

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 (IHNV), Family *Parvoviridae*, Genus *Penstyldensovirus*.

The International Committee on Taxonomy of Viruses has assigned IHNV 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

IHNV 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 a 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 IHNV have been identified (Tang *et al.*, 2003): Type 1 is from the Americas and East Asia (principally the Philippines). Type 2 is from South-East Asia. These genotypes are infectious to *Penaeus vannamei* and *P. monodon*. Two sequences homologous to part of the IHNV 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 IHNV-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)

IHNV 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; 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 IHNV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn (*P. monodon*), northern white 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 IHNV 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 (*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*) 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, 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 2.2.1 and 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; 1996a; 2011; Lightner *et al.*, 1992b).

2.2.6. Persistent infection

Some members of *P. stylirostris* and *P. vannamei* populations that survive infection with IHHNV or 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; 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 (Lightner, 1996a; Lightner *et al.*, 1983a; 1983b; 1985), and vertical transmission via infected eggs (Motte *et al.*, 2003) have been demonstrated.

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; 1996a; 1996b; Lightner *et al.*, 1992a; 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; 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, 1996b; Lightner *et al.*, 1992a; Lightner & Redman, 1998a). 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 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).

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 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 10² times 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; 1996b; Weppe 1992; Zarian-Herzberg & Ascencio-Valle, 2001). 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. Restocking with resistant species

There has been some limited application and success with disease-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarin-Herzberg & Ascencio 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 IHNV blocking agents.

2.4.7. Disinfection of eggs and larvae

IHNV is 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 IHNV 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 IHNV (Motte *et al.*, 2003).

2.4.8. General husbandry practices

Some husbandry practices have been successful in preventing the spread of IHNV. 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 (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; 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 infection with IHNV (Jaenike *et al.*, 1992; Lightner, 2005; Pruder *et al.*, 1995).

3. Sampling

3.1. Selection of individual specimens

While infection with IHNV 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 IHNV.

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

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and postlarvae, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 postlarvae depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs and tissues

IHNV infects tissues of ectodermal and mesodermal origin. The principal target tissues for IHNV 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 postlarvae) 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 or tissues that are not suitable

IHNV 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 IHNV (Lightner, 1996a; 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 disease in *Penaeus stylirostris*

Infection with IHHNV is often acute with very high mortalities in juveniles 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 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; Bondad-Reantaso et al., 2001; Brock et al., 1983; Brock & Main, 1994; Lightner, 1983; 1988; 1993; 1996a; 2011; Lightner et al., 1983a, 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 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 infection with IHHNV, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Bondad-Reantaso et al., 2001; Lightner et al., 1983a; 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 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 & Quintino, 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 is 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 4.3.1.2.3 of this chapter) of such sections with a DNA probe specific to IHHNV provides a definitive diagnosis of infection with IHHNV (Lightner, 1996a; 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

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) 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 (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).

In-situ hybridisation (see Section 4.3.1.2.3 below).

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 or artificial media*

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

DNA probes for dot-blot and ISH applications: 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.

Dot-blot hybridisation procedure for IHHNV: the dot-blot hybridisation method given below uses a digoxigenin-11-dUTP (DIG)-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al.* (1993) and 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 a 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 30 minutes 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 Diagnostics¹) 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

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*.

- 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.

In-situ hybridisation (ISH) procedure: the ISH method given below uses a DIG-labelled DNA probe for IHNV and generally follows the methods outlined in Mari *et al.* (1993) and 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 x	5 minutes each
Absolute alcohol	2 x	1 minute each
95% alcohol	2 x	10 dips each
80% alcohol	2 x	10 dips each
50% alcohol	1 x	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 $\mu\text{g ml}^{-1}$ 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.
- iv) Return slides to slide rack. Fix sections in 0.4% cold formaldehyde for 5 minutes at room temperature.
- v) Incubate slides in 2 x 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 3–5 minutes 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 x SSC	2 x	5–30 minutes at 37°C
1 x SSC	2 x	5 minutes at 37°C
0.5 x SSC	2 x	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 alkaline phosphatase conjugate at a ratio of 1/1000 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 x	10 dips each
Absolute alcohol	3 x	10 dips each
Xylene (or suitable substitute)	4 x	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.

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 ₂ O | 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 H₂O.
- 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 ₂ O | 1000 ml (qs) |
- pH to 7.5 with HCl. Autoclave.
To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml H₂O.
- 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 |
| H ₂ O | 100 ml (qs) |
- pH to 9.5 with HCl
Then add:
- | | |
|-------------------------|---|
| 50 mM MgCl ₂ | 1.02 g MgCl ₂ ·6H ₂ O |
|-------------------------|---|
- vii) Development solution
- Mix 90 ml Buffer III with 10 ml of 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 ₂ O (disodium salt) |
| H ₂ O | 1000 ml |
- pH to 8.0 with HCl.
- ix) 0.5% Bismarck Brown Y
- | | |
|------------------|--------|
| Bismarck Brown Y | 2.5 g |
| H ₂ O | 500 ml |
- Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

Polymerase chain reaction for IHNV: several one-step PCR methods (Krabsetsve *et al.*, 2004; Nunan *et al.*, 2000; Shike *et al.*, 2000; Tang *et al.*, 2000; 2007), and a number of commercial PCR kits are available for IHNV detection. Nested methods are also available from commercial sources.

There are multiple geographical variants of IHNV, 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 IHNV (Tang *et al.*, 2000; 2007). However these tests also detect IHNV-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 (Saksmerprom *et al.*, 2011; Tang & Lightner, 2006; Tang *et al.*, 2007). PCR primers have been developed that can detect the IHNV sequence but do not react with IHNV-related sequences present in the *P. monodon* stocks from Africa, Australia (Tang *et al.*, 2007), or Thailand (Saksmerprom *et al.*, 2011). Primer set 309F/R amplifies only a genomic segment of IHNV types 1 and 2 (the infectious forms of IHNV), 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 IHNV 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 IHNV

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 <i>et al.</i> , 2007)
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 <i>et al.</i> , 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 <i>et al.</i> , 2000)
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 <i>et al.</i> , 2007)
MG831F	831 bp	5'-TTG-GGG-ATG-CAG-CAA-TAT-CT-3'	45%/58°C	DQ228358
MG831R		5'-GTC-CAT-CCA-CTG-ATC-GGA-CT-3'	55%/62°C	(Tang <i>et al.</i> , 2007)

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region of the IHNV 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 IHNV: the PCR method described below for IHNV 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 4.1), 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), 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. 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 IHNV: (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 IHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHNV-infected material. Prepare primers at 10 μM in distilled water.
- iv) If PuReTaq™ 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 0.5 $\mu\text{g ml}^{-1}$ 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 IHNV.

Real-time PCR method for IHNV: real-time PCR methods have been developed for the detection of IHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHNV genome (Dhar *et al.*, 2001; Tang & Lightner, 2001). The real-time PCR method using TaqMan chemistry described below for IHNV generally follows the method used in Tang & Lightner (2001).

- i) The PCR primers and TaqMan probe are selected from a region of the IHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The primers and TaqMan probe are designed by the Primer Express software (Life Technologies). The upstream (IHNV1608F) and downstream (IHNV1688R) 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-carboxyfluorescein (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 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) Amplification is performed with the StepOnePlus PCR System (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-off cycles 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 IHNV-infected tissue.

Sequencing: PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHNV, 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 IHNV genome in host DNA (Tang & Lightner, 2006).

Through PCR, IHNV was detected in *P. monodon* from South-East Asia. Some of these IHNV PCR primers also reacted to IHNV-related sequences in *P. monodon* populations in Africa, Australia

and Thailand (Tang & Lightner, 2006; Saksmerprome *et al.*, 2011). To discriminate the IHNV-related sequences from the actual virus, PCR assays using primers that detect the IHNV and do not react with IHNV-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 IHNV 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 IHNV are not available.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of infection with IHNV 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
<i>In-situ</i> hybridisation	d	c	c	b	a	a
Dot-blot hybridisation	d	d	c	c	a	a
PCR, Real-time PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = 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 IHNV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with IHNV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

- i) Clinical signs indicative of infection with IHNV

or

- ii) Histopathology indicative of infection with IHNV

or

- iii) Positive result by PCR

7.2. Definition of confirmed case

Infection with IHNV 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 IHNV 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: <https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

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.