Annex 23. Item 10.1.2. – Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)

CHAPTER 2.2.3.

infection with Hepatobacter penaei (Necrotising hepatopancreatitis)

1. Scope

~~Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR)-deleted ISAV, or the non-pathogenic HPR0 (non-deleted HPR) ISAV of the Genus~~ *~~Isavirus~~* ~~and Family~~ *~~Orthomyxoviridae~~*~~.~~

Infection with *~~Candidatus~~**Hepatobacter penaei* means infection with the pathogenic agent *Candidatus* H. penaei*,* an obligate intracellular bacterium of the Family Holosporaceae, Order Rickettsiales ~~α-~~*~~Proteobacteria~~*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

*Hepatobacter penaei* is a pleomorphic, Gram-negative, intracytoplasmic bacterium (Nunan *et al.,* 2013)*.* It is a member of the α-*Proteobacteria* (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). More recently it has been suggested that it belongs to the Family Holosporaceae ~~family~~ within the Order Rickettsiales (Leyva *et al.,* 2018). The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). Genetic analysis of *H. penaei* associated with North and South American outbreaks suggests that the isolates are either identical or very closely related subspecies (Loy *et al.,* 1996). ~~Recently~~ Analysis based on the 16S rRNA confirms the high similarity among different *H. penaei* isolates in the Americas (99–100%) (Aranguren & Dhar, 2018).

2.1.2. Survival and stability in processed or stored samples

*Hepatobacter penaei*-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. *Hepatobacter penaei* frozen at –20°C to –70°C and –80°C have been shown to retain infectivity in experimental transmission trials with *Penaeus vannamei* (Crabtree *et al.*, 2006; Frelier *et al.,* 1992). Flash freezing *H. penaei* at –70°C to –80°C does not significantly affect the infectivity (Aranguren *et al*., 2010; Crabtree *et al.,* 2006).

2.1.3. Survival and stability outside the host

No information available*.*

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *H. penaei* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) ~~include~~ are: whiteleg 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 *H. penaei* according to Chapter 1.5. of the *Aquatic Code* ~~include~~ are: aloha prawn (*P. marginatus*), banana prawn (*P. merguiensis*), blue shrimp (*P. stylirostris*), giant tiger prawn (*P. monodon*), northern brown shrimp (*P. aztecus*), northern pink shrimp (*P. duorarum*) and northern white shrimp (*P. setiferus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: American lobster (*Homarus americanus*) (Avila-Villa *et al.,* 2012; Bekavac *et al.,* 2022).

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

Infection with *H. penaei* has been demonstrated in postlarvae (PL), juveniles, adults and broodstock of *P. vannamei* (Aranguren *et al.*, 2006).

2.2.4. Distribution of the pathogen in the host

The target tissue is the hepatopancreas: infection with *H. penaei* has been reported in all hepatopancreatic cell types (Lightner 2012). *Hepatobacter penaei* is also present in the faeces (Brinez *et al.,* 2003).

2.2.5. Aquatic animal reservoirs of infection

Some members of *P. vannamei* populations that survive infection with *H. penaei* may carry the intracellular bacteria for life and transmit it to other populations by horizontal transmission (Aranguren *et al.,* 2006; Lightner, 2005; Morales-Covarrubias, 2010; Vincent & Lotz, 2005).

2.2.6. Vectors

No vectors are known in natural infections.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Infection with *H. penaei* often causes ~~an~~ acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adults and broodstock, the incubation period and severity of the disease are somewhat size or age dependent, with juveniles always being the most severely affected. Infection with *H. penaei* results in the mortalities approaching 100% in *P. vannamei*, 5.6–15% in *P. duorarum*, and 5–17% in *P. aztecus* (Aguirre-Guzman *et al.,* 2010).

The prevalence was reported as 0.77% in cultured *P. vannamei* and 0.43% in cultured *P. stylirostris* in Peru (Lightner & Redman, 1994), 5–86.2% in Mexico (Ibarra-Gamez *et al.,* 2007), and 0.6–1.3% in *P. vannamei* in Belize, Brazil, Guatemala, Honduras, Mexico, Nicaragua and Venezuela (Morales-Covarrubias *et al.*, 2011).

~~NHP-affected broodstock ponds in Colombia reported mortalities of up to 85%, while non NHP-affected broodstock ponds in the same farm experienced mortalities of 40–50% (Aranguren~~ *~~et al.,~~* ~~2006).~~

2.3.2. Clinical signs, including behavioural changes

A wide range of gross signs can be used to indicate the possible presence of infection with *H.* *penaei*. These include lethargy, reduced food intake, atrophied hepatopancreas, anorexia and empty guts, noticeably reduced growth and poor length weight ratios (‘thin tails’)~~; soft shells and flaccid bodies; black or darkened gills; heavy surface fouling by epicomensals organisms; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic. (Lightner, 1996; Loy~~ *~~et al.,~~* ~~1996)~~.

2.3.3 Gross pathology

~~Infection with~~ *~~H. penaei~~* ~~often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adult and broodstock, the incubation period and severity of the disease are somewhat size or age dependent.~~ Gross signs are not specific, but shrimp with acute infection with *H. penaei* show atrophied hepatopancreas, empty guts, soft shells and flaccid bodies; black or darkened gills; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic. (Lightner, 1996; Loy *et al.,* 1996) ~~a marked reduction in food consumption, followed by changes in behaviour and appearance including pale discoloration of the hepatopancreas with further size reduction~~.

2.3.4. Modes of transmission and life cycle

Horizontal transmission of *H. penaei* can be through cannibalism or by contaminated water (Aranguren *et al.,* 2006; 2010; Frelier *et al.,* 1993; Gracia-Valenzuela *et al.*, 2011; Vincent *et al.*, 2004). *Hepatobacter penaei* in faeces shed into pond water has also been suggested as a source of contamination (Aranguren *et al.,* 2006; Briñez *et al.,* 2003; Morales-Covarrubias *et al.*, 2006). *Hepatobacter penaei*-positive broodstock females produce PL that were also *H. penaei*-positive, which suggests that a transmission from broodstock to progeny can occur~~s~~ (Aranguren *et al.,* 2006).

2.3.5. Environmental factors

The occurrence of infection with *H. penaei* in farms may increase during long periods of high temperatures (>29°C) and high salinity (20–38 ppt) (Morales-Covarrubias, 2010). In the months when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

2.3.6. Geographical distribution

*Hepatobacter penaei* appears to have a Western Hemisphere distribution in both wild and cultured penaeid shrimp (Aguirre-Guzman *et al.,* 2010; Del Rio-Rodriguez *et al.,* 2006). In the Western Hemisphere, *H. penaei* is commonly found in cultured penaeid shrimp in the Americas (Aranguren *et al.,* 2010; Frelier *et al.,* 1992; Ibarra-Gamez *et al.,* 2007; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2011). *Hepatobacter penaei*, was introduced into Africa from North America via movement of infected *P.vannamei* broodstock, however NHP was later eradicated by fallowing (Lightner *et al.,* 2012).

See WOAH WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

Early detection (initial phase) of clinical infection with *H. penaei* is important for successful treatment because of the potential for cannibalism to amplify and transmit the disease. Shrimp starvation and cannibalism of infected shrimp, and positive conditions for *H. penaei* ~~enaei~~ multiplication, are important factors for the spread of *H. penaei* in *P. vannamei*. Preventive measures include raking, tilling, and removing sediments from the bottom of the ponds, prolonged drying (through exposure to sunlight) of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, extensive liming of ponds and the use of ponds liners. The use of specific pathogen-free (SPF) broodstock is an effective preventive measure. NHP, particularly in the initial phase, can be treated by using antibiotics in medicated feeds. *~~Hepatobacter penaei~~* ~~is sensitive to oxytetracycline (Lightner & Redman, 1994).~~

2.4.1. Vaccination

No scientifically confirmed reports.

2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Breeding resistant strains

One population from Latin America that has been selected for several generations for resistance to Taura syndrome virus in the presence of infection with *H. penaei*, seems to be more resistant to NHP disease than the Kona line under experimental conditions (Aranguren *et al.,* 2010).

2.4.5. Inactivation methods

The use of hydrated lime (Ca(OH)2) to treat the bottom of ponds during pond preparation before stocking can help reduce infection with *H. penaei*.

2.4.6. Disinfection of eggs and larvae

Disinfection of eggs and larvae is a good management practice and is recommended for its potential to reduce *H. penaei* contamination of spawned eggs and larvae (and contamination by other disease agents).

2.4.7. General husbandry

The prevalence and severity of infection with *H. penaei* may be increased by rearing shrimp in relatively crowded or stressful conditions. Some husbandry practices have been successfully applied to the prevention of infection with *H. penaei*. Among these has been the application of PCR to pre-screening of wild or pond-reared broodstock.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Suitable specimens for testing for infection with *H. penaei* are the following life stages: PL, juveniles and adults.

3.2. Selection of organs or tissues

*Hepatobacter penaei* infects most enteric tissue. The principal target tissue for *H. penaei* is the hepatopancreas and this organ should be selected preferentially (Lightner, 2012).

3.3. Samples or tissues not suitable for pathogen detection

*Hepatobacter penaei* does not replicate in the midgut, caeca, connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells. Samples of pleopods or haemolymph are not recommended for *H. penaei* detectionby PCR.

3.4. Non-lethal sampling

*Hepatobacter penaei* can be detected in faeces samples collected from clinically affected populations of *Penaeus vannamei*~~may be collected and used for testing (usually by PCR),~~ when non-lethal testing of ~~valuable~~ broodstock is necessary (Brinez *et al.,* 2003~~; Frelier~~ *~~et al.,~~* ~~1993; Lightner, 1996~~). However, the use of faeces samples to detect *H. penaei* ~~NHP~~ in apparently healthy shrimp has not been evaluated. ~~Faeces samples have not been validated to the same level as hepatopancreas samples.~~

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 *General information* (*diseases of crustaceans*)

3.5.1. Samples for pathogen isolation

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

3.5.2. Preservation of samples for molecular detection

Tissue samples of hepatopancreas or faeces for PCR testing should be preserved in 70–95% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen, but repeated freezing and thawing should be avoided.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 5.3 of Chapter 2.2.0.

3.5.4. Samples for other tests

No scientifically confirmed reports.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger shrimp should be processed and tested individually. Small life stages such as PL or specimens up to 0.5 g can be pooled to obtain the minimum amount of material for *H. penaei* molecular detection.

4. Diagnostic methods

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

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

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

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

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

Shaded boxes = Not appropriate for this purpose.

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

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

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

| Method | 1. Surveillance of apparently healthy animals
 | 1. Presumptive diagnosis of clinically affected animals
 | 1. Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis
 |
| --- | --- | --- | --- |
| Early life stages2 | Juveniles2 | Adults | LV | Early life stages2 | Juveniles2 | Adults | LV | Early life stages2 | Juveniles2 | Adults | LV |
| Wet mounts |  |  |  |  |  | + | + | NA |  |  |  |  |
| Histopathology |  |  |  |  |  | ++ | ++ | NA |  |  |  |  |
| Cell culture |  |  |  |  |  |  |  |  |  |  |  |  |
| Real-time PCR | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 | ++ | ++ | ++ | 1 |
| Conventional PCR | ++ | ++ ~~+~~ | ++ ~~+~~ | 1 | ++ | +++ | +++ | 1 | ~~++~~ | ~~+++~~ | ~~+++~~ | ~~1~~ |
| Conventional PCR followed by amplicon sequencing |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 1 |
| *In-situ* hybridisation |  |  |  |  | + | ++ | ++ | NA | + | ++ | ++ | NA |
| Bioassay |  |  |  |  | + | + | + | NA | ~~+~~ | ~~+~~ | ~~+~~ | ~~NA~~ |
| LAMP |  |  |  |  |  |  |  |  |  |  |  |  |
| Ab-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| Ag-ELISA |  |  |  |  |  |  |  |  |  |  |  |  |
| Other antigen detection methods~~3~~ |  |  |  |  |  |  |  |  |  |  |  |  |
| Other methods~~3~~ |  |  |  |  |  |  |  |  |  |  |  |  |

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

4.1. Wet mounts

Wet mount squash examination of hepatopancreas tissue is generally conducted to detect presumptive infection with *H. penaei*. The hepatopancreas may be atrophied and have any of the following characteristics: soft and watery; fluid filled centre; pale colour with or without black stripes (melanised tubules). Hepatopancreatic tubules show deformity at the distal portion; multifocal melanisation initially at the distal portion of the tubule and, later on, in the medial and proximal portion; reduced or absence of lipid droplets (Lightner, 2012).

4.2. Histopathology and cytopathology

Histological methods can be useful for indicating acute and chronic infection with *H. penaei*.

Initial infection with *H. penaei* is difficult to diagnose using routine H&E histological methods. Therefore, molecular methods are recommended for screening populations for infection with ~~initial~~ *H. penaei* detection (e.g. by PCR or application of *H. penaei*-specific DNA probes or *in-situ* hybridisation ~~[ISH]~~ of histological sections).

Acute infection with *H. penaei* is characterised by atrophied hepatopancreas with moderate atrophy of the tubule epithelia, presence of bacterial cells and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations). Hypertrophic cells, individual epithelial cells, appeared to be separated from adjacent cells, undergo necrosis and desquamation into the tubular lumen. The tubular epithelial cell lipid content is variable.

The transitional phase of infection with *H. penaei* is characterised by haemocytic inflammation of the intertubular spaces in response to necrosis, cytolysis, and sloughing of hepatopancreas tubule epithelial cells. The hepatopancreas tubule epithelium is markedly atrophied, resulting in the formation of large oedematous (fluid filled or ‘watery’) areas in the hepatopancreas. Tubule epithelial cells within multifocal encapsulation are typically atrophied and reduced from simple columnar to cuboidal morphology. They contain little or no stored lipid vacuoles, markedly reduced or no secretory vacuoles and masses of bacteria. At this phase haemocyte nodules are observed in the presence of masses of bacteria in the centre of the nodule.

In the chronic phase of infection with *H. penaei*, tubular lesions, multifocal encapsulation and oedematous areas decline in abundance and severity and are replaced by infiltration and accumulation of haemocytes at the sites of necrosis. There are areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells with masses of bacteria in the cytoplasm and low numbers of haemocyte nodules.

4.3. Cell culture for isolation

*Hepatobacter penaei* has not been grown *~~in vitro~~* in cell culture. No crustacean cell lines exist (Vincent & Lotz, 2007).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

PCR methods including PCR and real-time PCR have been developed that target several *H. penaei* genes including 16S rRNA and flagella hook ~~Flg~~ E genes (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Loy *et al*., 1996).

*Extraction of nucleic acids*

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

*~~DNA extraction~~*

~~A general DNA extraction method may be used to extract DNA from the hepatopancreatic tissue of putatively infected shrimp. The amount of template DNA in a 10–25 µl PCR reaction volume should be in the range of 10–100 ng of total DNA~~

4.4.1. Real-time PCR

Real-time PCR methods for detection of *H. penaei* have the advantages of speed, specificity and sensitivity. The sensitivity of real-time PCR is ~100 copies of the target sequence from the *H. penaei* genome (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Vincent & Lotz, 2005).

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/target gene | Primer/probe (5’–3’) | Concentration | Cycling parameters |
| Method 1: Aranguren *et al.,* 2010; GenBank U65509 |
| *H. penaei*/16S rRNAgene | Fwd NHP1300F: CGT-TCA-CGG-GCC-TTG-TAC-ACRev NHP1366R: GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-AProbe: CCG-CCC-GTC-AAG-CCA-TGG-AA | 300 nM 100 nM | 40 cycles:95°C/15 sec and 60°C/1 min |
| Method 2: Aranguren & Dhar 2018; GenBank JQAJ01000001.1 |
| *H. penaei*/ Flagella hook gene ~~protein~~ | Fwd NHP FlgE3qF: AAC-ACC-CTG-TCT-CCC-CAA-TTCRev FlgE3qR: CCA-GCC-TTG-GAC-AAA-CAC-CTTProbe: CGC-CCC-AAA-GCA-TGC-CGC | 500 nM 100 nM | 40 cycles:95°C/1 sec and 60°C/20 sec |

~~The real-time PCR method using TaqMan chemistry described below for~~ *~~H. penaei~~* ~~based on the 16S rRNA gene generally follows the method used in Aranguren~~ *~~et al.~~* ~~(2010).~~

~~i) The PCR primers and TaqMan probe are selected from the 16S, rRNA gene of~~ *~~H. penaei~~* ~~(GenBank U65509) (Loy & Frelier~~*~~,~~* ~~1996). The primers and TaqMan probe were designed by the Primer Express software version 2.0 (Applied Biosystems). The upstream (NHP1300F) and downstream (NHP1366R) primer sequences are: 5’-CGT-TCA-CGG-GCC-TTG-TACAC-3’ and 5’-GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A-3’, respectively. The TaqMan probe NHP: 5’-CCG-CCC-GTC-AAG-CCA-TGG-AA-3’, which corresponds to the region from nucleotides 1321–1340, is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5’ and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3’ end.~~

~~ii)~~ *~~The real-time PCR reaction mixture contains:~~* ~~TaqMan One-step real-time~~~~PCR SuperMix (Quanta, Biosciences), 0.3 μM of each primer, 0.1 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.~~

~~iii) Amplification is performed with the master cycler Realplex 2.0 (Eppendorf). The cycling consists of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. After each cycle, the levels of fluorescence are measured.~~

~~iv) 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 and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from~~ *~~H. penaei~~*~~-infected hepatopancreas.~~

~~Protocol 2~~

~~Another real-time PCR method using TaqMan chemistry described below for~~ *~~H. penaei~~* ~~is based on the flagella gene (flagella hook protein, flgE) (Aranguren~~ *~~&~~* ~~Dhar,~~~~2018).~~

~~i) The PCR primers and TaqMan probe were selected from the Flg E gene of~~ *~~H. penaei~~* ~~(GenBank JQAJ01000001.1) (Aranguren & Dhar, 2018). The primers and TaqMan probe were designed by the Primer Express software version 3.0 (Applied Biosystems). The upstream (NHP FlgE3qF) and downstream (FlgE3qR) primer sequences are: 5’-AAC-ACC-CTG-TCT-CCC-CAA-TTC-3’; and 5’-CCA-GCC-TTG-GAC-AAA-CAC-CTT-3’, respectively. The TaqMan probe NHP: 5’-CGC-CCC-AAA-GCA-TGC-CGC-3’, is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5’ and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3’ end.~~

~~ii)~~ *~~The real-time PCR reaction mixture contains:~~* ~~The amplification reactions were conducted as follows: 0.5 μM of each primer, 0.1 μM TaqMan probe, 1× TaqMan Fast Virus 1-Step Master Mix (Life Technologies), 5–50 ng DNA template and HPLC water in a reaction volume of 10 μl. For optimal results, the reaction mixture should be vortexed and mixed well.~~

~~iii) The real-time PCR profile consists of 20 seconds at 95°C followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Amplification detection and data analysis for real-time PCR assays are carried out with the StepOnePlus real-time PCR system (Life Technologies).~~

~~iv) 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 and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, or DNA extracted from~~ *~~H. penaei~~*~~-infected hepatopancreas.~~

4.4.2. Conventional PCR

Hepatopancreas may be assayed for *H. penaei* using PCR. Two different PCR methods have been developed for *H. penaei* detection using 16S rRNA gene and ~~Flg E~~ flagella hook gene separately.

|  |  |  |  |
| --- | --- | --- | --- |
| Pathogen/target gene | Primer~~/probe~~ (5’–3’) | Concentration | Cycling parameters |
| Method 1: Aranguren *et al.,* 2010; GenBank Accession No.: MH230908.1; amplicon size 379 bp |
| *H. penaei*/16S rRNA gene | Fwd NHPF2: CGT-TGG-AGG-TTC-GTC-CTT-CAG-TRev NHPR2: GCC-ATG-AGG-ACC-TGA-CAT-CAT-C | 200 nM  | 35 cycles: 95°C/30 sec, 60°C/30 sec and 72°C/30 sec |
| Method 2: Aranguren & Dhar, 2018; GenBank Accession No.: JQAJ01000001.1; amplicon size 333 bp |
| *H. penaei*/ Flagella hook gene ~~protein~~ | Fwd FlgE 1143F: AGG-CAA-ACA-AAC-CCT-TGRev FlgE 1475R: GCG-TTG-GGA-AAG-TT | ~~0.2 μM~~ 200 nM  | 35 cycles,:95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec |

~~Protocol 1~~

~~The PCR based on 16S rRNA is based on Aranguren~~ *~~et al.~~* ~~(2010). Primers designated as NHPF2: 5’-CGT-TGG-AGG-TTC-GTC-CTT-CAGT-3’ and NHPR2: 5’-GCC-ATG-AGG-ACC-TGA-CAT-CAT-C-3’, amplify a 379-base pair (bp) fragment corresponding to the 16S rRNA of~~ *~~H. penaei~~*~~. The PCR method outlined below generally follows the method described in Aranguren~~ *~~et al.~~* ~~(2010).~~

~~i) The following controls should be included when performing the PCR assay a) known~~ *~~H. penaei~~* ~~negative tissue sample; b) a known~~ *~~H. penaei~~* ~~positive sample (hepatopancreas); and c) a ‘no template’ control.~~

~~ii) The PuReTaq~~~~TM~~ ~~Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.~~

~~iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 μl total volume) for detection of~~ *~~H. penaei~~* ~~in shrimp hepatopancreas samples are: primers (0.2 μM each), dNTPs (200 μM each), Taq polymerase (0.1 U μl~~~~–1~~~~), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.~~

~~iv) The cycling parameters are: Step 1: 95°C for 5 minutes, 1 cycle; Step 2: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, 35 cycles; Step 3: 60°C for 1 minute, 72°C for 2 minutes, 1 cycle; 4°C infinite hold.~~

~~Protocol 2~~

~~The PCR based on flagella gene (flagella hook protein, flgE) is based on Aranguren & Dhar (2018). Primers designated as NHP FlgE 1143F (5’-AGG CAA ACA AAC CCT TG-3’) and and the NHP FlgE 1475R (5’- GCG TTG GGA AAG TT-3’) amplify a 333-base pair (bp) fragment corresponding to the Flg E of~~ *~~H. penaei~~*~~.~~

~~i) The following controls should be included when performing the PCR assay a) known~~ *~~H. penaei~~* ~~negative tissue sample; b) a known~~ *~~H. penaei~~* ~~positive sample (hepatopancreas); and c) a ‘no template’ control.~~

~~ii) The PuReTaq~~~~TM~~ ~~Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.~~

~~iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 μl total volume) for detection of~~ *~~H. penaei~~* ~~in shrimp hepatopancreas samples are: primers (0.2 μM each), dNTPs (200 μM each), Taq polymerase (0.1 U μl~~~~–1~~~~), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.~~

~~iv) The cycling parameters are: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes followed by 4°C infinite hold.~~

~~Note: The conditions should be optimised for each thermal cycler using known positive controls.~~

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

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

~~PCR products may be cloned and sequenced or sequenced directly when necessary to confirm infection~~ ~~with~~ *~~H. penaei~~* ~~or to identify false positives or nonspecific amplification (Aranguren~~ *~~et al.,~~* ~~2010; Aranguren & Dhar, 2018; Vincent & Lotz, 2005).~~

4.6. *In-situ* hybridisation

The ISH method of Loy & Frelier (1996) and Lightner (1996) provides greater diagnostic sensitivity than do more traditional methods for *H. penaei* detection and diagnosis of infection that employ classical histological methods (Lightner, 1996; Morales-Covarrubias, 2010). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled DNA probe to *H. penaei* 16S rRNA provides a definitive diagnosis of infection with *H. penaei* (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.,* 2006). Pathognomonic *H. penaei* positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the DNA probes. (See Chapter 2.2.4 *Infection with infectious hypodermal and haematopoietic necrosis virus* for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson’s AFA fixative.)

4.7. Immunohistochemistry

Immunohistochemistry (IHC) tests using monoclonal antibodies (MAbs) to *H. penaei*, according to the methods described in Bradley-Dunlop *et al.* (2004), ~~are available~~ exist for *H. penaei* detection.

4.8. Bioassay

Confirmation of infection with *H. penaei* may be accomplished by bioassay of suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the intracellular bacteria (Aranguren *et al.,* 2010; Lightner, 2005). Oral protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped hepatopancreas of suspect shrimp to SPF juvenile *P. vannamei* in small tanks. The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the hepatopancreas feeding (*per os*) protocol is used to bioassay for *H. penaei*, *positive* indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of infection with *H. penaei* and unusual mortalities.

4.9. Antibody- or antigen-based detection methods

Serological tests are not applicable because shrimp are invertebrate animals that do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to *H. penaei*.

4.10. Other methods

No scientifically confirmed reports.

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

Real-time PCR ~~are~~ is the recommended test for surveillance to demonstrate freedom from infection with *H. penaei* in apparently healthy populations as described in Section 4.4.1 ~~and 4.4.2~~.

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status3F3F3F6F6F[[1]](#footnote-1)

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

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

i) A positive result by real-time PCR

ii) A positive result by conventional PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *H. penaei* is considered to be confirmed if at least one of the following criteria is met:

i) A positive result by two different probe-based real-time PCR tests targeting different region of the *H. penaei* genome

ii) A positive result by real-time PCR and conventional PCR targeting different region of the *H. penaei* genome followed by amplicon sequencing

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

6.2. Clinically affected animals

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

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs consistent with *H. penaei* infection

ii) Histopathology consistent with *H. penaei* infection

iii) A positive result by real-time PCR

iv) A positive result by conventional PCR

v) A positive result by *in-situ* hybridisation

vi) A positive result by bioassay

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *H. penaei* is considered to be confirmed if at least at least one of the following criteria is met:

i) A positive result by two different probe-based real-time PCR tests targeting different regions of the *H. penaei* genome

ii) A positive result by real-time PCR and conventional PCR targeting different regions of the *H. penaei* genome followed by amplicon sequencing

iii) ~~Histopathology consistent with~~ *~~H. penaei~~* ~~and positive~~ *~~in-situ~~* ~~hybridisation test~~ A positive result by *in-situ* hybridisation and real-time PCR

iv) A positive result by *in-situ* hybridisation and conventional PCR followed by amplicon sequencing

~~v) A positive result by bioassay followed by real-time PCR~~

~~vi) A positive result by bioassay followed by conventional PCR followed by amplicon sequencing~~

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

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *H. penaei* are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). This information can be used for the design of surveys for infection with *H. penaei*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

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

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction, ND = Not determined.

6.3.2. For surveillance of apparently healthy animals

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

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

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\*
\* \*

**NB:** There is a WOAH Reference Laboratory for infection with *Hepatobacter penaei*(necrotising hepatopancreatitis)
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
infection with *Hepatobacter penaei* (necrotising hepatopancreatitis).

**NB:** First adopted in 2012; Most recent updates adopted in 2017.

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