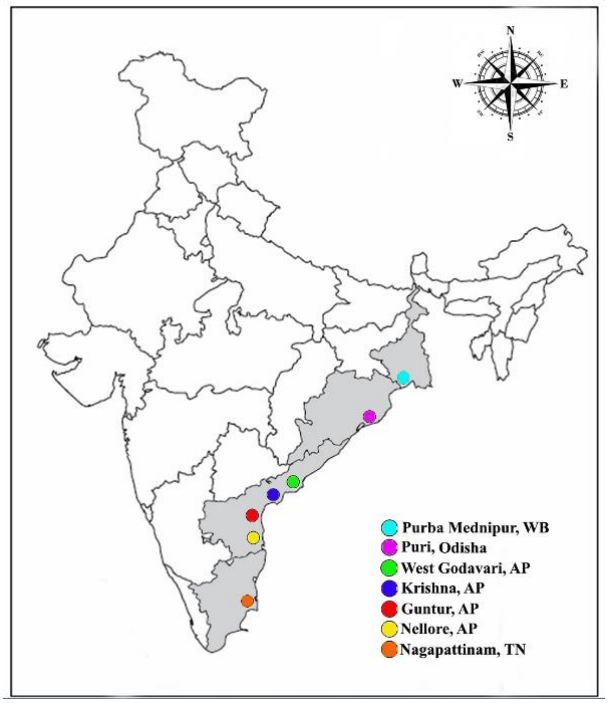

Figure 1. Map of India showing the study area and sampling sites of *P. vannamei* farms along the east coast.

Table 1. *Classification of IHHNV infected farms based on clinical signs.*

Farm Types	Criteria for classification
Type A farms	IHHNV positive farms with runt deformity, stunted growth and size variation. Degree of infection more.
Type B farms	IHHNV positive farms with runt deformity, stunted growth and size variation. Degree of infection mild or less.
Type C farms	IHHNV positive farms with no deformities, decreased growth and size variation and infected with other diseases like WSSV.

For histological examination, morbid shrimps were collected, dissected and preserved in Davidson's fixative (Bell and Lightner, 1988). After fixation, samples were transferred to 70% ethanol and stored until processing for histology using standard methods with routine Haematoxylin and Eosin (H&E) staining. Tissue sections from these samples also used for *in situ* hybridisation (ISH) with IHHNV-specific probe. For ISH, few paraffin sections (4-5 µm thick) from the IHHNV positive animals placed on APES coated microscope slides, deparaffinised in xylene following the protocol recommended by OIE, 2018. The slides examined under a microscope for positive signals. Both H&E and ISH sections were viewed under a light microscope.

Farm reared *P. vannamei* positive for IHHNV infection, showing the classic symptoms of IHHNV infection like rostral defects, deformities in abdominal segments, poor growth rate and huge size variation were collected. They were fed to uninfected animals of weighing about 2-3 gm (n=100) maintained in 500 L FRP tanks (@ 25 each tank, three treatment groups, and one control) for 21 days to observe the course of the disease.

Table 2. *IHHNV positive* P. vannamei *farms based on the clinical signs.*

S. No.	Place of Collection	State	DOC	Type of Farms
1	Nellore	Andhra Pradesh	52	Type C
2	Nellore		60	Type C
3	Nellore		50	Type C
4	Nellore		40	Type C
5	Nellore		40	Type C
6	Nellore		65	Type C
7	Nellore		70	Type C
8	Nellore		62	Type C
9	Nellore		60	Type C
10	Nellore		60	Type C
11	Nellore		60	Type C
12	Nellore		60	Type C
13	Nellore		60	Type C
14	Nellore		61	Type C
15	Nellore		100	Type B
16	Nellore		60	Type B
17	Nellore		55	Type B
18	Nellore		80	Type B
19	Nellore		50	Type A
20	Nellore		90	Type A
21	Guntur		90	Type A
22	Guntur		90	Type A
23	Krishna		90	Type A
24	West Godavari		20	Type A
25	West Godavari		30	Type A
26	West Godavari		120	Type A
27	Purba Medinipur	West Bengal	55	Type A
28	Puri	Odisha	50	Type B
29	Nagapattinam	Tamil Nadu	108	Type A
30	Nagapattinam		122	Type B

RESULTS

Table 2 gives details of IHHNV positive *P. vannamei* farms along the east coast of India. Out of 350 shrimp samples collected from various shrimp farms, 30 farms found to have typical clinical signs and also confirmed positive for IHHNV by various diagnostic methods (Figure 1). Of the 30 IHHNV positive farms, Type C farms (n=14) were mainly found in Nellore district of Andhra Pradesh, India. While Type A (n=10) and Type B (n=6) farms were seen scattered in other states. Figure 2 indicates the abdominal deformities and size variation observed in the infected farms.

Figure 2. IHHNV positive shrimp showing abdominal deformities and size variation.

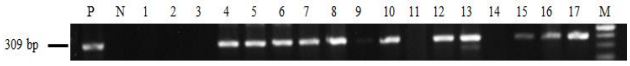

Figure 3. Gel picture showing IHHNV positive amplification from representative farms, Lanes: P-Positive control, N-Negative control, 1 to 17-Shrimp samples, M- Marker (100 bp).

The total DNA extracted from gills and pleopod were screened for IHHNV specific PCR. Out of 350 samples, analysed 30 samples gave positive amplification for IHHNV and presented in Figure 3. Organs like gills, eye, antennal gland, heart, hepatopancreas and gut from the morbid shrimps were screened and classical Cowdry Type A inclusion bodies were seen in almost all these tissues of Type A and Type B farms (Figures 4-5), while Type C farms did not show any inclusion bodies.

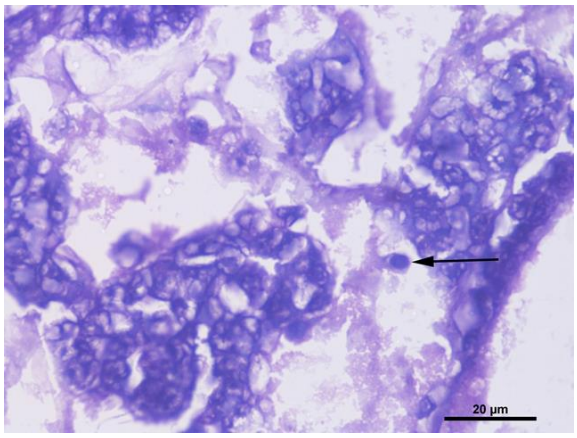

Figure 4. Degeneration of haematopoietic tissue with the presence of IHHNV inclusion bodies - H&E, 100X.

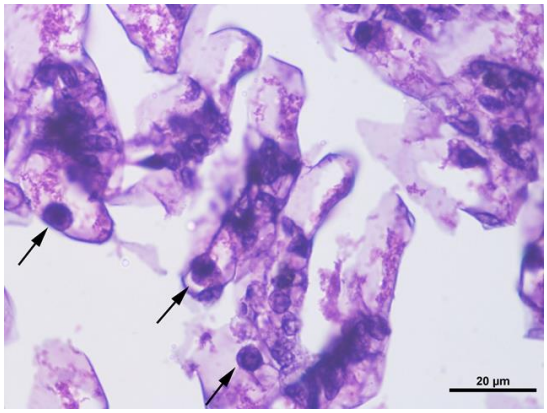

Figure 5. IHHNV inclusion bodies in gill tissue - H&E, 100X.

Viral inclusion bodies in the sections taken from the positive animals stained dark-blue to dark-purple when subjected to *in situ* hybridization (ISH), indicating the presence of hybridized IHHNV DNA or positive signals for the disease (Figure 6). The animals fed with IHHNV positive tissue samples could reproduce the disease on fifth-day post infection. The animals exhibited normal feeding, swimming behaviour. No specific clinical signs pertaining to this disease were observed in the animals. The DNA extracted from the gill and pleopod of these animals gave positive amplification for IHHNV (Figure 7).

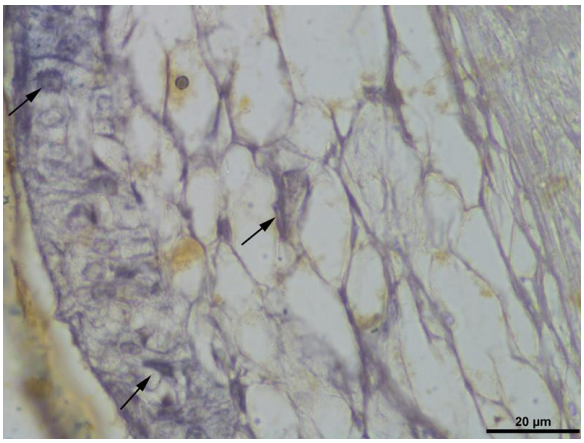

Figure 6. Presence of IHHNV positive signals in gut epithelium by *in situ* hybridization- 100X.

DISCUSSION

Runt deformity syndrome reported to cause rostral defects, muscular deformity, variable growth rate are seen mostly in *P. vannamei*, and these clinical signs are less apparently observed in *P. monodon* infected with IHHNV (Chayaburakul *et al.*, 2005). In the present study, 350 shrimp samples from various *P. vannamei* farms situated all over the East Coast of India were screened. Thirty farms were found to be positive for IHHNV infection.

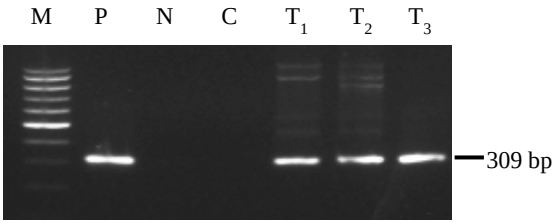

Figure 7. Gel picture showing IHHNV positive amplification in experimentally infected shrimps.
Lanes: M- Marker (100 bp), P- Positive control N- Negative control, C- Control group (T_0), T_1 to T_3- Treatment Groups 1 to 3.

Nellore district of Andhra Pradesh had more number of positive cases indicating more prevalence of IHHNV in that area. Similar to the present findings, Srinivas, Venkatrayalu, and Laxmappa (2016) correlated the presence of IHHNV infection in a pond to the infected post-larvae produced from broodstock raised in local shrimp farms. They have also reported that IHHNV is one of the seven major diseases encountered by the *P. vannamei* farms in Nellore district. In another observation, the majority of the Type C farms were recorded during 50-70 DOC, whereas Type A and Type B farms (except four farms) were above 90 DOC. The four farms which belong to Type B (DOC of 50, 55, 60, 80) and Type A (DOC of 20, 30, 50, 55) category might have received the infection vertically that would have resulted in the development of clinical signs earlier. Contrary to the present findings, Otta *et al*. (2014) have observed no external symptoms specific to IHHN disease in IHHNV infected animals even though the viral load was found to be high.

The degree of infection by IHHNV is highly variable among penaeid shrimps. Initially, the disease gave rise to mortalities in *P. stylirostris* and latter this species developed resistance. *Penaeus vannamei* and *P. monodon* act as chronic, asymptomatic to the infection and several other species act as carriers and reservoirs for this infection (Lightner, 1999). IHHNV will be more prone to *P. vannamei* than *P. monodon*, based on cellular interaction and development of physical deformities (Kalagayan *et al.*, 1991). In the present study, the variation in clinical signs across the infected farms was not uniform as deformities were not observed in all farms. This could be attributed to the genetic selection done by SPF facilities (Pruder *et al.*, 1995). The selection process might have conferred protection against IHHNV infection in some and chronic or asymptomatic stage in others. The *P. vannamei* culture in India is bred from SPF broodstocks. Absence or presence of deformities in the infected shrimps is due to several factors like genetic selection, vertical transmission, farming factors, and several other unexplained factors.

IHHNV can easily be detected by PCR. The present study also utilises the same primer in OIE protocol to diagnose the disease in the animal. Moreover, it also detects the infectious form of the disease in the animal and does not diagnose the IHHNV- related sequence in the animal (Tang, Navarro, and Lightner, 2007). Hence the farms which were diagnosed with IHHNV using the above described primers were predicted to be infectious. However, the histological examination of all the farms did not reveal the presence of typical Cowdry Type A inclusion bodies (CAI). This might be due to reduced virulence of the virus as well as the chronic stage of the disease.

Moreover, the farms wherein animals showed runt deformity, *i.e*. Type A and Type B farms had prominent CAI while Type C farms had less CAI bodies. Contrary to our findings Chayaburakul *et al*. (2005) found that CAI was much more rarely seen in *P. monodon* than in *P. vannamei* and may relate to the fact that IHHNV infection in *P. monodon* is generally less severe than in *P. vannamei*. Histopathology of the gills of experimentally infected samples did not show the presence of any inclusion bodies. This also might be due to the less virulence of the virus and the shorter duration of the experiment. Besides, experimental studies utilised 2-3 gm of shrimp, which may not be an ideal size for this disease to study. Moreover, vertical transmission of the disease might also influence the development of CAI. More insights into the development of these classical deformities with respect to IHHNV infections should be studied further.

CONCLUSIONS

Although IHHNV does not cause any mortality to *P. vannamei*, it remains a significant disease to shrimp farmers**.** Though the economic impact of this disease is not very clear still, it remains a challenge. Size variation, as well as variable growth rate, remains a regulating factor for the economic growth of the farm. Avoidance of this disease can be achieved through stocking good quality disease-free seeds, optimum rearing conditions as well as good management practices in the farm.

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