CHAPTER 2.2.4.

**infection with INFECTIOUS HYPODERMAL
AND HAEMATOPOIETIC NECROSIS virus**

**1. Scope**

Infection with infectious hypodermal and haematopoietic necrosis virus means infection with the pathogenic agent infectious hypodermal and haematopoietic necrosis virus (IHHNV), Family *Parvoviridae,* subfamily *Hamaparvovirinae,* Genus *Penstylhamaparvovirus* with IHHNV (*Decapod penstylhamaparvovirus 1)* as the Type species (Penez *et al.,* 2020).

**2. Disease information**

**2.1. Agent factors**

**2.1.1. Aetiological agent**

IHHNV is the smallest of the known penaeid shrimp viruses. The virion is a 20–22 nm, non-enveloped icosahedron, with a density of 1.40 g ml–1 in CsCl that contains linear single-stranded DNA with an estimated size of 3.9 kb, and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (Bonami *et al.,* 1990; Nunan *et al.,* 2000; GenBank NC\_002190).

At least two distinct genotypes of IHHNV have been identified (Tang *et al.,* 2003): Type 1 is from the Americas and East Asia (principally the Philippines) and Type 2 is from South-East Asia. These genotypes are infectious to *Penaeus* *vannamei* and *P.* *monodon*. Two sequences homologous to part of the IHHNV genome are found embedded in the genome of penaeids. These were initially described as Type 3A from East Africa, India and Australia, and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al*., 2007). Tissues containing the IHHNV-homologous sequences (also known as endogenous viral elements; Taengchaiyaphum *et al*., 2021) in the *P.* *monodon* genome are not infectious to susceptible host species (Lightner *et al*., 2009; Tang & Lightner, 2006; Tang *et al*., 2007).

**2.1.2. Survival and stability in processed or stored samples**

IHHNV is believed to be the most stable virus of the known penaeid shrimp viruses. Infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner *et al.,* 1987; Lightner *et al.,* 2009).

**2.1.3. Survival and stability outside the host**

No data.

For inactivation methods, see Section 2.4.5.

**2.2. Host factors**

**2.2.1. Susceptible host species**

Species that fulfil the criteria for listing as susceptible to infection with IHHNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: yellowleg shrimp (*Penaeus californiensis*)*,* giant tiger prawn (*P. monodon*), northernwhite shrimp (*P. setiferus*),blue shrimp (*P. stylirostris*)*,* and white leg shrimp (*P. vannamei*).

**2.2.2. Species with incomplete evidence for susceptibility**

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with IHHNV according to Chapter 1.5 of the *Aquatic Code* are: northern brown shrimp (*Penaeus aztecus*). Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: giant river prawn (*Macrobrachium rosenbergii)*, northern pink shrimp (*Penaeus duorarum*)*,* western white shrimp(*P. occidentalis*), *kuruma prawn* (*P. japonicus*)*,* green tiger prawn(*P. semisulcatus*)*, Hemigrapsus penicillatus,* Argentine stiletto shrimp (*Artemesia longinaris*)*,* Cuata swimcrab (*Callinectes arcuatus),* Mazatlan sole (*Achirus mazatlanus*)*,* yellowfin mojarra(*Gerres cinereus*)*,* tilapias (*Oreochromis* sp.)*,* Pacific piquitinga (*Lile stolifera*)andblackfin snook (*Centropomus medius*)*.*

**2.2.3. Likelihood of infection by species, host life stage, population or sub-populations**

IHHNV has been detected in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei.* Nauplii produced from infected broodstock have a high prevalence of infection with IHHNV (Motte *et al.,* 2003).

**2.2.4. Distribution of the pathogen in the host**

IHHNV targets gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, lymphoid organ, parenchymal cells, connective tissue cells and ovaries ([Chayaburakul](https://pubmed.ncbi.nlm.nih.gov/?term=Chayaburakul+K&cauthor_id=16408834), 2005; Lightner, 1996; Lightner & Redman, 1998).

**2.2.5. Aquatic animal reservoirs of infection**

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infection may carry the virus subclinically and infect their progeny or other populations by vertical and horizontal transmission (Bell & Lightner, 1984; Lightner, 1996; Motte *et al*., 2003).

**2.2.6. Vectors**

IHHNV was found in wild crabs (*Hemigrapsus penicillatus*, *Neohelice granulata*), but there were no clinical signs. Adults of *Macrobrachium rosenbergii* are carriers of IHHNV without apparent signs. Although the mussel *Mytilus edulis* is an important reservoir of IHHNV (Wei *et al.,* 2017), its capacity to transmit virus is unknown.

**2.3. Disease pattern**

**2.3.1. Mortality, morbidity and prevalence**

The effects of infection with IHHNV varies among shrimp species and populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with IHHNV results in acute, usually catastrophic, disease with mortalities approaching 100%. In contrast, in populations of *P. vannamei*, some selected lines of *P. stylirostris*, and some populations of *P. monodon*, infection with IHHNV results in a more subtle, chronic disease, runt-deformity syndrome (RDS), in which high mortalities are unusual, but where growth suppression and cuticular deformities are common (Kalagayan *et al*., 1991; Sellars *et al.,* 2019).

Infection with IHHNV interferes with normal egg, larval, and postlarval development. When broodstock are used from wild or farmed stocks where the disease is enzootic, hatching success of eggs may be reduced, and survival and culture performance of the larval and postlarval stages lowered (Motte *et al.,* 2003).

In the past, stocks of *P. stylirostris*, juveniles, subadults, and adults showed persistently high mortality rates due to infection with IHHNV. However, selected lines of *P. stylirostris* do not show mortality and appear to be tolerant to this virus. *Penaeus vannamei* and *P. monodon* stocks infected with IHHNV show poor and highly disparate growth and cuticular deformities, particularly bent rostrums and deformed sixth abdominal segments (Jagadeesan *et al.,* 2019; Sellars *et al.,* 2019).

In regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *P. stylirostris* in the lower and upper Gulf of California, respectively (Pantoja *et al.,* 1999); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (Morales-Covarrubias *et al.,* 1999); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (Nunan *et al.,* 2001); from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte *et al.,* 2003), and from 6 to 63% in *P. vannamei* broodstock and 49.5% in post-larvae fromMexico (Fernando *et al*., 2016). In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence is typical (Aly *et al*., 2021; Chayaburakul *et al.,* 2004; Lightner, 1996; Lightner *et al.,* 1983).

**2.3.2. Clinical signs, including behavioural changes**

Certain cuticular deformities, specifically a deformed rostrum bent to the left or right, which may be presented by *P. vannamei* and *P. stylirostris* with RDS, are indicative of infection with IHHNV (see Section 2.3.3 *Gross pathology: Infection with IHHNV in* Penaeus vannamei). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV.

In acute disease, *P. stylirostris* may present behavioural changes (see Section 2.3.3 *Gross pathology:* *Infection with IHHNV in* Penaeus stylirostris) but with RDS, no consistent behavioural changes have been reported for affected shrimp.

**2.3.3. Gross pathology**

*Infection with IHHNV in* Penaeus stylirostris

Infection with IHHNV may result in acute disease with very high mortalities in juveniles. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size- or age-dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Lightner, 1996; Lightner *et al.,* 1983). Gross signs are non-specific, but juvenile *P. stylirostris* with acute infection with IHHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHHNV have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. *Penaeus stylirostris* at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *P. stylirostris* and individuals become more bluish. In *P. stylirostris* and *P. monodon* with terminal-phase infection with IHHNV, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Lightner *et al.,* 1983).

*Infection with IHHNV in* Penaeus vannamei

RDS, a chronic form of infection with IHHNV, occurs in *P. vannamei.* The severity and prevalence of RDS in infected populations of juvenile or older *P. vannamei* may be related to infection during the larval or early postlarval stages. RDS has also been reported in cultured stocks of *P. stylirostris* and *P. monodon*. Juvenile shrimp with RDS may display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness, ‘bubble-heads’, and other cuticular deformities. Populations of juvenile shrimp with RDS display disparate growth with a wide distribution of sizes and many smaller than expected (‘runted’) shrimp. The coefficient of variation (CV = the standard deviation divided by the mean of different size groups within a population) for populations with RDS is typically greater than 30% and may approach 90%, while populations of juvenile *P. vannamei* and *P. stylirostris* free from infection with IHHNV (and thus RDS-free) usually show CVs of 10–30% (Lightner, 1996; Primavera & Quinitio, 2000).

**2.3.4. Modes of transmission and life cycle**

Transmission of IHHNV can be by horizontal or vertical. Horizontal transmission has been demonstrated by cannibalism or by contaminated water (Lightner, 1996; Lightner *et al.,* 1983), and vertical transmission via infected eggs (Motte *et al.,* 2003).

**2.3.5. Environmental factors**

The replication rate of IHHNV at high water temperatures was significantly reduced in a study in which viral replication was compared in *P.* *vannamei* experimentally infected and held at 24°C and 32°C. After a suitable incubation period, shrimp held at 32°C had approximately 102 times lower viral load than shrimp held at 24°C (Montgomery-Brock *et al*., 2007).

**2.3.6. Geographical distribution**

Infection with IHHNV has been reported from cultured shrimp in most of the major shrimp-culturing regions of the world including Asia, Oceania, North and South America and the Middle East.

IHHNV homologous sequences have been found within the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.*, 2007). These sequences do not represent viral DNA (refer Section 2.1.1 *Aetiological agent*).

See OIE WAHIS (<https://wahis.oie.int/#/home>) for recent information on distribution at the country level.

**2.4. Biosecurity and disease control strategies**

**2.4.1. Vaccination**

None available.

**2.4.2. Chemotherapy including blocking agents**

No scientifically confirmed reports.

**2.4.3. Immunostimulation**

No scientifically confirmed reports.

**2.4.4. Breeding resistant strains**

Selected stocks of *P. stylirostris* that are resistant to infection with IHHNV have been developed and these have had some successful application in shrimp farms (Lightner, 1996). However, lines of *P. stylirostris* bred for resistance to infection with IHHNV (Tang *et al.,* 2000) do not have increased resistance to other diseases, such as white spot syndrome virus (WSSV), so their use has been limited. In some stocks a genetic basis for IHHNV susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.,* 1997).

**2.4.5. Inactivation methods**

IHHNV is a stable shrimp virus; infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner *et al.,* 2009).

**2.4.6. Disinfection of eggs and larvae**

IHHNV is transmitted vertically by the transovarian route (Motte *et al.,* 2003). Disinfection of eggs and larvae is good management practice (Chen *et al.,* 1992) that may reduce IHHNV contamination of spawned eggs and larvae but is not effective for preventing transovarian transmission of IHHNV (Motte *et al.,* 2003).

**2.4.7. General husbandry**

Some husbandry practices have been successful in preventing the spread of IHHNV. Among these has been the application of PCR pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Motte *et al.,* 2003), as well as the development of specific pathogen-free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 2005). The latter has proven to be the most successful husbandry practice for the prevention and control of infection with IHHNV (Lightner, 2005).

**3. Specimen selection, sample collection, transportation and handling**

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

**3.1. Selection of populations and individual specimens**

Infection with IHHNV may be below detection limits in spawned eggs and larval stages, so these life stages are not suitable for surveillance to demonstrate freedom from infection with IHHNV.

**3.2. Selection of organs or tissues**

IHHNV infects tissues of ectodermal and mesodermal origin. The principal target tissues for IHHNV include connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells (Lightner, 1996; Lightner & Redman, 1998). Hence, whole shrimp (e.g. larvae or postlarvae) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

**3.3. Samples or tissues not suitable for pathogen detection**

Enteric tissues (e.g. the hepatopancreas, the midgut or its caeca) are inappropriate samples for detection of IHHNV (Lightner, 1996; Lightner & Redman, 1998). Shrimp eyes contain PCR inhibitors and their use should be avoided.

**3.4. Non-lethal sampling**

Haemolymph or excised pleopods may be collected and used for testing when non-lethal testing of valuable broodstock is necessary (Lightner, 1996; Lightner & Redman, 1998).

**3.5. Preservation of samples for submission**

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

**3.5.1. Samples for pathogen isolation**

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

**3.5.2. Preservation of samples for molecular detection**

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.5 of Chapter 2.2.0 *General information* (diseases of crustaceans).

**3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation**

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.2 of Chapter 2.2.0 *General information* (diseases of crustaceans).

**3.5.4. Samples for other tests**

Not relevant.

**3.6. Pooling of samples**

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger shrimp should be processed and tested individually. Small life stages such as PL can be pooled to obtain the minimum amount of material for virus isolation or molecular detection.

**4. Diagnostic methods**

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

**Ratings for purposes of use.** For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ = Methods are most suitable with desirable performance and operational characteristics.

++ = Methods are suitable with acceptable performance and operational characteristics under most circumstances.

+ = Methods are suitable, but performance or operational characteristics may limit application under some circumstances.

Shaded boxes = Not appropriate for this purpose.

**Validation stage**. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

OIE Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

***Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals***

| **Method** | 1. **Surveillance of apparently healthy animals**
 | 1. **Presumptive diagnosis of clinically affected animals**
 | 1. **Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis**
 |
| --- | --- | --- | --- |
| **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** |
| **Wet mounts** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Histopathology** |  |  |  |  |  | ++ | ++ | NA |  | ++ | ++ | NA |
| **Cell culture** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Real-time PCR** | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 | ++ | ++ | ++ | 1 |
| **Conventional PCR** | + | ++ | ++ | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing** |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** |  |  |  |  |  | + | + | 1 |  | ++ | ++ | 1 |
| **Bioassay** |  |  |  |  |  |  |  |  |  |  |  |  |
| **LAMP** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ab-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ag-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other antigen detection methods3** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other methods3** |  |  |  |  |  |  |  |  |  |  |  |  |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction;
LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). 2Susceptibility of early and juvenile life stages is described in Section 2.2.3.
3Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

**4.1. Wet mounts**

No reliable methods have been developed for direct microscopic pathology.

**4.2. Histopathology and cytopathology**

Presumptive acute infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained sections whereas chronic infection are much more difficult to diagnose using these staining methods. For diagnosis of chronic infections and confirmation of acute infections however, the use of molecular methods is required for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or *in-situ* hybridisation [ISH] of histological sections).

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of infection with IHHNV. These characteristic IHHNV inclusion bodies are eosinophilic and often haloed (with H&E stains of tissues preserved with fixatives that contain acetic acid, such as Davidson’s AFA and Bouin’s solution) (Bell & Lightner, 1988; Lightner, 1996), intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies caused by infection with IHHNV may be easily confused with developing intranuclear inclusion bodies caused by WSSV infection. ISH assay (see Section 4.6 In-situ *hybridisation*) of such sections with a DNA probe specific to IHHNV provides a definitive diagnosis of infection with IHHNV (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

The use of Davidson’s fixative (containing 33% ethyl alcohol [95%], 22% formalin [approximately 37% formaldehyde], 11.5% glacial acetic acid and 33.5% distilled or tap water) is highly recommended for all routine histological studies of shrimp (Bell & Lightner, 1988; Lightner, 1996a). To obtain the best results, dead shrimp should not be used. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are killed by injection of fixative directly into the hepatopancreas; the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline is opened with fine-pointed surgical scissors to enhance fixative penetration (the abdomen may be removed and discarded), the whole shrimp (or cephalothorax) is immersed in fixative for 24 to 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags.

**4.3. Cell culture for isolation**

IHHNV has not been grown *in vitro*. No crustacean cell lines exist.

**4.4. Nucleic acid amplification**

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (Tang *et al*., 2000; 2007). However, these tests also detect non-infectious endogenous viral elements (EVE) within the *P. monodon* genome (previously known as types 3A and 3B), which are inserted into the genome of certain stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (Saksmerprome *et al.,* 2011; Tang & Lightner, 2006; Tang *et al.,* 2007). As these PCR methods may result in positive test results in uninfected *P. monodon,* positive results should be confirmed by a method that detects IHHNV but not the IHHNV-related EVEs.

PCR primers have been developed that can detect the IHHNV sequence but do not amplify IHHNV-related EVEs present in the *P. monodon* stocks from Africa, Australia (Tang *et al.,* 2007), or Thailand (Saksmerprome *et al.,* 2011). Primer set 309F/R amplifies only a genomic segment of IHHNV types 1 and 2 (the infectious forms of IHHNV), but not the non-infectious EVEs within the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Primer set MG831F/R reacts only with non-infectious EVEs within the *P. monodon* genome (Tang *et al.,* 2007). Hence, confirmation of unexpected positive or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. histology, bioassay, ISH) is highly recommended.

**4.4.1. Real-time PCR**

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

**Real-time** PCR methods have been developed for the detection of IHHNV (Dhar *et al.,* 2001; Tang & Lightner, 2001). A highly sensitive SYBR Green real-time PCR targeting a segment of the IHHNV genome that is less susceptible to endogenisation was developed (Encinas-Garcia *et al.,* 2015). More recently, a TaqMan real-time assay capable of differentiating endogenous virus element from infectious form of IHHNV in *P. monodon* has been reported (Cowley *et al.,* 2018)*.* The real-time PCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (2001).

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5’-TAC-TCC-GGA-CAC-CCA-ACC-A-3’ and 5’-GGC-TCT-GGC-AGC-AAA-GGT-AA-3’, respectively. The TaqMan probe 5’-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3’), is synthesised and labelled with FAM on the 5’ end and TAMRA on the 3’ end.

ii) Preparation of DNA template: DNA extracted from tissues or haemolymph that was preserved in 95% ethanol and then dried. A control consisting of tissues or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods. Commercial DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega), or DNazol (Life Technologies). Spectrophotometric readings of the final DNA will indicate the purity of the DNA and the amount of total DNA extracted from the sample.

iii) The real-time PCR reaction mixture contains: TaqMan Fast virus 1-step Master Mix (Life Technologies, or commercially-available equivalent reagents), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 20 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) The cycling profile is: initial denaturation of 20 seconds at 95°C, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.

**4.4.2. Conventional PCR**

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Several one-step PCR methods (Krabsetsve *et al.,* 2004; Nunan *at al.,* 2000; Shike *et al.,* 2000; Tang *et al.,* 2000; 2007), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available.

***Table 4.4.2.1.*** ***Recommended primer sets for one-step PCR detection of IHHNV***

| **Primer**  | **Product** | **Sequence (5’-3’)** | **G+C%/Temp.** | **GenBank & References** | **Specificity** |
| --- | --- | --- | --- | --- | --- |
| 389F | 389 bp | CGG-AAC-ACA-ACC-CGA-CTT-TA  | 50%/72°C | AF218266 | All genetic variants of IHHNV  |
| 389R |  | GGC-CAA-GAC-CAA-AAT-ACG-AA | 45%/71°C | (Tang *et al.,* 2007) | and IHHNV-related EVEs |
| 77012F | 356 bp | ATC-GGT-GCA-CTA-CTC-GGA | 50%/68°C | AF218266 | Not given in the reference |
| 77353R |  | TCG-TAC-TGG-CTG-TTC-ATC | 55%/63°C | (Nunan *et al.,* 2000) |  |
| 392F | 392 bp | GGG-CGA-ACC-AGA-ATC-ACT-TA | 50%/68°C | AF218266 | All genetic variants of IHHNV and IHHNV-related EVEs |
| 392R |  | ATC-CGG-AGG-AAT-CTG-ATG-TG | 50%/71°C | (Tang *et al.,* 2000) |  |
| 309F | 309 bp | TCC-AAC-ACT-TAG-TCA-AAA-CCA-A | 36%/68°C | AF218266  | IHHNV but not IHHNV-related EVEs |
| 309R |  | TGT-CTG-CTA-CGA-TGA-TTA-TCC-A | 40%/69°C | (Tang *et al.,* 2007) |  |
| MG831F | 831 bp | TTG-GGG-ATG-CAG-CAA-TAT-CT | 45%/58°C | DQ228358 | IHHNV-related EVEs but not IHHNV |
| MG831R |  | GTC-CAT-CCA-CTG-ATC-GGA-CT | 55%/62°C | (Tang *et al.,* 2007) |  |

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region of the IHHNV genome. Primers 77012F/77353R are from a region in between the nonstructural and capsid protein-coding region of the genome. In the event that results are ambiguous using the 389F/R ‘universal’ primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012F/77353R or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.

General PCR method for IHHNV:the PCR method described below for IHHNV generally follows the methods outlined in Tang *et al.* (2007) and Nunan *et al.* (2000). However, recent minor modifications including the sources of the reagents and the use of an automated DNA extraction instrument are acceptable. The modifications include DNA extraction method, choice of primers (Table 4.4.2.1), and the volume of reaction. These slightly modified methods have been validated in accordance with Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases* and do not affect the diagnostic performance of the assay.

i) Use as a template, the extraction of DNA templates is the same as that described above. Use 1–5 µl of extracted DNA as a template per 25 µl reaction volume.

ii) The following controls should be included in every PCR assay for IHHNV: (a) DNA from a known negative tissue sample; (b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and (c) a ‘no template’ control.

iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV-infected material. Prepare primers at 10 µM in distilled water.

iv) If PuReTaqTM Ready-To-Go PCR Beads (GE Healthcare) are used, the PCR profile involves a 3–5 minutes at 95°C to denature DNA followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

v) Prepare a ‘Master Mix’ consisting of water and primers.

vi) For a 25 µl reaction mix, add 24 µl Master Mix to each tube and then add 1 µl of the DNA template to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. Insert tubes into the thermal cycler and start the PCR program.

viii) After PCR, run 6–10 µl of the sample in a 1.5% agarose gel (containing SYBRTM Safe (Thermo Fisher Scientific) or equivalent to stain the DNA). Look for the 389 bp band (if using primers 389F and 389R) or for the 356 bp band (if using primers 77012F and 77353R). Bands are not always seen, as it is necessary to have at least 10 ng DNA µl–1 to see DNA in a gel. A direct sequencing of amplified products can be performed through gel extraction of a PCR band with correct size and the sequencing primer(s) used for amplification to confirm the presence of IHHNV.

**4.4.3. Other nucleic acid amplification methods**

Loop-mediated isothermal amplification (LAMP) assays and real-time isothermal recombinase polymerase amplification (RPA) assay are available to detect and confirm IHHNV infection (Arunrut *et al*., 2011; Sun *et al*., 2006; Xia *et al.*, 2015), however, they are currently not recommended as they are not sufficiently validated.

**4.5. Amplicon sequencing**

PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified products from the infectious form of the virus and demonstrate the presence of the non-infectious IHHNV-related EVEs in the host genome (Tang & Lighter, 2006).

**4.6. *In-situ* hybridisation**

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. The ISH method uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al*. (1993) and Lightner (1996).

Gene probe and PCR methods provide greater diagnostic specificity and sensitivity than traditional techniques that employ classic histological approaches. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp. A haemolymph sample may be taken with a tuberculin syringe, or an appendage (a pleopod for example) may be biopsied (Bell *et al*., 1990), and used as the sample for a dot-blot hybridisation test.

**4.7. Immunohistochemistry**

Not relevant.

**4.8. Bioassay**

If SPF shrimp are available, the following bioassay method is based on Tang *et al.* (2000), is suitable for IHHNV diagnosis.

i) For bioassay, feed the minced shrimp tissue suspected of being infected with IHHNV to the indicator shrimp species (e.g. SPF *P. vannamei* and *P. stylirostris* at the PLs or juvenile stage) at 10% of their body weight twice daily for 1 days.

ii) For the following, the indicator shrimp were maintained on a pelletised ration.

iii) Examine moribund shrimp grossly or by using the methods described above. There may be no apparent mortality during the experimental period.

iv) If at 30 days after feeding there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

Known IHHNV positive and negative control groups should be included in the bioassay.

**4.9. Antibody- or antigen-based detection methods**

None has been successfully developed.

**4.10. Other methods**

Not available.

**5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations**

Conventional PCR and/or real-time PCR are the recommended test for surveillance to demonstrate freedom from infection with IHHNV in apparently healthy populations as described in Sections 4.4.1 and 4.4.2.

**6. Corroborative diagnostic criteria**

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

**6.1. Apparently healthy animals or animals of unknown health status**4F4F**[[1]](#footnote-1)**

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

**6.1.1. Definition of suspect case in apparently healthy animals**

The presence of infection with IHHNV shall be suspected if at least one of the following criteria is met:

i) Positive result by conventional PCR

ii) Positive result by real-time PCR

**6.1.2. Definition of confirmed case in apparently healthy animals**

The presence of infection with IHHNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time PCR and conventional PCR targeting non-overlapping regions of the viral genome and amplicon sequencing

ii) Histopathology consistent with IHHNV infection coupled with *in-situ* hybridisation and detection of IHHNV by real-time PCR

iii) Histopathology consistent with IHHNV infection coupled with *in-situ* hybridisation and detection of IHHNV by conventional PCR and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

**6.2 Clinically affected animals**

Clinical signs are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

**6.2.1. Definition of suspect case in clinically affected animals**

The presence of infection with IHHNV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality

ii) Positive result by conventional PCR

iii) Positive result by real-time PCR

iv) Histopathology consistent with IHHNV infection

v) Positive result by *in-situ* hybridisation

**6.2.2. Definition of confirmed case in clinically affected animals**

The presence of infection with IHHNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time PCR and conventional PCR targeting a non-overlapping region of the genome and amplicon sequencing

ii) Histopathological changes characteristic of infection with IHHNV with a positive result by *in-situ* hybridisation and detection of IHHNV by real-time PCR

iii) Histopathological changes characteristic of infection with IHHNV or positive result by *in-situ* hybridisation and detection of IHHNV by conventional PCR and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

**6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]**

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHHNV is provided in Table 6.3.1. This information can be used for the design of surveys for infection with IHHNV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

**6.3.1. For presumptive diagnosis of clinically affected animals**

| **Test type** | **Test purpose** | **Source populations** | **Tissue or sample types** | **Species** | **DSe (*n*)** | **DSp (*n*)** | **Reference test** | **Citation** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |

* DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.

**6.3.2. For surveillance of apparently healthy animals**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Test type** | **Test purpose** | **Source populations** | **Tissue or sample types** | **Species** | **DSe (*n*)** | **DSp (*n*)** | **Reference test** | **Citation** |
|  |  |  |  |  |  |  |  |  |

* DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.

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\* \*

**NB:** There are OIE Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/> ).
Please contact the OIE Reference Laboratories for any further information on
infection with infectious hypodermal and haematopoietic necrosis virus

**NB:** First adopted in 1995 as infectious hypodermal and haematopoietic necrosis;
Most recent updates adopted in 2018.

1. For example transboundary commodities. [↑](#footnote-ref-1)