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*, Genus *Penstyldensovirus*.

The International Committee on the Taxonomy of Viruses has assigned IHHNV with the species name of Decapod penstyldensovirus 1 (King et al., 2012).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

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, 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 AF218266).

At least two distinct genotypes of IHHNV have been identified (Tang et al., 2003): Type 1 from the Americas and East Asia (principally the Philippines). Type 2 from South-East Asia. These genotypes are infectious to *P. 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 in the *P. monodon* genome are not infectious to the susceptible host species *P. vannamei* and *P. monodon* (Lightner et al., 2009; Tang & Lightner, 2006; Tang et al., 2007).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective 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, 1996a; Lightner et al., 1987; Lightner et al., 2009).

2.1.4. Life cycle

Not applicable.

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 the *Aquatic Animal Health Code* (*Aquatic Code*) include: yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*Penaeus stylirostris*), and white leg shrimp (*Penaeus 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* include: northern brown shrimp (*Penaeus aztecus*).

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 (*Penaeus occidentalis*), kuruma prawn (*Penaeus japonicus*), green tiger prawn (*Penaeus semisulcatus*), *Hemigrapsus penicillatus*, Argentine stiletto shrimp (*Artemesia longinaruis*), Cuata swimcrab (*Callinectes arcuatus*), Mazatlan sole (*Archirus mazatlanus*), yellowfin mojarra (*Gerres cinereus*), tilapias (*Oreochromis* sp.), Pacific piquitinga (*Lile stolifera*) and blackfin snook (*Centropomus medius*).

2.2.3. Susceptible stages of the host

IHHNV has been detected in all life stages (i.e. eggs, larvae, postlarvae [PL], juveniles and adults) of *P. vannamei.* Eggs produced by IHHNV-infected females with high virus loads were found to generally fail to develop and hatch. Those nauplii produced from infected broodstock that do hatch have a high prevalence of infection with IHHNV (Motte et al., 2003).

2.2.4. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1and 2.2.3

2.2.5. Target organs and infected tissue

IHHNV infects and has been shown to replicate (using *in-situ* hybridisation [ISH] with specific DNA probes) in tissues of ectodermal and mesodermal origin from the embryo. Thus, the principal target organs include: the gills, cuticular epithelium (or hypodermis), all connective tissues, the haematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, and striated muscle show no histological signs of infection with IHHNV and are usually negative by ISH (Lightner, 1993; Lightner, 1996a; Lightner, 2011; Lightner et al., 1992b).

2.2.6. Persistent infection

Some members of *P. stylirostris* and *P. vannamei* populations that survive infection with IHHNV epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (Bell & Lightner, 1984; Lightner, 1996a; Lightner, 1996b; Morales-Covarrubias & Chavez-Sanchez, 1999; Motte et al., 2003).

2.2.7. Vectors

No vectors are known.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of IHHNV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (Lightner, 1996a; Lightner et al., 1983a; Lightner et al., 1983b; Lightner et al., 1985), as has vertical transmission via infected eggs (Motte et al., 2003).

2.3.2. Prevalence

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); and from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte et al., 2003). Other penaeids collected during some of these surveys and found to be IHHNV positive included the brown shrimp, *P. californiensis* and the Western white shrimp

P. occidentalis. In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence, approaching 100%, is typical (Chayaburakul et al., 2004; Lightner, 1988; Lightner, 1996a; Lightner, 1996b; Lightner et al., 1992a; Lightner et al., 1983a; Martinez-Cordova, 1992).

2.3.3. Geographical distribution

IHHNV appears to have a world-wide distribution in both wild and cultured penaeid shrimp (Brock & Lightner, 1990; Lightner, 1996a; Lightner, 1996b; Owens et al., 1992). Although infection with IHHNV has been reported from cultured *P. vannamei* and *P. stylirostris* in most of the shrimp-culturing regions of the Western Hemisphere and in wild penaeids throughout their range along the Pacific coast of the Americas (Peru to northern Mexico), the virus has not been reported in wild penaeid shrimp on the Atlantic coast of the Americas (Bondad-Reantaso et al., 2001; Brock & Main, 1994; Lightner, 1996a; Lightner, 1996b; Lightner et al., 1992a; Lightner & Redman, 1998a; Lightner & Redman, 1998b). infection with IHHNV has also been reported in cultured penaeid shrimp from Pacific islands including the Hawaiian Islands, French Polynesia, Guam, and New Caledonia. In the Indo-Pacific region, the virus has been reported from cultured and wild penaeid shrimp in East Asia, South-East Asia, and the Middle East (Bondad-Reantaso et al., 2001; Lightner, 1996a).

An IHHN-like virus sequence has been reported from Australia (Krabsetsve et al., 2004; Owens et al., 1992), and the presence of infection with IHHNV in farmed prawns in Australia was reported to the OIE in 2008. As discussed in Section 2.1.1, IHHNV-related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang et al., 2007)

2.3.4. Mortality and morbidity

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 an 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, RDS, in which high mortalities are unusual, but where growth suppression and cuticular deformities are common (Kalagayan et al., 1991).

Infection with IHHVV 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 is reduced, and survival and culture performance of the larval and postlarval stages is lowered (Motte et al., 2003).

Stocks of *P. stylirostris*, juveniles, subadults and adults show persistently high mortality rates. In *P. vannamei* and possibly *P. monodon* stocks infected with IHHNV show poor and highly disparate growth, and cuticular deformities, particularly bent rostrums and deformed sixth abdominal segments.

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 10^2 lower viral load than shrimp held at 24°C (Montgomery-Brock et al., 2007).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods for IHHNV have been developed.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Breeding for resistance

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 (Clifford, 1998; Lightner, 1996a; Lightner, 1996b; Weppe, 1992; Zarain-Herzberg & Ascensio-Valle, 2001). However lines of *P. stylirostris* that were 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 IHHN susceptibility in *P. vannamei* has been reported (Alcivar-Warren et al., 1997).

2.4.5. Restocking with resistant species

There has been some limited application and success with disease-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarain-Herzberg & Ascensio-Valle, 2001). The relative resistance of *P. vannamei* to infection with IHHNV is considered to be among the principal factors that led to *P. vannamei* being the principal shrimp species farmed in the Western Hemisphere and, since 2004, globally (Lightner, 2005; Lightner et al., 2009).

2.4.6. Blocking agents

There are no reports to date for IHHNV blocking agents.

2.4.7. Disinfection of eggs and larvae

IHHNV has been demonstrated to be transmitted vertically by the transovarian route (Motte et al., 2003). Hence, while disinfection of eggs and larvae is good management practice (Chen et al., 1992) and is recommended for its potential to reduce IHHNV contamination of spawned eggs and larvae produced from them (and contamination by other disease agents), the method is not effective for preventing transovarian transmission of IHHNV (Motte et al., 2003).

2.4.8. General husbandry practices

Some husbandry practices have been successful in preventing the spread of IHHNV. Among these has been the application of polymerase chain reaction (PCR) pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001; Motte et al., 2003), as well as the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; Lightner, 2005; Lotz et al., 1995; Pruder et al., 1995; Wyban, 1992). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (Jaenike et al., 1992; Lightner, 2005; Pruder et al., 1995).

3. Sampling

3.1. Selection of individual specimens

While infection with IHHNV may affect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages are not suitable samples for detection or certification of disease freedom from infection with IHHNV.

3.2. 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.

3.3. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing.

3.4. Best organs and 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, 1996a; Lightner & Redman,

1998a). Hence, whole shrimp (e.g. larvae or PLs) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

Haemolymph or excised pleopods may be collected and used for testing (usually for PCR, or dot-blot hybridisation with specific probes) when non-lethal testing of valuable broodstock is necessary (Lightner, 1996a; Lightner & Redman, 1998a).

3.5. Samples/tissues that are not suitable

IHHNV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of IHHNV (Lightner, 1996a; Lightner, 2011; Lightner & Redman, 1998a).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

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 pathognomonic for infection with IHHNV (see Section 4.2.1.2). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV. As *P. vannamei*, *P. stylirostris*, and *P. monodon* can be infected by IHHNV and not present obvious signs of infection (e.g. they may show markedly reduced growth rates or 'runting'), molecular tests are recommended when evidence of freedom from infection with IHHNV is required.

4.1.2. Behavioural changes

In acute disease, *P. stylirostris* may present behavioural changes (see Section 4.2.1.1) but with RDS, no consistent behavioural changes have been reported for affected shrimp.

4.2. Clinical methods

4.2.1. Gross pathology

4.2.1.1. Infection with IHHNV in *Penaeus stylirostris*

Infection with IHHNV is often acute with very high mortalities occurring in juvenile life stages of this species. 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 and/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; Bell & Lightner, 1987; Bondad-Reantaso et al., 2001; Brock et al., 1983; Brock & Main, 1994; Lightner, 1988; Lightner, 1993; Lightner, 1996a; Lightner, 2011; Lightner et al., 1983a; Lightner et al., 1983b). Gross signs are not 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 with acute infection 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* as such individuals become more bluish. In *P. stylirostris* and *P. monodon* with terminal-phase IHHNV infections, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Bondad-Reantaso et al., 2001; Lightner et al., 1983a; Lightner et al., 1983b).

4.2.1.2. 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 that occurred 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% (Bray et al., 1994; Brock & Lightner, 1990; Brock et al., 1983; Brock & Main, 1994; Browdy et al., 1993; Carr et al., 1996; Lightner, 1996a; Primavera & Quinitio, 2000; Pruder et al., 1995).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained sections (see Section 4.2.6). Chronic infection with IHHNV and RDS are much more difficult to diagnose using routine H&E histological methods. For diagnosis of chronic infections, the use of molecular methods are recommended for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or 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, 1996a), 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 4.3.1.2.3of this chapter) of such sections with a specific DNA probe to IHHNV provides a definitive diagnosis of infection with IHHNV (Lightner, 1996a; Lightner, 2011; Lightner & Redman, 1998a).

4.2.4. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

4.2.6.1. Histopathology

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 less the abdomen) is immersed in fixative for from 24 to no more than 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported (via post or courier to the diagnostic laboratory) by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags (see Section 4.2.3).

4.2.6.2. *In-situ* hybridisation

See Section 4.3.1.2.3below.

4.2.7. Electron microscopy/cytopathology

Electron microscopy is not recommended for routine diagnosis of IHHNV.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4

4.3.1.1.2. Smears

See Section 4.2.5

4.3.1.1.3. Fixed sections

See section 4.2.6

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

IHHNV has not been grown *in vitro*. No crustacean cell lines exist (Lightner, 1996a; Lightner & Redman, 1998a; Lightner & Redman, 1998b).

4.3.1.2.2. Antibody-based antigen detection methods

None has been successfully developed.

4.3.1.2.3. Molecular techniques

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. PCR tests for IHHNV have been developed and a number of methods and commercial PCR detection kits are readily available.

4.3.1.2.3.1. DNA probes for dot-blot and ISH applications

Gene probe and PCR methods provide greater diagnostic specificity and sensitivity than traditional techniques that employ classical 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.

4.3.1.2.3.2. Dot-blot hybridisation procedure for IHHNV

The dot-blot hybridisation method given below uses a digoxigendin-11-dUTP (DIG)-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari et al., 1993and Lightner, 1996a. Formulas for the required reagents are given after the protocols.

i) Prepare a positively charged nylon membrane: cut to a size to fit samples and controls and mark with soft-lead pencil making 1 cm squares for each sample. Include a positive and a negative control on each filter. Lay out on to a piece of filter paper (Whatman 3MM).

ii) If necessary, samples can be diluted in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) plus 50 µg ml^{-1} salmon sperm DNA. Samples for dot-blot hybridisation can be haemolymph, tissues homogenised in TN buffer (20 mM Tris-HCl, pH 7.4, 0.4 M NaCl), or extracted DNA in 10 mM Tris/HCl.

iii) Boil samples for 5 minutes and quench on ice for 1–2 minutes. Keep on ice until samples are dotted on to the membrane.

iv) Dot 1–3 µl of each sample on to an appropriate place on the filters. Allow to air-dry and then fix samples on to the membrane by baking at 80°C for 30 minutes or by UV cross-linking using a DNA transilluminator for 3 minutes.

v) Remove membranes from the oven or transilluminator and put into a heat-seal bag with 4 ml per membrane of prehybridisation solution. Seal the bags and put into a 68°C water bath for 0.5–1 hour.

vi) Boil the DIG-labelled probe for 3–5 minutes and then keep on ice. Remove the prehybridisation solution from the bags. Add 2 ml of fresh prehybridisation solution to each bag and then add the predetermined amount of DIG-labelled probe to each, mixing well as it is being added. Seal the bags, place back in the 68°C water bath and incubate for 8–12 hours.

vii) Wash membranes well with:

2 × SSC/0.1% SDS	2 ×	5 minutes at room temperature
0.1 × SSC/0.1% SDS	3 ×	15 minutes at 68°C
Buffer I	1 ×	5 minutes at room temperature

viii) React the membrane in bags with anti-DIG alkaline phosphatase conjugate (Roche Diagnostics[1]) diluted 1/5000 in Buffer I Incubate for 30–45 minutes at room temperature on a shaker platform.

ix) Wash membrane well with:

Buffer I	2 ×	15 minutes at room temperature
Buffer III	1 ×	5 minutes at room temperature

x) Develop the membranes in bags using a development solution (nitroblue tetrazolium salt [NBT]/X-phosphate in Buffer III) made up just prior to use. React in the dark at room temperature for 1–2 hours. Stop the reactions in Buffer IV and dry the membranes on 3MM filter paper.

xi) Photograph the results (colour fades over time).

xii) Store dry membranes in heat-seal bags.

4.3.1.2.3.3. In-situ *hybridisation (ISH) procedure*

The ISH method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari et al., 1993and Lightner, 1996a.

i) Embed tissue in paraffin and cut sections at 4–6 µm thickness. Place sections on to positively charged microscope slides (do not put gelatine in water to float sections; just use water).

ii) Put slides in a slide rack, such as a Tissue-Tek rack. Heat the slides in an oven for 45 minutes at 60°C. In the staining centre, rehydrate the tissue as follows:

Xylene (or suitable substitute)	3 ×	5 minutes each
Absolute alcohol	2 ×	1 minute each
95% alcohol	2 ×	10 dips each
80% alcohol	2 ×	10 dips each
50% alcohol	1 ×	10 dips
Distilled water	Six rinses (do not let slides dry out)	

iii) Wash the slides for 5 minutes in PBS (or Tris/NaCl/EDTA [TNE] buffer). Prepare fresh proteinase K at 100 µg ml^{-1} in PBS (or TNE). Place slides flat in a humid chamber, pipette on 500 µl of the proteinase K solution and incubate for 10–15 minutes at 37°C. Drain fluid onto blotting paper.

1 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

iv) Return slides to slide rack. Fix sections in 0.4% cold formaldehyde for 5 minutes at room temperature.

v) Incubate slides in 2 × SSC for 5 minutes at room temperature.

vi) With slides flat, add 0.5–1 ml prehybridisation buffer and incubate in a humid chamber for 15–30 minutes at 37°C.

vii) Boil the DIG-labelled probe for 3–5minutes and quench on ice; spin briefly in the cold and keep on ice. Dilute the probe to 25 ng ml^{-1} in prehybridisation solution and cover the tissue with 250 µl of the solution. Incubate the slides for 2–4 hours at 42°C or overnight at 37°C in a humid chamber.

viii) Place slides in slide rack. Wash the slides as follows:

2 × SSC	2 ×	5–30 minutes at 37°C
1 × SSC	2 ×	5 minutes at 37°C
0.5 × SSC	2 ×	5 minutes at 37°C

ix) Wash the slides for 1–3 minutes in Buffer I at room temperature. Put the slides flat in a humid chamber and block with 0.5 ml per slide of Buffer II. Incubate for 15 minutes at 37°C.

x) Dilute the anti-DIG AP conjugate at a ratio of1/1000 in Buffer II. Cover tissue with 500 µl of diluted conjugate and incubate in a humid chamber for 30 minutes at 37°C.

xi) Place the slides in a slide rack. Wash in Buffer I twice for 5–10 minutes each time at room temperature. Wash once with Buffer III for 1–2 minutes.

xii) Prepare the development solution by first adding 4.5 µl NBT per 1 ml buffer III. Mix well. Then add 3.5 µl X-phosphate per ml of solution and mix well. Pipette on 500 µl per slide and incubate in a humid chamber in the dark for 2–3 hours at room temperature.

xiii) Stop the reaction by returning the slides to a slide rack and washing in Buffer IV for 15 minutes at room temperature.

xiv) Counterstain the slides by dipping for 5 minutes in 0.5% aqueous Bismarck brown Y.

xv) Dehydrate the slides in the staining centre as follows:

95% alcohol	3 ×	10 dips each
Absolute alcohol	3 ×	10 dips each
Xylene (or suitable substitute)	4 ×	10 dips each

Do not allow the slides to dry out - leave them in the last xylene (or xylene substitute) container until ready for cover-slips.

xvi) Mount with cover-slips and mounting medium (Permount).

xvii) Examine the slides under bright-field for a dark-blue or black precipitate that marks sites where IHHNV DNA is present. Pathodiagnostic intranuclear Cowdry type A inclusions are well marked with the probe. Also often marked are host cell nuclei without obvious inclusions, cytoplasmic inclusions, and accumulation of free virus in the tissue spaces and haemolymph.

4.3.1.2.3.3.1. Reagent formulas for ISH method

i) 10 × Tris/NaCl/EDTA (TNE) buffer

Tris base	60.57 g
NaCl	5.84 g
EDTA	3.72 g
H_2O	900 ml (qs to 1 litre)

pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 × TNE in 900 ml H_2O.

ii) Proteinase K, 100 µg ml^{-1} (prepare just prior to use)

PBS	10 ml 1 × PBS
Proteinase K	1 mg

iii) Prehybridisation buffer (50 ml final volume)

4 × SSC	10 ml 20 × SSC
50% formamide	25 ml 100% formamide
1 × Denhardt's	2.5 ml 20 × Denhardt's
5% dextran sulphate	10 ml 25% dextran sulphate

Warm to 60°C.

Boil 2.5 ml of 10 mg ml^{-1} salmon sperm DNA and add to buffer for final concentration of 0.5 mg ml^{-1} salmon sperm DNA.

iv) 10 × Buffer I

1 M Tris/HCl	121.1 g Tris base
1.5 M NaCl	87.7 g NaCl
H_2O	1000 ml (qs)

pH to 7.5 with HCl. Autoclave. To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml H_2O.

v) Buffer II (blocking buffer)

Blocking reagent	0.25 g Blocking reagent (Roche Diagnostics)
Buffer I	50 ml 1 × Buffer I

vi) Buffer III

100 mM Tris/HCl	1.21 g Tris base
100 mM NaCl	0.58 g NaCl
DD H_2O	100 ml (qs)

pH to 9.5 with HCl. Then add:

50 mM $MgCl_2$	1.02 g $MgCl_2.6H_2O$

vii) Development solution

Mix 90 ml Buffer III with 10 ml 10% PVA. Store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

4.5 µl NBT	75 mg NBT ml^{-1} in 70% dimethylformamide
3.5 µl X-phosphate	5-bromo-4-chloro-3-indoyl phosphate, toluidine salt

viii) Buffer IV

10 mM Tris/HCl	1.21 g Tris base
1 mM EDTA	0.37 g EDTA.2H_2O (disodium salt)
H_2O	1000 ml
pH to 8.0 with HCl.	

ix) 0.5% Bismarck Brown Y

Bismarck Brown Y	2.5 g
H_2O	500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

4.3.1.2.3.4. Polymerase chain reaction for IHHNV

Several single-step PCR methods (Krabsetsve et al., 2004; Nunan et al., 2000; Shike et al., 2000; Tang et al., 2000; Tang et al., 2003), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available from commercial sources.

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; Tang et al., 2007). However, these tests also detect IHHNV-related sequences called 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 (Tang & Lightner, 2006; Tang et al., 2007; Saksmerprome et al., 2011). PCR primers have been developed that can detect the IHHNV sequence but do not react with IHHNV-related sequences 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 segment from IHHNV types 1 and 2 (the infectious forms of IHHNV), but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang & Lightner, 2006; Tang et al., 2007). Primer set MG831F/R reacts only with types 3A and 3B, which are non-infectious and part of 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.

Table 4.1. *Recommended primer sets for one-step PCR detection of IHHNV*

Primer	Product	Sequence	G+C% / Temp.	GenBank & References
389F	389 bp	5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3'	50%/72°C	AF218266
389R		5'-GGC-CAA-GAC-CAA-AAT-ACG-AA-3'	45%/71°C	(Tang et al., 2000)
77012F	356 bp	5'-ATC-GGT-GCA-CTA-CTC-GGA-3'	50%/68°C	AF218266
77353R		5'-TCG-TAC-TGG-CTG-TTC-ATC-3'	55%/63°C	(Nunan et al., 2000)
392F	392 bp	5'-GGG-CGA-ACC-AGA-ATC-ACT-TA-3'	50%/68°C	AF218266
392R		5'-ATC-CGG-AGG-AAT-CTG-ATG-TG-3'	50%/71°C	(Tang et al., 2000; Tang et al., 2007)
309F	309 bp	5'-TCC-AAC-ACT-TAG-TCA-AAA-CCA-A-3'	36%/68°C	AF218266
309R		5'-TGT-CTG-CTA-CGA-TGA-TTA-TCC-A-3'	40%/69°C	(Tang et al., 2007)
MG831F	831 bp	5'-TTG-GGG-ATG-CAG-CAA-TAT-CT-3'	45%/58°C	DQ228358

Table 4.1. *Recommended primer sets for one-step PCR detection of IHHNV*

Primer	Product	Sequence	G+C% / Temp.	GenBank & References
MG831R		5'-GTC-CAT-CCA-CTG-ATC-GGA-CT-3'	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 regions of the genome. In the event that results are ambiguous using the 389F/R 'universal' primer set, it s 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 wth sequencing of PCR amplicons for confirmation.

4.3.1.2.3.5. General PCR method for IHHNV

The PCR method described below for IHHNV generally follows the methods outlined in Tang et al., 2007and 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 4.1), 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 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. Other DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), 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. Use 1–5 µl of extracted DNA as a templateper 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 profileinvolves a 3–5-minute step 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 programme.

viii) After PCR,run 6–10 µl of the sample in a 1.5% agarose gel (containing 0.5 µg ml^{-1} ethidium bromide 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.

4.3.1.2.3.6. Real-time PCR (qPCR) method for IHHNV

qPCR methods have been developed for the detection of IHHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHHNV genome (Dhar et al., 2001; Tang & Lightner, 2001).

The qPCR 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 non-structural protein. The primers and TaqMan probe are designed by the Primer Express software (Life Technologies). 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 fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

ii) Preparation of DNA template: the extraction of DNA template is the same as that described above .

iii) The qPCR 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) Amplification is performed with the StepOnePlus PCRSystem (Life Technologies or equivalent PCR systems). 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.

v) At the end of the reaction, fluorescence intensity is measured. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. A cut-off Ct value is set through the analyses of several independent runs of negative and positive controls. Samples with a Ct value lower than 40 cut-offcycles are considered to be positive.

vi) It is necessary to include a 'no template' control in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture . A positive control should also be included, and it can be a plasmid containing the target sequence, or purified virions, or DNA extracted from IHHNV-infected tissue.

4.3.1.2.3.6.1. Sequencing

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 insertion of non-infectious IHHNV genome in host DNA (Tang & Lightner, 2006).

Through PCR, IHHNV was detected in *P. monodon* from South-East Asia. Some of these IHHNV PCR primers also reacted to IHHNV-related sequences in *P. monodon* populations in Africa, Australia and Thailand (Saksmerprome et al., 2011; Tang & Lightner, 2006). To discriminate the IHHNV-related sequences from the actual virus, PCR assays using primers that detect the IHHNV and do not react with IHHNV-related sequences present in the *P. monodon* stocks from Africa or Australia (Tang et al., 2007), or Thailand (e.g. Saksmerprome et al., 2011) have been developed.

PCR commercial kits are available for detection of IHHNV and can be acceptable provided they have been validated as fit for such purpose. The OIE validation procedure is described in Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases*.

4.3.2. Serological methods

Shrimp are invertebrate animals and do not produce antibodies. Therefore, serological methods for detection of infection with IHHNV are not available.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of infection with IHHNV are listed in Table 5.1.The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the

tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for surveillance, detection and diagnosis*

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	d	d
Bioassay	d	d	d	d	c	c
Direct LM (wet mount)	d	d	d	d	d	d
Histopathology	d	d	c	c	a	b
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	d	c	d	d
***In-situ*hybridisation**	d	c	c	b	a	a
Dot-blot hybridisation	d	d	c	c	a	a
PCR, qPCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; qPCR = real-time polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with infectious hypodermal and haematopoietic necrosis virus

As indicated in Table 5.1., PCR or real-time PCR are the recommended methods for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic IHHNV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with IHHNV-specific DNA probes) is a suitable method (Table 5.1.).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with IHHNV is suspected if at least one of the following criteria is met:

i) Clinical signs indicative of infection with IHHNV

or

ii) Histopathology indicative of infection with IHHNV

or

iii) Positive result by PCR.

7.2. Definition of confirmed case

Infection withIHHNV is considered to be confirmed if two of the following criteria are met:

i) Positive result by *in-situ* hybridisation

ii) Positive result by PCR (always genotype specific)

iii) Sequence analysis to confirm IHHNV nucleic acid sequence.

The two methods must target different areas of the genome.

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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 HEAMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2018.