Genomics, Molecular Epidemiology and Diagnostics of Infectious hypodermal and hematopoietic necrosis virus

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Abstract Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is one of the major viral pathogens of penaeid shrimps worldwide, which has resulted in severe mortalities of up to 90 % in cultured *Penaeus* (*Litopenaeus*) *stylirostris* from Hawaii and hence designated *Penaeus stylirostris* densovirus (PstDNV). IHHNV is distributed in shrimp culture facilities worldwide. It causes large economic loss to the shrimp farming industry. Our knowledge about the natural reservoirs of IHHNV is still scarce. Recent studies suggest that there is sufficient sequence variation among the isolates from different locations in Asia, suggesting multiple geographical strains of the virus. Four complete genomes and several partial sequences of the virus are available in the GenBank. Complete genome information would be useful for assessing the specificity of diagnostics for viruses from different geographical areas. Comparisons of complete genome sequences will help us gain insights into point mutations that can affect virulence of the virus. In addition, because of unavailability of shrimp cell lines for culturing IHHNV in vitro, quantification of virus is difficult. The recent progress in research regarding clinical signs, geographical distribution, complete genome sequence and genetic variation, transmission has made it possible to obtain information on IHHNV. A comprehensive understanding of IHHNV infection process, pathogenesis, structural proteins and replication is essential for developing prevention

measures. To date, no effective prophylactic measure for IHHNV infection is available for shrimp to reduce its impact. This review provides an overview of key issues regarding IHHNV infection and disease in commercially important shrimp species.

Keywords IHHNV · PstDNV · Virus · Genetic diversity · Diagnosis · Shrimp · *Penaeus* · PCR

Introduction

Viral diseases have been seriously impacting the sustainability and economic success of shrimp aquaculture as they have been resulting in heavy mortalities. About 20 different shrimp viruses have been reported [28], which have been implicated in mass mortalities in cultured shrimp, such as *White spot syndrome virus* (WSSV), yellow head virus (YHV), infectious hypodermic and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) [33, 41, 60]. IHHNV, *Penaeus monodon* densovirus (PmoDNV), formerly referred to as hepatopancreatic parvovirus (HPV) and lymphoidal parvovirus (LPV) associated with parvoviral infections in penaeid shrimps [2, 50, 62].

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) of shrimp, one of the major viral pathogens of penaeid shrimps has recently been classified as *Penaeus stylirostris* densovirus (PstDNV) [84] under the family Parvoviridae and is a DNA virus. IHHNV infection caused severe mortalities up to 90 % during 1981 in cultured *P. (Litopenaeus) stylirostris* postlarvae and juveniles in Hawaii, which were imported from commercial hatcheries in Costa Rica and Ecuador [41, 50, 51]. It has been reported to cause Runt deformity syndrome (RDS) in *P. (Litopenaeus) vannamei* [34] and *P. monodon* which is a

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chronic, non-lethal disease [51, 66]. In this review, we discuss the recent progress on IHHNV, a major viral pathogen of penaeid shrimps worldwide. For the ease of understanding we will continue to use the old nomenclature of referring to the virus as infectious hypodermal and hematopoietic necrosis virus (IHHNV).

Clinical Signs

IHHNV infection does not produce any gross clinical signs of disease [50]. The infected juvenile of *P. vannamei* and *P. monodon* show runt-deformity syndrome [11, 13, 16, 34, 66] whereas acute epidemics cause heavy mortalities in juveniles and sub-adults of *P. stylirostris* [6]. Like all other parvoviruses, IHHNV do not encode a DNA polymerase and depend on host cells for DNA replication and multiplication. Therefore, they need rapidly proliferating cells of the host for their replication. The target organs for IHHNV infection include tissues of ectodermal (cuticular epidermis, hypodermal epithelium of the fore and hind gut, nerve cord and nerve ganglia) and mesodermal (hematopoietic organs, antennal gland, tubule epithelium, gonads, lymphoid organ, connective tissue and striated muscles) origin [41]. The virus does not infect organ systems of endodermal origin (i.e., hepatopancreas, mid-gut epithelium, anterior mid-gut caecum or posterior midgut caecum). IHHNV infection has been demonstrated in all life stages of penaeid species including eggs, larvae, postlarvae (PL), juveniles and adults [3]. PL and juvenile shrimps are more susceptible to IHHNV infection than adults due to the presence of actively dividing cells [7, 34, 42]. IHHNV-infected females fail to develop embryos and also fail to hatch the eggs [3, 59]. This virus is highly species-specific, particularly in terms of gross manifestations.

In case of *P. stylirostris*, the vertically infected early larvae do not show any clinical signs, but postlarvae (PL35) or adults show gross signs of the disease, often followed by mass mortalities. For the horizontal transfer of infection, the severity of the disease is size/age dependant and the young juveniles were observed to be more susceptible than the adults [7]. Even though the gross signs of infectious hypodermal and hematopoietic necrosis (IHHN) are not specific, the juvenile *P. stylirostris* with acute IHHN show reduced food consumption, followed by changes in behaviour and appearance [48, 63]. *P. stylirostris* with acute IHHN, infected shrimp become motionless and roll-over and slowly sink to the pond bottom. Finally they are cannibalized by healthy shrimps. At this stage of infection, *P. stylirostris* may often show white or buff-coloured spots in the cuticular epidermis, mainly at the junction of the tergal plates of the abdomen that gives a mottled appearance to the shrimp [41]. In recent years, it

has been noted that *P. stylirostris* has become more tolerant to IHHNV infection and no significant mortalities have been reported [58, 79]. The *P. stylirostris* that survive IHHNV epidemics can carry the virus for life and cause its spread by vertical and horizontal transmission [41] despite the fact that they show no gross signs of disease.

In *P. vannamei*, IHHN is typically a chronic disease [50] and the infection results in development and growth abnormalities known as runt deformity syndrome (RDS), [6, 14, 15, 34, 66]. Typical signs of RDS include deformed rostrum, wrinkled antennal flagella, cuticular roughness, 'bubble-heads' and deformation of 6th abdominal segment and tail fan. Growth retardation due to IHHNV infection in this species has ranged between 30 and 90 % and resulted in heavy economic losses between 10 and 50 % [92]. Despite publications suggesting no mortality in *P. vannamei* in Ecuador, Dr. Lachlan Harris [unpublished data] has good evidence of a spike in mortality up to 10 % in virus-infected shrimp in the last couple of weeks of grow out which depresses the profit margin of farms. RDS has also been reported in cultured stocks of *P. monodon*. In India, we have recently presented molecular evidence of the presence of IHHNV associated with slow growth in *P. monodon* (Fig. 1) [67].

Geographical Distribution

IHHNV has been reported in many regions around the world including North, South, Central America, the Caribbean and the Indo-Pacific region and is now considered cosmopolitan in distribution. IHHNV was first recognized in 1981, in Hawaii, in *P. stylirostris* imported from South and Central America [50] and later in 1987, IHHNV was introduced to western Mexico that bordered the Gulf of California through an imported post-larval stock of *P. vannamei* [46]. In 1986 it was found in imported quarantined stocks of *P. vannamei* in Taiwanese shrimp culture facilities [52]. Owens et al. [63] reported the natural occurrence of IHHNV in the Australian Indo-West Pacific region. In 2008, the infectious form of IHHNV was found in farmed *P. monodon* in Australia [71]. IHHNV has been detected in *P. stylirostris* imported to French Polynesia and Guam for aquaculture [21, 79]. The virus was reported from wild and cultured *P. monodon* in east and SE Asian countries like Singapore, Malaysia, Indonesia, Thailand, Taiwan, India and the Philippines [26, 41, 66, 67, 82]. An epidemiological survey conducted in China during 2001–2004 revealed a prevalence of IHHNV in shrimps and crabs collected from the culture area of 51.5 and 8.3 % respectively [96]. High prevalence of IHHNV has been reported in shrimp farms in northeastern Brazil ranging from 9.4 to 81 % [12]. Teixeira-Lopes et al. [85] reported the occurrence of IHHNV and IMNV co-infection in *P. vannamei* farmed in northeast Brazil for the first time. The recent

Fig. 1 Size variations observed in 50 days old *P. monodon* infected by IHNV [67]

study in Brunei Darussalam confirmed the presence of IHNV virus in wild *P. monodon* with a low prevalence of 14.1 % [19]. For the first time IHNV was found in wild *Artemesia longinaris* from Argentina by PCR and real time quantitative PCR (qPCR) with a fairly high prevalence of 30 % [56]. A low prevalence rate (1.1–3.3 %) was found in cultured *P. vannamei* in Venezuela [8]. IHNV has been also recently reported from South Korea in *P. vannamei* and its pond water, which had a high nucleotide similarity to the Ecuador strain (AY362548) [37]. Figure 2 shows the geographical distribution of IHNV isolates. A recent study in Malaysia documented the presence of IHNV infection in wild berried broodstock of freshwater prawn, *Macrobrachium rosenbergii* with a prevalence of 20 % [29].

Host Range and Transmission

IHNV was first reported in *P. stylirostris* and *P. vannamei* in early 1980s in the Americas [50, 51] and later in *P. monodon* from Asia. Eventually, it was found to have been introduced by way of live black tiger shrimp [41, 43, 47]. Since the initial discovery of IHNV in cultured shrimp in Hawaii in 1981, IHNV is considered as a cosmopolitan virus and has been reported from most penaeid shrimps [41]. Natural infections have been reported in *P. vannamei*, *P. stylirostris*, *P. occidentalis*, *P. monodon*, *P. semisulcatus*, *P. californiensis*, *P. schmitti* and *P. japonicus* throughout the world [25, 26, 40, 47, 58, 64]. Natural infection of IHNV in postlarvae and sub-adults of *M. rosenbergii* were reported from southern Taiwan [32] and in Malaysia [29]. Experimental infections have also been reported in *P. setiferus*, *P. aztecus* and *P. duorarum* [41]. *P. (Fenneropenaeus) indicus* and *P. (Fenneropenaeus) merguensis* appear to be non-susceptible to IHNV infection [15, 41, 45]. During 2003, a study in China [96] shows the presence of the virus in Harry-clawed shore crab, *Hemigrapsus penicillatus*, collected from the culture areas

of northern China. An experimental study conducted on native wild specimens of *F. subtilis* collected in the estuary of the Pacoti River on the coast of northeastern Brazil showed them to be susceptible to IHNV [20]. *Artemesia longinaris* as a new wild host for IHNV was recorded from Argentina [56]. In Australia, in a survey of 328 wild caught prawns (7 spp.), crabs and lobsters only *P. monodon* was positive for IHNV or IHNV-like amplicons [Kraetsche and Owens, unpublished].

The transmission of IHNV in the natural environment happens both by vertical and horizontal [6, 41, 54, 57–59]. The horizontal transmission of IHNV in culture system occurs mainly through the ingestion of dead infected shrimp and by contact with water containing the virus. Vanpatten et al. [89] demonstrated the presence of infectious IHNV particles in the feces of juvenile laughing gulls (*Larus atricilla*) and domestic chickens (*Gallus domesticus*) after consuming viral-infected shrimp tissue. Vertical transmission occurs due to transfer of this virus from mother to offspring. This could be through the shedding of viral particles at the time of spawning and then ingestion by larvae at first feeding, or by the transmission of virus from oocytes to larvae [54]. The vertical transmission of IHNV through female *P. vannamei* was confirmed by nested-PCR analysis of embryos and larvae produced by IHNV-infected females fertilized by IHNV-free males [59]. Zhang and Sun also reported IHNV in ovarian tissues and fertilized egg of *P. (Fenneropenaeus) chinensis* [97].

Morphology

IHNV is the smallest of the known penaeid shrimp viruses. Based on physicochemical and ultrastructural properties of the virion and genomic organization, IHNV is taxonomically placed under the *Parvoviridae* family [9, 55, 74]. These are small icosahedral, nonenveloped viral particles 20–22 nm

Fig. 2 Geographical distribution of infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates. Asterisks indicates country and year of emergence

in diameter containing a single-stranded, linear DNA of approximately 3.9 kb [44, 69]. The average buoyant density of this virus by CsCl density gradient centrifugation is 1.40 g/ml [9]. By SDS-PAGE analysis, it was estimated that the purified virions contains four polypeptides of 74, 47, 39, and 37.5 kDa [9]. Recently Hou et al. [31] reported the self-assembly of recombinantly expressed IHHNV capsid protein (CP) into virus-like particles (VLP's) in its size and shape and IHHNV-VLP's to encapsidate RNA and DNA of 0.5 kb size. By indirect immunofluorescence microscopy, the entry of the VLPs into primary hemocytes of shrimp was investigated and it paved the way of using these VLPs as vehicle for antiviral drug delivery. By X-ray crystallography, the three-dimensional (3D) structure of recombinant, empty virus-like particles (VLPs) of IHHNV have been reported [35]. The capsid protein of IHHNV consists of an eight-stranded, antiparallel β -barrel "jelly roll" motif which remains in the same position relative to the icosahedral symmetry axes as in other parvoviruses. The near-atomic resolution structure of IHHNV is compatible with the low-resolution 3D structure features of *Aedes albopictus* densovirus; hence the classification of these two viruses within the same Brevdensovirus genus is appropriate [35]. When combined with the molecular data, the evidence is indisputable and compelling. Therefore, we suggest here that the virus should be named *P. stylirostris* Brevdensovirus (*PstBDNV*). Therefore, we will continue to use the older, designation for the virus in this review.

Genome Organization

To date only four complete genome sequence of IHHNV are available: Hawaii (GenBank Accession No. AF218266; 3,909 bp), India (GenBank Accession No. GQ411199; 3,908 bp) [69], Korea (GenBank Accession No. JN377975; 3,914 bp) [38] and China (GenBank Accession No. EF633688; 3,833 bp). In addition to these partial sequences are also available from Mexico (GenBank Accession No. AF273215; 3,873 bp), Taiwan (GenBank Accession Nos. AY355307, AY355306, and AY355308), Thailand (GenBank Accession Nos. AY362547, and AY102034), Ecuador (GenBank Accession No. AY362548) and Australia (GenBank Accession No. GQ475529). In addition to infectious IHHNV, virus-related sequences (non-infectious IHHNV) integrated into the genome of *P. monodon* has been reported from Africa, Australia, Madagascar and India [39, 68, 80, 82]. The Indian IHHNV genome had a base composition of 36.4 % A, 20.6 % T, 23.5 % C and 19.4 % G. Thus, the G + C and A + T content of the genome is 42.9 and 57.1 %, respectively [69].

The overall organization and size of the coding sequences of the Indian IHHNV genome is very similar to those described from Hawaii (GenBank Accession No. AF218266) and Mexico [74]. Nucleotide sequence analysis of the Indian IHHNV genome reveals three major coding domains: a left ORF (NS1) of 2,001 bp, a mid ORF (NS2) of 1,092 bp and a right ORF (VP) of 990 bp (Fig. 3). The

right and middle ORFs are in the same reading frame whereas the left ORF (NS1) is in a different reading frame. The left ORF comprising about 50 % of the genome codes for non-structural protein 1 (NS1) of IHNV which starts at nt 648 and terminates with a TAA codon at 2648 nt. This ORF encodes a protein of 666 amino acids, with a molecular weight of 75.77 kDa [69]. The ORF1 protein sequence contains highly conserved replication initiator motifs (rolling-circle replication (RCR) motifs) and NTP-binding and helicase domains (ATPase motifs) common to all parvoviruses which is located between 257 and 315 aa and 480 and 579 aa, respectively [1, 10, 69, 74].

The mid ORF of Indian IHNV strain consists of 1,092 bp. ORF2 starts with ATG codon at nt 591 and terminates with a TAG codon at 1681. This ORF encodes a protein of 363 aa with a molecular mass of 42.11 kDa. The multiple alignment of amino acid sequences of ORF2 of Indian IHNV isolate shows 3 amino acid substitutions compared to the closest relatives from Thailand (AY102034) and Taiwan (AY355307) at 36th, 107th and 227th positions [69]. The potential function of nonstructural protein 2 (mid ORF) is unknown. The ORF3 is the smallest (990 bp) among the three ORFs in the Indian IHNV genome which starts with ATG at nt 2590, and terminates with the TAA codon at nt 3577. This ORF encodes a protein of 329 aa with a molecular mass of 37.48 kDa. The multiple alignment of amino acid sequences of ORF3 of the Indian IHNV isolate shows 11 amino acid substitutions compared to its closest relatives (Taiwan; AY355307 and Thailand; AY102034) [69]. Clearly, there is a discrepancy between the number of polypeptides visualized by SDS-PAGE at 4 [9] and the number of ORFs at 3 [69]. SDS-PAGE sizes compared to molecular weight are very similar; NS1 at 74 vs 75.77; NS2 at 47 and 39 vs 42.11 and VP1 at 37.5 vs 37.48 kDa respectively. Perhaps there is some cleavage and processing of the NS proteins which gives the smaller, extra protein band.

The three putative IHNV promoters (P2, P11 and P61) are located upstream of the left, middle and right ORFs, respectively [23, 24, 74]. A recent study in USA mapping these promoters [24] demonstrated the P2 promoter transcribes the nonstructural protein 1 gene (left ORF), P11 promotes the expression of nonstructural protein 2 gene

(middle ORF) and P61 drives the expression of structural protein gene (right ORF). They also compared the functional activities of these promoters by luciferase assay, which revealed that luciferase expression driven by the P2 promoter was 3.7-fold higher than P11, over fivefold higher than P61 promoter while the luciferase expression driven by the P11 promoter was 1.4-fold higher than the P61 promoter.

Genotype Variation

Molecular studies show considerable variation among Asian isolates of the virus and little variation among American isolates [79, 82]. All IHNV isolates from the Americas are nearly identical with IHNV from the Philippines. Considering aspects of history and epidemiology of IHNV in the Americas, this suggests that IHNV might have been introduced into America from Philippines through import of live *P. monodon* as a candidate aquaculture species during the very early development of shrimp farming [42, 82].

Four IHNV genotypes have been documented: 1. Americas and East Asia (principally the Philippines); 2. South-East Asia; 3. Madagascar, India, Mauritius and Australia (designated as Type A); and 4. East Africa, Mozambique and Tanzania (designated as Type B) [3, 90]. The first two genotypes are infectious to the representative penaeids, *P. vannamei* and *P. monodon*, while the latter two genetic variants are not infectious to these species [39, 79, 82]. Both types (Type A and Type B) of IHNV-related sequences have been found inserted into the genome of *P. monodon* from East Africa, Tanzania and Mozambique [80, 83].

Based on nucleotide sequence analysis of a 2.9 kb fragment of the IHNV genome (containing all three ORFs of IHNV), very low sequence variation was found amongst the 14 IHNV isolates collected from Hawaii and various regions of Americas. Even though there were no deletions or insertions in this region of the viral genome, 30 nucleotide substitutions were noticed viz. 12, 5 and 13 substitutions in ORF1, ORF2 and ORF3, respectively [79]. Among 30 nucleotide substitutions in these ORFs, only 15 nucleotide substitutions resulted in amino acid changes (7 in the left ORF, 1 in the middle ORF and 7 in the right

Fig. 3 Genomic organization of coding sequences of Indian strain of infectious hypodermal and hematopoietic necrosis virus (IHNV) genome. The genome is shown in plus strand. The three open reading frames (ORF1, ORF2 and ORF3) are indicated in boxes with their positions

ORF) but there was no correlation between substitution and virulence [79]. On comparison of the sequence homology of the same 2.9 kb region of IHHNV genome of the Hawaiian isolate to the Philippine IHHNV isolate, there was a high nucleotide identity of 99.8 % whereas the Thailand and Taiwan IHHNV isolates showed 96 % identity. The putative IHHNV sequences detected in *P. monodon* from Tanzania and Madagascar showed a divergence of 8.2 % and 14.1 % respectively from Hawaiian isolate [82]. A non-infectious form of IHHNV was later reported from *P. monodon* samples from Tanzania and Madagascar [80]. We surmise this to be the possible reason for the high sequence divergence of these two isolates from the Hawaiian isolate. In contrast to the report of Tang and Lightner [79], there was an unexpectedly high mean rate of nucleotide substitution (1.39×10^{-4} substitutions/site/year) was reported by Robles-Sikisaka et al. [70] who compared sequences of the IHHNV capsid protein from 89 penaeid shrimp, along with 14 sequences isolated from *P. stylirostris* and *P. monodon* from different geographic locations. These studies indicate that IHHNV haplotype and nucleotide diversity is higher than previously reported and the genetic diversity is not uniform across geographic regions. IHHNV is the first marine invertebrate parvovirus for which such high rates of nucleotide substitutions have been reported.

Diagnosis of IHHNV

Due to the importance of penaeid shrimp culture, the availability of easy and rapid methods that allow early diagnosis is essential for routine monitoring of the animal health status and to restrain further disease outbreaks.

Histopathology

Traditionally, the diagnosis of IHHNV infection is by examining histological sections wherein prominent Cowdry type A, eosinophilic, intranuclear inclusion bodies surrounded by margined chromatin in hypertrophied nuclei of cells is seen (Fig. 4) in tissues of ectodermal and mesodermal origin [50]. These inclusion bodies of IHHNV can be confused with the developing WSSV inclusion bodies [44].

Genome-Based Diagnostic Tools

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a highly sensitive and robust technique for detection of IHHNV infection in shrimps. Apart from its specificity, the sensitivity of this detection system helps diagnosis of early infection. The

sensitivity of a PCR assay is a function of several factors such as primer composition, structure and homology to the target molecule. Several single step, nested, duplex, multiplex and real-time PCR methods have been reported for the detection of IHHNV in shrimp samples worldwide [3, 27, 36, 49, 59, 61, 67, 74, 78, 79, 81–83, 93, 95] and a number of commercial PCR kits are available. These methods are useful for routine diagnostic applications in disease outbreaks and for use in surveillance and screening of IHHNV free shrimp stocks. However, since there are multiple geographic variants of IHHNV, some of the variants are not detected by some methods.

A rapid and reliable polymerase chain reaction (PCR) method was developed for the detection of IHHNV in penaeid shrimps in 2000 [61]. The primer pairs (77012/2553) used for this study was designed based on the sequence of IHHNV genome from Hawaii (GenBank Accession No. AF218266) and amplified a 1,681 bp fragment of the coat protein of IHHNV. This primer set was able to detect IHHNV obtained from *P. stylirostris*, *P. monodon* and *P. vannamei* from several different geographic regions including Panama, Philippines, Mexico, Ecuador, Hawaii, and Texas. Later Tang et al. [81] designed a pair of primer (IHHNV392F/R) based on the Hawaiian isolate (GenBank AF218266) which amplified a product of 392 bp of the nonstructural protein coding region. A PCR assay was also been described in the

Fig. 4 Histopathology of IHHNV infected adult *P. monodon*. Note the Cowdry type A hypertrophied nuclei with eosinophilic intranuclear inclusion bodies (H&E. 1000x) [68]

Manual of diagnostic tests for aquatic animals [3] utilizing two sets of primers for the detection of IHHNV. The primer set 77012F/77353R binds to a region in between the non-structural and structural (coat protein) protein coding regions and amplifies a 356 bp product. The second primer set IHHNV 389F/R is designed from the nonstructural protein-coding region (ORF 1) of the IHHNV genome and amplifies a 389 bp product [3]. These primer pairs, 392F/R, 389F/R and 77012/77353 successfully detected all the known genetic variants of IHHNV including Types A and B [3, 39, 79, 82].

In 2002, two sets of primers were designed; IHHNV721F/IHHNV2860R and IHHNVF/IHHNVR1 based on the Hawaiian isolate [79]. These primers helped to analyze the variation in the viral genome by examining 70 % of IHHNV genome from 14 isolates collected during 1982–1997. Tang et al. [82] designed a set of primers IHHN3065F/R based on IHHNV sequence from Hawaii (GenBank AF218266) which amplified approximately 70 % of the entire viral genome including the full length ORF1 and ORF3. Braz et al. [12] described another PCR assay using the primers which amplified a 185 bp product in the nonstructural protein region that overlaps the structural protein region. Specific primer sets were designed to distinguish infectious IHHNV from a non-infectious variant of IHHNV that is incorporated in the genome. A nested PCR has also been developed in India [67]. A different nested PCR based commercial kit was also developed for improved sensitivity of detection [59]. For the specific detection of infectious IHHNV, the primers IHHNV309F/R having 9–12 nucleotide mismatches with virus-related sequence can be used [83]. However, a recent study in Thailand, revealed that relatively common occurrence of random and variable inserts of IHHNV genome fragments found in the genome of *P. monodon*, which may lead to false positive results for infectious IHHNV using commonly recommended methods such as IQ2000™ kit and PCR with discriminatory primers IHHNV309F/R [72].

For the specific detection of Type A virus-related sequences, MG831F/R primers can be used (Table 1) [80]. Research from Australia showed that in the years immediately after the IHHNV outbreak of 1990 [63] (1991–1995), most *P. monodon* were negative for amplicons of the MG831F/R primer set, but since 1996, 90 % of prawns have been amplicon positive [Krabsetsve and Owens unpublished]. This is clear evidence of both a recent introduction of IHHNV into Australia and the phenomenon of viral accommodation in action. Also many of these MG831F/R amplicons were double amplicons confirming that multiple inserts of partial IHHNV had occurred. These MG831F/R amplicons are not produced in IHHNV-infected *P. stylirostris* nor *P. vannamei* demonstrating that this insertion site is specific for *P. monodon*. Despite the fact

that wild type IHHNV was circulating in 1990 [63], [Krabsetsve and Owens unpublished] and again in 2008 [71], the inserted virus-related sequence is ~15 % divergent from the wild-type virus, suggesting an ancient exposure to a IHHNV-like virus. If the DNA mutation rates found by Robles-Sikisaka et al. [70] hold for Australian IHHNV-like sequences, then the Australian IHHNV-like strain that produced the integrated sequences last shared a common ancestor with IHHNV ~4.3 million years ago. Furthermore, a 950 bp IHHNV-like sequence incorporating all of NS2 and most of NS1 has been found in freshwater crayfish *Cherax quadricarinatus* which has 70 % identity to Australian virus-like sequence [Rusaini and Owens unpublished]. This suggests an even older exposure of crayfish to a IHHNV-like virus or another similar brevidensovirus.

A multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was also developed for detection of six different viruses in penaeid shrimp including IHHNV [36]. Yang et al. [95] developed a single-step multiplex PCR for simultaneous detection of WSSV and IHHNV in penaeid shrimp. Recently, a multiplex PCR was developed for co-detection of IHHNV and WSSV with a sensitivity of 1×10^3 copy numbers [18]. Recent studies suggest that multiple geographical strains of this virus exist and hence it is necessary to use specific primers for the routine diagnosis of IHHNV in shrimp culture.

Real-Time PCR Methods

Real-time PCR which is used to amplify and simultaneously quantify a targeted DNA molecule enables detection and quantification of the viral pathogen in the tissues of infected shrimp. By using this technique the viral load in infected shrimp can be accurately determined which in turn helps in risk assessment as well as disease monitoring during culture. Real-time PCR assays has been successfully applied for detection and quantification of IHHNV infection in shrimps. In 2001, Tang and Lightner developed a real-time PCR method using a TaqMan probe in a 5' nuclease assay to detect the virus in penaeid shrimps [78]. A pair of PCR primers that amplified an 81 bp DNA fragment and a fluorogenic probe (TaqMan probe) were selected from a conserved region of ORF1 (open reading frame 1) of the IHHNV genomic sequence (GenBank AF218266). Dhar et al. [22] developed a duplex real-time PCR for detection and quantification of both IHHNV and WSSV using SYBR Green chemistry. Real-time PCR has also been used for quantification of IHHNV in different tissues of infected samples and subsequently, help to develop IHHNV-resistant line of shrimps (Super Shrimp®). In 2008, a real-time multiplex PCR for detection of three viral pathogens viz. WSSV, IHHNV and TSV

Table 1 PCR primer pairs used for detection of IHNV infection in penaeid shrimps

Primer code	Primer sequence	Product length (bp)	References
IHHNV392F	5'-GGGCGAACCAGAATCACTTA-3'	392	[81]
IHHNV392R	5'-ATCCGGAGGAATCTGATGTG-3'		
77012F	5'-ATCGGTGCACTACTCGGA-3'	356	[3]
77353R	5'-TCGTACTGGCTGTTTCATC-3'		
IHHNV389F	5'-CGGAACACAACCCGACTTTA-3'	389	[3]
IHHNV389R	5'-GGCCAAGACCAAAATACGAA-3'		
IHHNV309F	5'-TCCAACACTTAGTCAAAAACCAA-3'	309	[83]
IHHNV309R	5'-TGTCTGCTACGATGATTATCCA-3'		
IHHNV648F	5'-GAACGGCTTTCGTATTTTGG-3'	648	[67]
IHHNV648R	5'-AGCGTAGGACTTGCCGATTA-3'		
MG831F	5'-TTGGGGATGCAGCAATATCT-3'	831	[80]
MG831R	5'-GTCCATCCACTGATCGGACT-3'		
IHHNVF	5'-ATGTGCGCCGATTCAACAAG-3'	1,200	[79]
IHHNVR1	5'-CTAAGTGACGGCGGACAATA-3'		
IHHNV721F	5'-TCTACTGCCCTCTGCAACGAG-3'	2,000	[79]
IHHNV2860R	5'-GTGGGTCTGGTCCACTTGAT-3'		
IHHNV3065F	5'-GACGACGAAGAATGGACAGA-3'	3,000	[82]
IHHNV3065R	5'-TGCCTGGGTAGCTGGTATGTATA-3'		
77012F	5'-ATCGGTGCACTACTGGGA-3'	1,681	[61]
2553R	5'-CGGACAATATCCCTGACT-3'		
I2814F	5'-TAATGAAGACGAAGAACACGCCGAAGG-3'	703	[96]
I3516R	5'-TGGGTAGACTAGGTTTCCAAGGGATGGTT-3'		

of penaeid shrimp was developed and sensitivity in comparison to routine PCR was 10, 1,000 and 10 times higher respectively for the three viruses [94].

Dot-Blot and In situ Hybridization (ISH)

DNA probes are useful for the diagnosis of various viral diseases and provide greater diagnostic sensitivity than conventional histopathology. They find application in the non-lethal testing of valuable broodstock. Dot-blot and in situ hybridization (ISH) methods for detection of IHNV have been developed and commercial kits are available [3]. The first generation gene probe was developed by Mari et al. [55]. The specificity of BS4.5 probe labeled with non-radioactive digoxigenin-11-dUTP (DIG-11-dUTP) was investigated by dot blot, in situ hybridization and Southern blotting. Results confirmed to be highly specific for IHNV since it did not react with insect parvovirus DNA and HPV infected tissues [41, 55]. A non lethal serodiagnostic field kit was used for screening of candidate specific pathogen-free *P. vannamei* broodstock by using specific gene probe [17] and in 2007, a DIG-labeled probe I703 was developed for detection of IHNV infection in *P. vannamei* by dot-blot and in situ hybridization [96].

Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a relatively new DNA amplification technique, developed in 2000. It is a simple technique and has high specificity, efficiency and rapidity under isothermal conditions which can be applied for disease diagnosis in shrimp aquaculture. In 2006, a diagnostic LAMP based method was developed for IHNV detection and the four sets of primers were designed targeting the IHNV genome sequence (GenBank Accession No. AF218266) [77]. The target DNA is amplified and visualized using gel electrophoresis within 60 min under isothermal condition at 64 °C. The assay was 100-fold more sensitive than the traditional PCR. Recently, Arunrut et al. [4] successfully developed a LAMP assay combined with a chromatographic lateral flow dipstick (LFD) for the specific, rapid and simple visual detection of IHNV-specific amplicons. In this method, in addition to the four sets of primers normally used, one extra set of primer was required to accelerate the LAMP reaction. The technique developed in this study was faster than the usual LAMP reaction described in earlier study [77]. A real-time LAMP assay was developed for the detection of IHNV infection in penaeid shrimps by employing four primers

with a sensitivity of 10^2 – 10^3 copies/ μ l [76]. Recently, a multiplex LAMP (mLAMP) assay was developed for the detection of two penaeid shrimp viruses, IHHNV and WSSV. The sensitivity of this assay was 10^3 and 10^2 times higher on the detection limits for IHHNV and WSSV, respectively, when compared to nested and classical PCR [30].

Ramification Amplification

Ramification amplification (RAM), an isothermal nucleic acid amplification method, was first described in 1998 [98]. This method utilizes a specially designed circular probe (C-probe) as an amplifiable target. Once the C-probe hybridizes to the target region, the 5' and 3' ends of the probe are brought together and subsequent ligation of the two termini results in a closed circular molecule. By using specific primer pairs, the closed circular C probe is amplified under isothermal condition. After its initial discovery [98], it became widely used for diagnosis and single nucleotide polymorphism analysis [5, 53, 73, 87, 88, 91]. In 2006, a simple and sensitive RAM assay was developed for detection of IHHNV by Teng et al. [86]. The sensitivity of this assay was found similar to that of the IQ2000 IHHNV detection system.

Antigen-Based Detection

Although PCR-based methods are used widely for shrimp virus detection in the laboratory, they are not quite suitable for field detection. The detection and identification of IHHNV with an immunoassay is a simple and low-cost alternative that can be specific and sensitive. An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of IHHNV using 6 IgM Murine monoclonal antibodies (MAbs) generated against purified IHHNV by Poulos et al. [65]. Unfortunately, results of detection were not reliable since some of the shrimp samples that gave negative results by histology and gene probing gave positive results for ELISA and vice versa. More recently, based on MAbs generated against recombinant capsid protein of IHHNV, simple immunodetection methods have been developed for the detection of IHHNV in infected shrimp [75].

Concluding Remarks

IHHNV is one of the major viral pathogens of penaeid shrimps worldwide that has resulted in mass mortalities in *P. stylirostris*. In addition, it also causes abnormal deformities of cuticle, abdominal segments, tail fan and rostrum, wrinkled antennal flagella, bubble-heads along with wide

size variations and reduced growth that finally affects the quality of the commodity shrimp. Several important issues related to the process of infection, propagation and interaction of IHHNV with hosts at the cellular and molecular level, remain to be elucidated. Studies are needed as well, to determine the true risks of this viral infection on wild populations of crustaceans. Since IHHNV is widely distributed in shrimp culture facilities throughout the world, further studies are required to document its distribution among wild populations.

Recent reports show that genetic diversity of IHHNV is not uniform across geographic regions. For investigation on mutations within a geographic area or individual hosts, genome sequencing is the method of choice. Genetic characterization of multiple isolates from separate sites can clarify whether the viruses are evolving and/or whether exogenous sources account for the presence of different strains in an area. The sequencing of the complete genome of IHHNV strains is allowing great advances in the study of the biology of the organism and improving diagnosis and control of disease. Further studies are required to determine the relationship between the integrated and infectious form of IHHNV. Recent studies show that diagnosis by using currently recommended methods may lead to false positive test results for infectious IHHNV since some forms get integrated into the host genome. Hence, research should be focused on development of improved detection methods that would reduce or eliminate the possibility of false positive results.

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