CHAPTER 2.2.2.

# INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS

## 1. Scope

Infectious hypodermal and haematopoietic necrosis (IHHN) disease is caused by infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) (6, 7, 29, 38, 39, 42, 60). A large portion of the IHHNV genome has been found to be inserted in the genome of some genetic lines of *Penaeus monodon.* There is no evidence that this variant of IHHNV is infectious (60, 61).

Synonyms: the International Committee on the Taxonomy has assigned IHHNV (a parvovirus) as a tentative species in the genus *Brevidensovirus*, family *Parvoviridae* with the species name of PstDNV (for *Penaeus stylirostris* densovirus) (21). For the purpose of this *Aquatic Manual*, most references to the viral agent of IHHN will be as IHHNV.

## 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 IHHN 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 4.1 kb, and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (7, 49).

At least three distinct genotypes of IHHNV have been identified (60, 63): Type 1) from the Americas and East Asia (principally the Philippines); Type 2) from South-East Asia; Type 3A) East Africa, India and Australia; and Type 3B) the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (61, 62). The first two genotypes are infectious to the representative penaeids, *P. vannamei* and *P. monodon*, while the latter two genetic variants are not infectious to these species (60–63). IHHNV type 3A and type 3B related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (61, 62). The putative IHHNV sequences in the *P. monodon* genome are not infectious to the representative host species *P. vannamei* and *P. monodon* (37, 61, 62).

#### 2.1.2. Survival outside the host

No data.

#### 2.1.3. Stability of the agent (effective inactivation methods)

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 (29, 33, 37).

#### 2.1.4. Life cycle

Not applicable.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

Most penaeid species can be infected with IHHNV, including the principal cultured species, *P. monodon* (black tiger shrimp/prawn), *P. vannamei* (Pacific white shrimp), and *P. stylirostris* (Pacific blue shrimp).

IHHNV infections are most severe in the Pacific blue shrimp, *P. stylirostris*, where the virus can cause acute epizootics and mass mortality (> 90%). In *P. stylirostris,* the juvenile and subadult life stages are the most severely affected (2, 3, 11, 12, 29, 36, 38).

IHHNV causes the chronic disease runt-deformity syndrome (RDS) in *P. vanname*i in which reduced, irregular growth and cuticular deformities, rather than mortalities, are the principal effects (10, 14, 16, 24, 29, 47). IHHNV infection in *P. monodon* is usually subclinical, but RDS, reduced growth rates and reduced culture performance have been reported in IHHNV-infected stocks (17, 52).

#### 2.2.2. Susceptible stages of the host

IHHNV has been demonstrated 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 IHHNV infection (47).

#### 2.2.3. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1 and 2.2.2.

#### 2.2.4. 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 by IHHNV and are usually negative for IHHNV by ISH (28, 29, 34).

#### 2.2.5. Persistent infection with lifelong carriers

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infections and/or epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (2, 29, 30, 45, 47).

#### 2.2.6. Vectors

No vectors are known in natural infections.

#### 2.2.7. Known or suspected wild aquatic animal carriers

IHHNV is common in wild penaeid shrimp in South-East Asia (*P. monodon*) and in the Americas (*P. vannamei, P. stylirostris* and other Pacific side wild penaeid species) (22, 29, 37, 46, 48).

### 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 (29, 38–40, 58), as has vertical transmission via infected eggs (47).

#### 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 (51); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (46); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (48); and from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (47). 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 (17, 27, 29, 30, 32, 38, 43).

#### 2.3.3. Geographical distribution

IHHNV appears to have a world-wide distribution in both wild and cultured penaeid shrimp (11, 29, 50). In the Western Hemisphere, IHHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although 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 (8, 13, 29, 30, 32, 36). 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 (8, 29). An IHHN-like virus has been reported from Australia (25, 50), and the presence of IHHN 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 (61, 62).

#### 2.3.4. Mortality and morbidity

Depending on the host species and the genotype of the virus, IHHN may take three distinct forms: in unselected *P. stylirostris*, infection by IHHNV results in an acute, usually catastrophic disease with mortalities approaching 100%. In contrast, in *P. vannamei*, some selected lines of *P. stylirostris*, and in *P. monodon*, infection by IHHNV results in a more subtle, chronic disease, RDS, in which high mortalities are unusual, but significant growth suppression and cuticular deformities are common. In the third situation, a large portion of the IHHNV genome has been found to be inserted in the genome of some genetic lines *of P. monodon*. There is no evidence that this variant of IHHNV is infectious (60, 61).

#### 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. However, even at the higher temperature, significant (up to $10^5$ virus copies 50 $ng^{-1}$ of shrimp DNA) IHHNV replication still occurred in shrimp held at 32°C (44).

### 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.

#### 2.4.3. Immunostimulation

No scientifically confirmed reports.

#### 2.4.4. Resistance breeding

Selected stocks of *P. stylirostris* that are resistant to IHHN disease have been developed and these have had some successful application in shrimp farms (19, 30, 64, 67). Some selected lines of *P. stylirostris* that were bred for IHHN disease resistance, were found to be refractory to infection (58). However, such stocks have no increased resistance to diseases such as white spot syndrome virus (WSSV), and, hence, their use has been limited, although with some stocks a genetic basis for IHHN susceptibility in *P. vannamei* has been reported (1).

#### 2.4.5. Restocking with resistant species

There has been some limited application and success with IHHNV-resistant *P. stylirostris* (19, 30, 64, 67). The relative resistance of *P. vannamei* to IHHN disease, despite infection by 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 (31, 37, 56).

#### 2.4.6. Blocking agents

There are reports of shrimp with high viral loads of IHHNV being resistant to infection by WSSV (9). However, 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 (47). Hence, while disinfection of eggs and larvae is good management practice (18) 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 transmission of IHHNV (47).

#### 2.4.8. General husbandry practices

Some husbandry practices have been successfully applied to the prevention of IHHNV infections and disease. Among these has been the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (22, 47), as well as the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (30, 31, 41, 53, 66). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (23, 31, 53). Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status (37). The development of SPF *P. vannamei* that were free not only of IHHNV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its challenging *P. monodon* by 2004–2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (31, 37, 56).

## 3. Sampling

### 3.1. Selection of individual specimens

Suitable specimens for testing for infection by IHHNV are all life stages (eggs, larvae, PL, juveniles and adults) (47). While IHHNV may infect 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 may not be suitable samples for IHHNV detection or certification for IHHN disease freedom.

### 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 PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL 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 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 (29, 35). 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 (29, 35).

### 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 infection by IHHNV (29, 35).

## 4. Diagnostic methods

The prevalence and severity of IHHNV infections may be ‘enhanced’ in a contained population by rearing shrimps in relatively crowded or stressful conditions. The ‘crowding stress’ factors may include high stocking densities and marginal water quality (i.e. low dissolved oxygen, elevated water temperature, or elevated ammonia or nitrite) in the holding tank water. These conditions may encourage expression of low-grade IHHNV infections and the transmission of the agent from carriers to previously uninfected hosts in the population resulting in increased prevalence and severity of infections that can be more easily detected using the available diagnostic and detection methods for IHHNV (29).

## 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 by 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 only show markedly reduced growth rates or 'runting'), molecular tests are recommended when evidence of freedom from IHHN disease is required.

### 4.1.2. Behavioural changes

In acute IHHN 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. IHHN disease in *Penaeus stylirostris*

IHHNV often causes an acute disease with very high mortalities in juveniles of this species. Vertically infected larvae and early PL 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 (2, 3, 8, 12, 13, 26–30, 38, 39). Gross signs are not IHHN specific, but juvenile *P. stylirostris* with acute IHHN show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species with acute IHHN 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 (8, 26–30, 38, 39).

#### 4.2.1.2. IHHN disease in *Penaeus vannamei*

RDS, a chronic form of IHHN disease, occurs in *P. vannamei* as a result of IHHNV infection. 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 PL 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 IHHNV-free (and thus RDS-free) populations of juvenile *P. vannamei* and *P. stylirostris* usually show CVs of 10–30% (10–15, 29, 52, 53).

### 4.2.2. Clinical chemistry

Not applicable.

### 4.2.3. Microscopic pathology

Acute IHHNV infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained histological methods (see Section 4.2.6). Chronic IHHNV infections 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 IHHNV infection. These characteristic IHHN 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) (4, 29), 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 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 specific DNA probe to IHHNV provides a definitive diagnosis of IHHNV infection (4, 11, 27, 29, 35).

### 4.2.4. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

### 4.2.5. Smears

Not applicable.

### 4.2.6. 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 4.3.1.2.3.1 for dot-blot hybridisation methods.

*4.3.1.1.3. Fixed sections*

See section 4.2.3.

#### 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 (29, 35).

*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 products using these methods are readily available.

*DNA probes for dot-blot and ISH applications:* gene probe and PCR methods provide greater diagnostic sensitivity than do more traditional techniques for IHHN diagnosis 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 (5), and used as the sample for a direct dot-blot test.

*4.3.1.2.3.1 Dot-blot hybridisation detection of IHHNV with DNA probes*

The probe is labelled with a non-radioactive label, digoxigenin-11-dUTP (DIG-11-dUTP). The system using DIG to label nucleic acid probes was developed by Boehringer Mannheim Biochemicals (this company is now owned by Roche Diagnostic Corporation), which is described in the Roche *DIG Nonradioactive Labeling and Detection Product Selection Guide* and *DIG*

*Application Manual for Filter Hybridization™ System User's Guide for Membrane Hybridization* and from Boehringer Mannheim's *Nonradioactive In Situ Hybridization Application Manual*[1] (54, 55). The protocols given below use a DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (29, 42). An alternative method for producing DIG-labelled probes uses specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit™.

*Dot-blot hybridisation procedure:* the dot-blot hybridisation method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al.* (42) and Lightner (29). Formulas for the required reagents are given after the protocols.

i) Prepare a positively charged nylon membrane (Roche Diagnostics Cat. No. 1-209-299 or equivalent): cut pieces 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, dilute samples to be assayed in TE (Tris/EDTA [ethylene diamine tetra-acetic acid]) buffer plus 50 µg $ml^{-1}$ salmon sperm DNA, using 1 µl sample in 9 µl buffer in 1.5 ml microcentrifuge tubes. Samples for dot-blots can be haemolymph, tissues homogenised in TN (Tris/NaCl: 0.4 M NaCl and 20 mM Tris-HCl, pH 7.4) buffer, or extracted DNA in 10 mM Tris/HCl.

iii) Boil samples for 10 minutes and quench on ice for 5 minutes. Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein. 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) Adjust a water bath to 68°C and prepare the prehybridisation solution. For a 10 × 15 cm membrane, prepare 8 ml per membrane. Set a stirring hot plate to 'low' and stir while warming the solution for 30 minutes until the blocking agent has dissolved and the solution is cloudy. Also, prepare some heat-seal bags that are slightly larger in size than the membrane: five to six bags will be needed per membrane.

vi) 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.

vii) Boil the DIG-labelled probe for 10 minutes, quench on ice and then microfuge in the cold to bring all the liquid down in the microcentrifuge tube. Keep on ice. Remove the prehybridisation solution from the bags. Add 2 ml of fresh prehybridisation solution to each bag and then add the correct, 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.

viii) Wash membranes well with:

| | | |
|---|---|---|
| 2 × standard saline citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) | 2 × | 5 minutes at room temperature |
| 0.1 × SSC/0.1% SDS (use 4 ml/filter and seal in bags) | 3 × | 15 minutes at 68°C |
| Buffer I | 1 × | 5 minutes at room temperature |
| Buffer II | 1 × | 30 minutes at room temperature |
| Buffer I | 1 × | 5 minutes at room temperature |

(Buffers are prepared ahead of time).

ix) React the membrane in bags with anti-DIG AP conjugate (Roche Diagnostics 1-093-274) diluted 1/5000 in Buffer I. Use 3 ml per membrane, incubate for 30–45 minutes at room temperature on a shaker platform.

x) 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*.

xi) Develop the membranes in bags using 3 ml per membrane of 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.

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

xiii) Store dry membranes in heat-seal bags.

*4.3.1.2.3.2. In-situ hybridisation detection of IHHNV with DNA probes*

The ISH method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al.* (42) and Lightner (29). Formulas for the required reagents are given after the protocols.

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 phosphate buffered saline (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.

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 10 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. Drain fluid onto blotting paper. During this incubation, pre-warm the wash buffers at 37°C.

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 5 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. Drain the fluid on to blotting paper.

x) Dilute the anti-DIG AP conjugate (Roche Applied Science cat. 10686322) 1/1000 in Buffer II (1 µl anti-DIG AP per 1 ml buffer). 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 5–10 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.

NOTE: Always run a known positive and negative control.

Reagent formulas for ISH method:

i) 10 × phosphate buffered saline

| | |
|---|---|
| NaCl | 160 g |
| $KH_2PO_4$ | 4 g |
| $Na_2HPO_4$ | 23 g |
| KCl | 4 g |
| DD $H_2O$ | 1950 ml (qs to 2 litres) |

pH to 8.2 with NaOH; autoclave to sterilise; store at room temperature. To make 1 × PBS, dilute 100 ml 10 × PBS in 900 ml DD $H_2O$; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

ii) 10 × Tris/NaCl/EDTA (TNE) buffer

| | |
|---|---|
| Tris base | 60.57 g |
| NaCl | 5.84 g |
| EDTA | 3.72 g |
| DD $H_2O$ | 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 DD $H_2O$; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

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

| | |
|---|---|
| PBS | 10 ml 1 × PBS |
| Proteinase K | 1 mg |

iv) 0.4% formaldehyde

| | |
|---|---|
| 37% formaldehyde | 5.4 ml |
| DD $H_2O$ | 500 ml |

Store at 4°C; can be reused up to four times before discarding.

v) 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; store at 4°C.

vi) 20 × SSC buffer

| | |
|---|---|
| 3M NaCl | 175.32 g NaCl |
| 0.3 M $Na_3C_6H_5O_7.2H_2O$ | 88.23 g Na citrate.$2H_2O$ |
| DD $H_2O$ | 1000 ml (qs) |

pH to 7.0; autoclave; store at 4°C.

To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD $H_2O$; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD $H_2O$; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD $H_2O$. Filter solutions through a 0.45 µm filter; store at 4°C.

vii) 20 × Denhardt's solution

| | |
|---|---|
| BSA (Fraction V) | 0.4 g bovine serum albumin |
| Ficoll 400 | 0.4 g Ficoll |
| PVP 360 | 0.4 g polyvinylpyrollidine |
| DD $H_2O$ | 100 ml |

Filter solutions through a 0.45 µm filter; store at 4°C. Aliquot 2.5 ml into small tubes and store frozen.

viii) 25% dextran sulphate

| | |
|---|---|
| Dextran sulphate | 25 g |
| DD $H_2O$ | 100 ml |

Mix to dissolve; store frozen in 10 ml aliquots.

ix) Salmon sperm DNA (10 mg $ml^{-1}$)

| | |
|---|---|
| Salmon sperm DNA | 0.25 g |
| DD $H_2O$ | 25 ml |

To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18-gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.

x) 10 × Buffer I

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

pH to 7.5 with HCl. Autoclave; store at 4°C.

To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD $H_2O$. Filter through a 0.45 µm filter; store at 4°C.

xi) Buffer II (blocking buffer)

| | |
|---|---|
| Blocking reagent | 0.25 g Blocking reagent (Roche Diagnostics 1-096-176) |
| Buffer I | 50 ml 1 × Buffer I |

Store at 4°C for up to 2 weeks.

xii) 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 $MgCl_2.6H_2O$ |

Filter through a 0.45 µm filter; store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)

| | |
|---|---|
| Polyvinyl alcohol | 10 g |
| DD $H_2O$ | 100 ml |

To prepare, slowly add PVA to water while stirring on low heat. (It takes 2–3 hours for PVA to go into solution.) Dispense 10 ml per tube and store frozen at –20°C.

xiv) 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 (Roche Diagnostics 1-383-213) |
| 3.5 µl X-phosphate | 5-bromo-4-chloro-3-indoyl phosphate, toluidine salt (50 mg $ml^{-1}$ in dimethylformamide) (Roche Diagnostics 1-383-221) |

xv) Buffer IV

| | |
|---|---|
| 10 mM Tris/HCl | 1.21 g Tris base |
| 1 mM EDTA | 0.37 g $EDTA.2H_2O$ (disodium salt) |

DD $H_2O$ 1000 ml

pH to 8.0 with HCl. Filter through a 0.45 µm filter; store at 4°C.

xvi) 0.5% Bismarck Brown Y

Bismarck Brown Y 2.5 g
DD $H_2O$ 500 ml

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

*4.3.1.2.3.3. Polymerase chain reaction*

Several single-step PCR methods (25, 49, 57–59, 63), and a number of commercial PCR kits are available for IHHNV detection. A nested method is also available, but only as a kit from a commercial source (47).

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods for IHHNV. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (25, 60, 63, 65), including types 3A and 3B, which are inserted into the genome of certain geographic stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (25, 61, 62). 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 (61, 62). Primer set MG831F/R reacts only with types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (62). Hence, confirmation of unexpected positive and/or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. PCR using primers from another region of the genome, real-time PCR, 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 | (58) |
| 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 | (49) |
| 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 | (58, 62) |
| 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 | (62) |
| 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 | (62) |

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region (ORF 1) of the IHHNV genome. Primers 77353/77012 are from a region in between the nonstructural and the structural (coat protein) protein-coding regions 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 77012/77353 or the 392F/R primer sets for confirmation.

*4.3.1.2.3.3.1. General PCR method for IHHNV*

The PCR method described below for IHHNV generally follows the methods outlined in Nunan *et al.* (49). Cumulative experience with the technique has led to modifications with respect to template (DNA extraction of clinical specimens), choice of primers (Table 4.1), and volume of reaction.

i) Use as a template, the DNA extracted from ground tissue homogenate (TN buffer, 0.4 M NaCl, 20 mM Tris, pH 7.4) or haemolymph (collected with a small amount of 10% sodium citrate) or from tissue or haemolymph that was fixed in 95% ethanol and then dried. A control consisting of tissue or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods, but excellent results have been obtained using kits from Roche Diagnostics (Cat. No. 1-796-828) or Qiagen (Cat. No. 51304), or reagents from Gibco Life Sciences (DNazol Cat. No. 10503-027). 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 per 50 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 100 ng $µl^{-1}$ in distilled water. Keep frozen at –70°C.

iv) Use a 'hot start' method for the polymerase: if Applied Biosystem's AmpliTaq Gold is used, this involves a 5-minute step at 95°C to denature DNA prior to the primers binding and activation of the enzyme. This programme is then linked to the cycling programme (35 cycles) and an extension programme. The programme is set as follows:

| | | | |
|---|---|---|---|
| Hot start | Programme 1 | 5 minutes 95°C | |
| Linked to | Programme 2 | 30 seconds 95°C | |
| | | 30 seconds 55°C | 35 cycles |
| | | 1 minute 72°C | |
| Linked to | Programme 3 | 7 minutes 72°C | |
| Linked to | Programme 4 | 4°C until off | |

v) Prepare a 'master mix' consisting of water, 10 × PCR buffer, the four dNTPs, the two primers, $MgCl_2$, AmpliTaq Gold and water (assume use of 1 µl of template; if using more, adjust water accordingly). Add mix to each tube. Use thin-walled tubes designed for PCR. Always run a positive and a negative control.

'Master Mix':

| | |
|---|---|
| DD $H_2O$ | 32.5 µl × number of samples |
| 10 × PCR buffer | 5 µl × number of samples |
| 10 mM dTTP | 1 µl × number of samples |
| 10 mM dATP | 1 µl × number of samples |
| 10 mM dCTP | 1 µl × number of samples |
| 10 mM dGTP | 1 µl × number of samples |
| 25 mM $MgCl_2$ | 4 µl × number of samples |
| Forward primer (100 ng $µl^{-1}$) | 1.5 µl × number of samples |
| Reverse primer (100 ng $µl^{-1}$) | 1.5 µl × number of samples |
| AmpliTaq Gold | 0.5 µl × number of samples |

Vortex this solution to mix all reagents well; keep on ice.

NOTE: The volume of the PCR reaction may be modified. Previously, the PCR reactions for IHHNV were run in 100 µl volumes, but it is not necessary to use that amount of reagents, therefore 50 µl volumes are described in this procedure. Likewise, the PCR reactions can also be run in volumes as small as 25 µl. To do this, increase or decrease the volume of the reagents accordingly.

vi) For a 50 µl reaction mix, add 49 µl Master Mix to each tube and then add 1 µl of the sample to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. If the thermal cycler does not have a heated lid to prevent condensation, then carefully overlay the top of each sample with 25–50 µl mineral oil and re-cap the tubes. Insert tubes into the thermal cycler and start programme 1 ('hot start'), which is linked to cycling, extension and soak cycles.

viii) If mineral oil was used, recover samples from under the mineral oil using a pipette set at 50 µl and transfer to a fresh tube. Using the long-tipped pipette tips (designed for loading gels) results in less oil being carried over with the sample.

ix) Run 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 Southern transfer of the gel or a dot-blot can be run for more sensitive detection. The DNA can also be precipitated (0.3 M sodium acetate and 2.5 volumes 100% ethanol, –70°C, for 1–3 hours, centrifuge for 20 minutes) and resuspended in 1/10th volume (i.e. 4 µl) TE (10 mM Tris, 1 mM EDTA, pH 7.5) or water and either re-run in the gel or tested in a dot-blot.

*4.3.1.2.3.3.2 Real-time PCR method for IHHNV*

Real-time PCR (RT-PCR) 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 (20, 59).

The RT-PCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (59).

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 (Applied Biosystems). 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'), which corresponds to the region from nucleotide 1632 to 1644, 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 (Applied Biosystems, part no. 450025).

ii) Preparation of DNA template: the extraction and purification of DNA template is the same as that described in the section of traditional PCR.

iii) The PCR reaction mixture contains: TaqMan Universal PCR Master Mix (Applied Biosystems, part no. 4324018), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 or equivalent can also be used). The cycling profile is: activation of AmpliTaq Gold for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

v) At the end of the reaction, real-time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. 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. Samples will be defined as negative if the Ct (threshold cycle) values exceed 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive. To confirm the real-time PCR results, an aliquot of PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. An 81-bp DNA fragment can be visualised in the samples that are positive for IHHNV.

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 or in the heat block of the thermal cycler. A positive control should also be included, and it can be a plasmid containing the target sequence, or purified virions, or DNA from IHHNV-infected tissue.

*4.3.1.2.3.4. Sequencing*

PCR products may be cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified product from the infectious form of the virus and demonstrate the presence of the insertion of non-infectious IHHNV genome in host DNA (60, 61).

*4.3.1.2.4. Agent purification*

While methods have been reported for isolation and purification of IHHNV, these methods are not applicable to the routine diagnosis of the disease.

### 4.3.2. Serological methods

Shrimp are invertebrate animals and do not produce antibodies. Therefore, serological methods for IHHN are not available.

## 5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of 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 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 targeted surveillance and diagnosis*

| Method | Targeted 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** | d | d | d | d | d | d |
| **Histopathology** | d | d | c | c | a | a |
| **Transmission EM** | d | d | d | d | c | c |
| **Antibody-based assays** | d | d | d | c | d | d |
| **DNA probes – *in situ*** | d | d | b | b | a | a |
| **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 infectious hypodermal and haematopoietic necrosis

As indicated in Table 5.1, PCR (Section 4.3.1.2.3.3) is the recommended method 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 our 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

Poor hatching success of eggs, and poor survival and culture performance of the larval and PL stages (47) when broodstock are used from wild or farmed stocks where IHHNV is enzootic.

In farmed stocks of *P. stylirostris*, juveniles, subadults and adults may show persistently high mortality rates. In *P. vannamei, P. stylirostris,* and possibly *P. monodon,* IHHNV-infected stocks may show poor and highly disparate growth, poor overall culture performance, and cuticular deformities, including especially bent rostrums and deformed sixth abdominal segments.

Demonstration of eosinophilic to pale basophilic intranuclear inclusion bodies in the typical target tissues for IHHNV. As IHHNV intranuclear inclusion bodies are nearly identical in appearance to those occurring in the early stages of WSSV infections, their presence in tissue sections should be considered as a presumptive

diagnosis of IHHNV until confirmed with a second test method, such as dot-blot or ISH with IHHNV-specific DNA probes or positive PCR test results for IHHNV.

### 7.2. Definition of confirmed case

Any combination of at least two of the following four methods (with positive results):

- Positive dot-blot hybridisation test results for IHHNV.
- ISH positive histological signal to IHHNV-type lesions.
- PCR positive results for IHHNV.
- Sequencing of PCR specific products may be required when the purpose is to determine the genotype of IHHNV.

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**NB:** There is an OIE Reference Laboratory for Infectious hypodermal and haematopoietic necrosis (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int)