Detection of IHHN virus in a Specific Pathogen Free bonafide *Penaeus vannamei* (Boone, 1931) broodstock consignment imported to India

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The World Organization for Animal Health (formerly Office International des Epizooties OIE) listed pathogenic shrimp virus, infectious hypodermal haematopoetic necrosis virus (IHHNV), has been detected in a specific pathogen free (SPF) declared consignment imported from an approved SPF *vannamei* broodstock supplier to India. The Aquatic Quarantine Facility (AQF), Chennai the only Government-approved quarantine premise for ensuring the SPF status of imported *Penaeus vannamei* broodstock detected this pathogen during its routine screening process. The AQF, which has so far screened about 1068 SPF batches of *vannamei* consignment, recorded that one particular batch imported to India was not SPF, though the exporting country declared the stock as SPF. The presence of the viral pathogen was confirmed by both nested PCR and quantitative PCR (qPCR). Sequencing data of the target viral genome revealed 100% similarity with the already published decapod penstyldensovirus-1 isolate IHHNV partial genome (GenBank accession no. KJ862253). The test results declared by AQF were further validated by the Central Institute of Brackishwater Aquaculture, Chennai, which is the referral laboratory of AQF. As the import of non-SPF stock into the country is a source of biosecurity threat, the entire batch was incinerated according to the standard operating procedure of AQF. Thus the stringent biosecurity measures and disease diagnostic strategies followed at AQF served to safeguard the industry from this virus, which would have otherwise gained entry into the country through the falsely claimed SPF stock. This communication highlights the importance of SPF stock and detection of IHHNV.

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SHRIMP aquaculture is an industry that has experienced a worldwide economic growth. In India, aquaculture is mainly shrimp-centric, and shrimp production is predominantly contributed by the exotic species, the Pacific white legged shrimp (*Penaeus vannamei*), which was first introduced into the country for culture in 2009 (ref. 1). The sprint in culture that the species had gained in India is evident from the shrimp export data recorded in 2014–15. Exports of *vannamei* from India increased in terms of quantity (26.90%) and value (18.98%) over the previous year, which was due to the intense *vannamei* culture in the country. The major reason for the wide spread culture being the ease of availability of imported Specific Pathogen Free (SPF) *vannamei* stock. The Food and Agriculture Organization (FAO)[3] has reported that adoption of the SPF concept was a milestone in the domestication of *P. vannamei* that has led to the industry's current explosive growth in the country and its apparent sustainability.

SPF stock is required to mitigate the risk of introduction of pathogens during trans-boundary shipment of exotic shrimp species. Currently, the Indian *vannamei* industry predominantly sources the brooders from SPF *vannamei* broodstock suppliers approved by the Coastal Aquaculture Authority (CAA)[4]. These centres are located in Mexico, USA (Florida and Hawaii) and Indonesia (Figure 1).

Permission to import *P. vannamei* to India was granted in 2009 based on the condition that only broodstock of this species should be imported and the imported stock should be certified as SPF[5]. The Government of India also made it mandatory that the stock should have only a single port of entry, i.e. Chennai, and all the imported stock should be retested for confirmation of SPF status of shrimps in a highly biosecured, Government-approved quarantine premise called the aquatic quarantine facility (AQF) for *L. vannamei*. This regulation was imposed as a step to mitigate the risks associated with the introduction of *vannamei* to India, as this species is considered exotic in the country. The broodstock was to be imported only by *vannamei* hatchery operators approved by the CAA from approved suppliers. The consignment at the time of import should be accompanied by health certificates and other relevant documents issued by a competent authority of the exporting country indicating clearly that the stock imported is SPF.

The following criteria have to be fulfiled for declaration of broodstock as SPF as identified by OIE: (i) the etiological agent should be known; (ii) availability of reliable methods for diagnosis, and (iii) significant impact of the disease at local, regional or international level. The pathogens, as listed by OIE, viz. white spot syndrome virus (WSSV), infectious hypodermal haematopoetic necrosis virus (IHHNV), taura syndrome virus

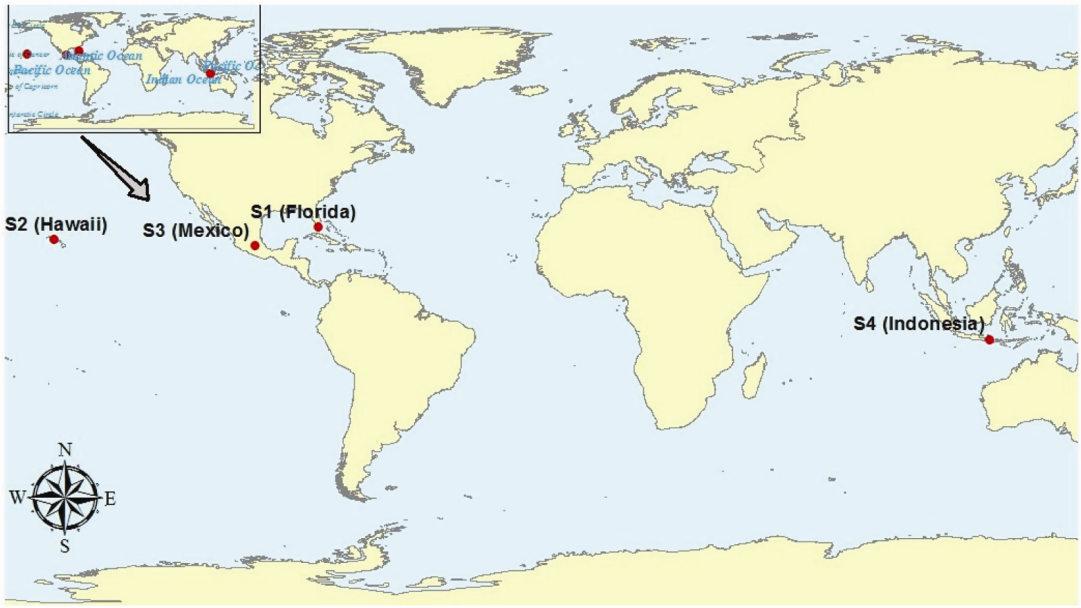

Figure 1. Location map of major Specific Pathogen Free *Penaeus vannamei* broodstock suppliers (S1–S4).

(TSV), yellow head virus/gill associated virus (YHV/GAV), infectious myonecrosis virus (IMNV) and necrotising hepatopancreatic proteo bacterium (NHPB) stand out as the most significant ones and cause huge loss to shrimp farmers. The estimated economic loss in penaeid shrimp aquaculture due to these pathogens had been documented[6], according to which, IHHNV was reported to incur a loss of US$ 1.0 billion. Therefore, considering the huge economic loss caused to the farmers who currently rely on only the imported broodstock, it is imperative that the stock should be free from these pathogens. Further, SPF status also ensures that the stock of the exotic species imported is safe for transboundary shipment and does not cause any biosecurity threat to the native species of the importing country[7]. Utilization of SPF shrimp stocks is also considered as a novel method of disease control[8].

At AQF, the SPF status of imported broodstock is assessed by non-lethal sampling and pathogen screening by highly sensitive double-step or nested PCR technique[9]. Fresh pleopod and faecal samples were collected at random according to the standard operating procedures of AQF (ref. 5). Sample collection was performed immediately on receipt of the broodstock consignment to the facility. Duplicate samples were also collected and stored at –20°C in appropriate preservatives for a duration of one crop cycle. The stored samples were also made available for cross-verification of the results, whenever required by the Aquatic Animal Health Division of Central Institute of Brackishwater Aquaculture (CIBA), Chennai which is the referral laboratory of AQF (ref. 5). The broodstock samples were then screened for the presence of six OIE-listed pathogens (WSSV, IHHNV, NHPB, IMNV, TSV, YHV/GAV) by nested PCR to ensure the SPF status of the imported consignment. Screening of each of the six pathogens was performed by subjecting five sets of samples for extraction and PCR analysis.

The samples were immediately processed for nucleic acid extraction following the DTAB–CTAB method. IQ 2000 kit was used as the test kit for pathogen screening[10]. The nucleic acid extracts were then subjected to nested PCR using specific primers designed (Farming Intelligene Tech. Corp., USA) for screening each viral disease[11]. The concentration of DNA used for screening IHHNV pathogen was approximately 40 ng/µl. DNA extracts of five randomly collected pleopod samples were amplified with three positive controls of dilution 2000, 200 and 20 copies/reaction respectively. An IHHNV negative sample obtained from an SPF stock was also subjected to PCR analysis along with the test samples and controls. Yeast tRNA was used as the template for negative control.

The extracted viral nucleic acids were amplified in a UNI IQ programmed thermal cycler (VeritiDx-Applied Biosystems, Singapore) specifically designed for a 'better-than-gradient' approach to PCR optimization with a precise control over six temperature zones. The conditions followed for amplification were: initial heating at 42°C for 30 min and at 94°C for 2 min, followed by 15 cycles of denaturation at 94°C for 20 sec, annealing at 62°C for 20 sec and extension at 72°C for 30 sec with a final extension at 72°C for 30 sec and at 20°C for 30 sec. The nested PCR reaction included 30 cycles of denaturation at 94°C for 20 sec, annealing at 62°C for 20 sec and extension at 72°C for 30 sec with a final extension of 72°C for 30 sec and at 20°C for 30 sec. The PCR products were

then separated in 2% agarose gel, stained with ethidium bromide (Sigma) and the results were documented using a gel documentation system (VilberLourmat QUANTUM ST4 Imaging Systems, France). The electrophoretic results indicated that all samples run were positive (Figure 2) as the viral amplicon was formed at 286 and/or 560 base pairs (bp). The amplified extract of the SPF stock (IHHNV negative sample) revealed the presence of only a single band which was formed at 848 bp.

The viral copy number/µl of the extracted DNA was further determined by real-time, quantitative qPCR (Roche Lightcycler 96, Germany). The test kit used for qPCR was IQREAL^{TM} IHHNV quantitative system[12], which quantifies both target pathogen (FAM) and Internal Control (IC) or reference genes (VIC) of shrimp genomic nucleic acid in a single reaction. FAM is a fluorescent dye (5′-5′-carboxyfluoroscein) that labels the hydrolysis probe. VIC serves as built-in IC and prevents any false negative interpretation of results due to failed extraction or inhibition. The reaction condition followed for amplification was 42°C for 30 min, then 92°C for 15 sec, and 60°C for 1 min, repeated for 40 cycles. The positive standards used were in the range 10^5, 10^4, 10^3 and 10^2 copies of IHHNV genome/µl of the standard. For absolute quantitation of the viral genome, the sample extracts were pooled and subjected to analysis. Copies of the viral genome were determined by interpolating the quantification cycle (C_q) into the standard curve generated from the standards and presented in log scale (Figure 3 *a*). C_q values predict the quantity of template[13]. The sample was run in triplicate along with triplicate sets of positive standards, negative template control and extract of IHHNV negative stock (SPF). The C_q values obtained were expressed as mean. Yeast tRNA was used as template for negative control to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler.

Analysis of the sample by both nested PCR and qPCR indicated that the particular batch of *vannamei* broodstock consignment was positive for the viral genome IHHN and hence non-SPF. Quantitative analysis by qPCR revealed that the viral genome was amplified at mean C_q value of 20.17 (Table 1) and the average number of genome copies obtained was 3.13×10^4 copies/µl of DNA. The mean quantification cycles of the target genome and reference genes (IC) of positive plasmid standards of dilution 10^5–10^2 ranged from 18.57 to 29.35 and 19.36 to 30.73 respectively. The reference gene of the SPF stock recorded amplification at mean C_q of 17.71.

According to the protocol followed for IHHNV screening[14], samples with C_q value lower than 40 cycles were considered positive for IHHNV. In the present study, the C_q value obtained for the IHHNV PCR product was 20.17 (Figure 3 *b*), which confirms that the tested sample was positive. The electrophoretic image of IHHNV genome indicated a strong band of 286 bp and a moderate band of 560 bp. This result suggests a moderate level of viral infection in the samples and the same was supported by the qPCR result, which indicated C_q value of 20.17. The samples were also sent to the NABL-accredited Aquaculture Pathology Laboratory of India, Sirkali, Tamil Nadu which declared similar results. Fresh samples were again collected and rechecked to confirm the obtained results.

Sequencing of the target viral genome was performed using Big Dye® Terminator v3.1 cycle sequence kit (Applied Biosystems) after purification of the PCR-generated amplicons with GeneJET PCR purification kit (Thermo Scientific). Clean-up of cycle sequence product was performed using ethanol–EDTA purification protocol (Big Dye® Terminator v3.1 cycle sequence kit). The resultant DNA was then sequenced in forward and reverse directions using Genetic Analyzer (Applied Biosystems 3500) following manufacture's instruction and analysed

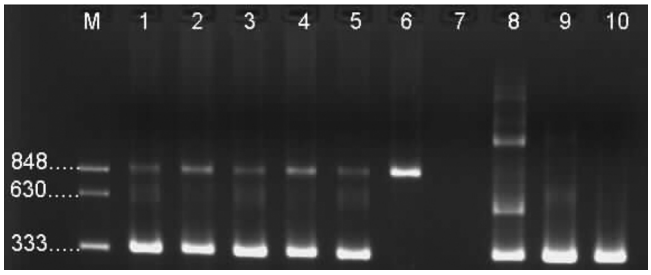

Figure 2. Electrophoretic image of the viral pathogen IHHNV screened in samples of imported *P. vannamei* broodstock consignment. (M, Molecular weight marker; lanes 1–5, Samples of IHHNV infection; lane 6, SPF sample; lane 7, Negative control; lane 8, IHHNV P_1(+) standard 2000 copies/reaction; lane 9, IHHNV P_2(+) standard 200 copies/reaction; lane 10, IHHNV P_3(+) standard 20 copies/reaction).

Table 1. Mean quantification cycles (C_q) of the target (IHHNV) pathogen, internal control (IC) of positive plasmid standards and unknown sample

Sample type	Gene	Mean (C_q)
Standard 1 (10^5)*	IHHNV	18.57
	IC	19.36
Standard 2 (10^4)	IHHNV	21.6
	IC	21.94
Standard 3 (10^3)	IHHNV	25.42
	IC	28.19
Standard 4 (10^2)*	IHHNV	29.91
	IC	30.73
NTC	IHHNV	…
	IC	34.58
SPF (extract)	IHHNV	…
	IC	17.71
Unknown	IHHNV	20.17
	IC	23.18

*Dilutions from 10^5 to 10^2 viral copies/µl of standard.

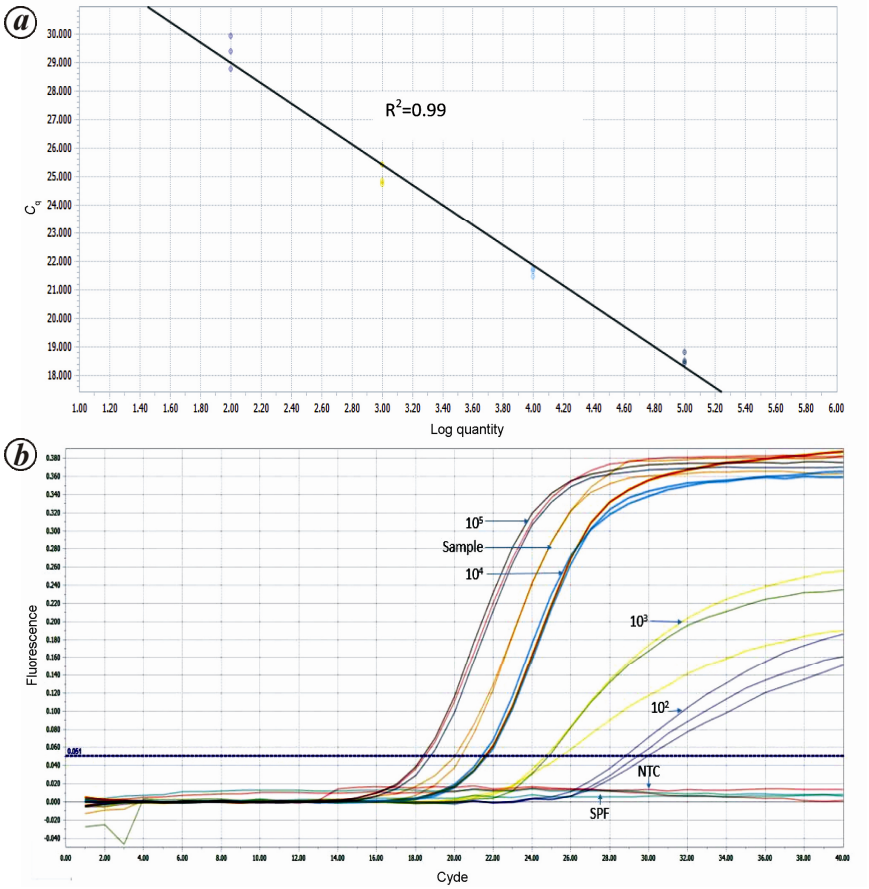

Figure 3. ***a***, Standard curve of IHHNV copy number versus quantification cycle (C_q). ***b***, Amplification plot of the tested IHHNV pathogen (value of 0.051 set as minimum threshold value; background fluorescence generated below this level was considered as noise).

using ClustalW and Mega software version 5 (ref. 15). The sequence obtained was as follows:

TGAAAGACTTAAAAGTATCAGGAGGCACATCA
TTTGAGACTCTCACATTTACAGACACCCCATAT
TTAGAAATATTTAAGGATACTACTGGACTACA
TAATCAACTATCAACTAAGGAAGCCGACGTAA
CATTGGCAAAATGGATACAAAATCCCCAACTT
GTGACCGTACAATCAACAGCAGCAAACTATGA
AGACCCAATCCAACAATTTGGATTCATGGAAC
AAATGCGAACCGGTGACAGAAAAGCCTATAC
AATCCATGGTGACACTAGAAATTGGTATGGCG
GAGAAATACCAACAACCGGACCCACCTTCATC
CCAAAATGGGGTGGTCAATTAAAAT.

It revealed 100% similarity with already published decapod penstyldensovirus-1 isolate IHHNV partial genome (GenBank accession no. KJ862253) and with many other sequences published for IHHNV isolate CTD2 capsid protein gene[16].

IHHNV is an important agent of disease in cultured penaeid shrimp. This virus was reported[17] to cause severe mortalities in *Penaeus stylirostris* from 1980 to 1990, but recent reports revealed that *P. stylirostris* has become tolerant to IHHNV infection and significant mortalities have not been noticed[18]. IHHNV is a parvo virus[19] categorized under C-2 pathogen group[20] and *P. vannamei* was considered as an asymptomatic carrier of this virus[19]. The pathogen was also reported in *P. monodon*, which revealed that a large portion of the IHHNV genome was found to be inserted in the genome of some genetic lines of this species. However, there is no evidence that this variant of IHHNV is infectious in *P. monodon*[18,21].

IHHNV caused physical deformities in *P. vannamei*, particularly in rostrum and cuticle, stunted growth, significant size variation and decreased production in farms[22]. It has also been documented that ponds when stocked with IHHNV-infected postlarvae typically had low production levels due to the development of IHHNV-caused runt-deformity syndrome (RDS). In India, ponds stocked with postlarvae produced from farm-reared broodstock reported severe economic and production impacts due to IHHNV infection, which was mainly due to reduced and irregular growth and small-sized shrimp at harvest[23]. Poor survival of infected larvae and production of poor-quality postlarvae from IHHNV-infected *P. vannamei* broodstock were also documented[9,24].

Existence of infectious and non-infectious genotypic variants of IHHNV has also been reported[16,25]. In the present study, the pathogen detected in the consignment was found to be infectious. The kit (IQ2000™ IHHNV) used

for the viral pathogen detection could detect only the pathogenic strain[26].

The PCR laboratory of AQF has so far screened 1068 batches of *vannamei* broodstock from 11 different suppliers, since the inception of the facility. Among the samples tested, all were found to be negative, except one which was imported from a newly approved supplier, from Mexico. Though the consignment was declared to be SPF for all the six OIE-listed pathogens by the competent authority of the country that exported the stock to India, the consignment screened positive for IHHNV and hence was incinerated according to the Standard Operating Procedures (SOP) of AQF. PCR results of the same set of samples showed the absence of the remaining five-tested pathogens (WSSV, NHPB, IMNV, TSV and YHV/GAV).

The AQF thus prevented the entry of the pathogen IHHNV and its further spread across the country through the imported brooders. This study clearly indicates the need for strict quarantine of imported *vannamei*, though the competent authority of the exporting country claimed the consignment as an SPF stock. The stringent measures followed in AQF caution the hatchery operators and as well as suppliers to be alert while importing or supplying the broodstock to India. According to the SOP of AQF, if two consignments exported by a supplier are found to be positive, then the supplier shall be blacklisted[5]. It is worth mentioning that the supplier took utmost care in declaring the SPF status while importing the second consignment to India. The tests conducted by the AQF ensured that the second batch of *vannamei* broodstock imported was SPF for all the six OIE-listed pathogens.

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