CHAPTER 2.2.1.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

AHPND has a bacterial aetiology (Kondo *et al*., 2015; Tran *et al*., 2013). It is caused by specific virulent strains of V. parahaemolyticus (*Vp*$_{AHPND}$) that contain a ~70-kbp plasmid with genes that encode homologues of the Photorhabdus insect-related (Pir) binary toxin, PirA and PirB (Gomez-Gil *et al*., 2014; Gomez-Jimenez *et al*., 2014; Han *et al*., 2015a; Kondo *et al*., 2014; Lee *et al*., 2015; Yang *et al*., 2014). The plasmid within *Vp*$_{AHPND}$ has been designated pVA1, and its size may vary slightly. Removal (or "curing") of pVA1 abolishes the AHPND-causing ability of *Vp*$_{AHPND}$ strains.

Within a population of *Vp*$_{AHPND}$ bacteria, natural deletion of the Pirvp operon may occur in a few individuals (Lee *et al*., 2015; Tinwongger *et al*., 2014). This deletion is due to the instability caused by the repeat sequences or transposase that flank the Pir toxin operon. When the deletion occurs, it means that a *Vp*$_{AHPND}$ strain will lose its ability to induce AHPND. However, if the Pir toxin sequence is used as a target for detection, then a colony that has this deletion will produce a negative result even though the colony was derived from an isolate of AHPND-causing *Vp*$_{AHPND}$. A recent report describes a naturally occurring deletion mutant of *Vp*$_{AHPND}$ that does not cause a clinical manifestation of AHPND (Aranguren *et al*., 2020a).

The plasmid pVA1 also carries a cluster of genes related to conjugative transfer, which means that this plasmid is potentially able to transfer to other bacteria.

2.1.2. Survival and stability in processed or stored samples

AHPND cannot be transmitted from infected samples that have been stored frozen (Tran *et al*., 2013). Some *Vibrio* species are sensitive to freezing (Muntada-Garriga *et al*., 1995; Thomson & Thacker, 1973).

2.1.3. Survival and stability outside the host

Vp$_{AHPND}$ is expected to possess similar properties to other strains of V. parahaemolyticus found in seafood that have been shown to survive up to 9 and 18 days in filtered estuarine water and filtered seawater at an ambient temperature of 28 ± 2°C (Karunasagar *et al*., 1987).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Code* are: fleshy prawn (*Penaeus chinensis*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: kuruma prawn (*Penaeus japonicus*).

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

Mortalities occur within 30–35 days, and as early as 10 days, of stocking shrimp ponds with postlarvae (PL) or juveniles (Joshi *et al*., 2014b; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013). De la Pena *et al*. (2015) reported disease outbreaks in the Philippines occurring as late as 46–96 days after pond-stocking.

2.2.4. Distribution of the pathogen in the host

Gut including stomach, and hepatopancreas.

2.2.5. Aquatic animal reservoirs of infection

None known.

2.2.6. Vectors

No vector is known, although as *Vibrio* spp. are ubiquitous in the marine environment, the possibility that there are vector species could be expected.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

AHPND is characterised by sudden, mass mortalities (up to 100%) usually within 30–35 days of stocking grow-out ponds with PLs or juveniles (Hong *et al*., 2016). Older juveniles may also be affected (de la Pena *et al*., 2015).

In regions where AHPND is enzootic in farmed shrimp, evidence indicates a near 100% prevalence (Tran *et al*., 2014).

2.3.2. Clinical signs, including behavioural changes

The onset of signs of disease and mortality can start as early as 10 days post-stocking. Signs of disease in moribund prawns may include pale to white hepatopancreas (HP) due to pigment loss in the connective tissue capsule (NACA, 2014). Behavioural changes such as frequent sinking to the bottom of tanks may also be noted.

2.3.3 Gross pathology

Gross pathological observations include pale-to-white HP, significant atrophy of the HP, soft shells, guts with discontinuous, or no contents and black spots or streaks visible within the HP (due to melanised tubules). In addition, the HP does not squash easily between the thumb and forefinger (probably due to increased fibrous connective tissue and haemocytes) (NACA, 2014).

2.3.4. Modes of transmission and life cycle

Vp$_{AHPND}$ has been transmitted experimentally by immersion, feeding (per os) and reverse gavage (Dabu *et al*., 2017; Joshi *et al*., 2014b; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013), simulating natural horizontal transmission via oral routes and co-habitation.

2.3.5. Environmental factors

Water sources with low salinity (<20 ppt) seem to reduce the incidence of the disease. Peak occurrence seems to occur during the hot, dry season from April to July. Overfeeding, poor seed quality, poor water quality, poor feed quality, algal blooms or crashes are also factors that may lead to occurrences of AHPND in endemic areas (NACA, 2014).

2.3.6. Geographical distribution

The disease was initially reported in Asia in 2010. It has since been reported in the Americas (2013) and Africa (2017).

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Not available.

2.4.2. Chemotherapy including blocking agents

Not available.

2.4.3. Immunostimulation

None known to be effective.

2.4.4. Breeding resistant strains

Not available.

2.4.5. Inactivation methods

Experimental studies have shown that *Vp*$_{AHPND}$ could not be transmitted via frozen infected shrimp (Tran *et al*., 2013). Similarly, other strains of V. parahaemolyticus are known to be sensitive to freezing, refrigeration, heating and common disinfectants (Muntada-Garriga *et al*., 1995; Thomson & Thacker, 1973).

2.4.6. Disinfection of eggs and larvae

Not available.

2.4.7. General husbandry

As with other infectious diseases of shrimp, established good sanitary and biosecurity practices, such as improvement of hatchery sanitary conditions and PL screening are likely to be beneficial; good broodstock management, use of high-quality post-larvae and good shrimp farm management including strict feeding rate control, appropriate stocking density etc. are all well-established practices that reduce the impact of disease, including AHPND. An AHPND-tolerant line of *P. vannamei* was recently reported, but at present (2022) no genetically improved lines are commercially available (Aranguren *et al*., 2020b).

3. Specimen selection, sample collection, transportation and handling

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

3.1. Selection of populations and individual specimens

Samples of moribund shrimp or shrimp that show clinical signs (see Section 2.3.2) should be selected for AHPND diagnosis. It is assumed that adults (broodstock) can carry strains of *Vp*$_{AHPND}$ (Lee *et al*., 2015; Nunan

et al., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013). Therefore, broodstock without clinical signs may also be selected for diagnostic testing.

3.2. Selection of organs or tissues

Samples may be taken from gut-associated tissues and organs, such as the hepatopancreas, stomach, midgut and hindgut.

3.3. Samples or tissues not suitable for pathogen detection

Samples other than gut-associated tissues and organs are not appropriate (NACA, 2014; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013).

3.4. Non-lethal sampling

Faecal matter may be collected from valuable broodstock for AHPND diagnosis. However, compared with tissue sampling, the relative utility of faecal samples for detecting AHPND-causing bacteria has not been evaluated.

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.

3.5.1. Samples for pathogen isolation

High quality samples are essential for successful pathogen isolation and bioassay. Sample quality depends mainly on the time since collection and time spent in storage. Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animals and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. Alternatively, samples can be preserved in a DNA preservative for PCR testing. If material cannot be fixed it may be frozen, but repeated freezing and thawing of samples 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

Tissue samples for histopathology, immunohistochemistry or *in-situ* hybridisation can be preserved in Davidson's AFA fixative for histology (Joshi *et al*., 2014a; 2014b; Nunan *et al*., 2014; Sirikharin *et al*., 2015; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013).

3.5.4. Samples for other tests

Not applicable.

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 specimens should be processed and tested individually. Small life stages can be pooled to obtain the minimum amount of material for bacterial isolation or molecular detection.

4. Diagnostic methods

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

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

+++ =	Methods are most suitable with desirable performance and operational characteristics.
++ =	Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

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

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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology		+	+	NA		+	+	NA				
Isolation					+	+	+	NA				
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	++	++	++	2	++	++	++	2				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	2
***In-situ* hybridisation**												
Bioassay					+	+	+	NA				
LAMP		++	++	1								
Ab-ELISA												
Ag-ELISA		+	++	1		+	++	1		+	++	1
Other antigen detection methods												
Other methods												

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

4.1. Wet mounts

Not applicable.

4.2. Histopathology and cytopathology

Histological examination of AHPND infected shrimp reveals that pathological changes are limited to the hepatopancreas. The disease has three distinct phases:

i) The acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach. No B-, F- and R-cells are seen in the hepatopancreatic tubule and some nuclei of tubule epithelial cells are enlarged (karyomegaly). No significant bacterial involvement appears during this phase (Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

ii) The terminal phase is characterised by marked intra-tubular haemocytic inflammation and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells (NACA, 2014; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

iii) In *Penaeus vannamei* AHPND tolerant lines, a chronic phase can be observed. The chronic phase is characterised by only a few tubules with epithelial necrosis accompanied by bacteria and inflammation. This phase resembles a septic hepatopancreatic necrosis (SHPN) (Aranguren *et al*., 2020b).

4.3. Isolation

4.3.1. Enrichment of samples prior to DNA extraction

Preliminary enrichment culture for detection of *Vp*$_{AHPND}$ may be carried out using any suitable bacteriological medium (e.g. tryptic–soy broth or alkaline peptone water containing 2.5% NaCl supplement) incubated for 4 hours at 30°C with shaking. Then, after letting any debris settle, the bacteria in the culture broth are pelleted by centrifugation. Discarding the supernatant, DNA can be extracted from the bacterial pellet in preparation for PCR analysis.

4.3.2. Agent isolation

Vp$_{AHPND}$ may be isolated from diseased shrimp using standard microbiological media for isolation of *Vibrio* species from such sources (Lightner, 1996; Tran *et al*., 2013). Confirmation of identification of *Vp*$_{AHPND}$ may be undertaken by PCR analysis.

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.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

PCR methods have been developed that target the *Vp*$_{AHPND}$ toxin genes. The AP3 method is a single-step PCR that targets the 12.7 kDa PirAvp gene (Sirikharin *et al*., 2015). It was validated for isolates of *Vp*$_{AHPND}$ and non-pathogenic bacteria (including other *Vibrio* and non-*Vibrio* species) that had previously been tested by bioassay (Sirikharin *et al*., 2015). Subsequently, Soto-Rodriguez *et al*. (2015), using 9 *Vp*$_{AHPND}$ and 11 non-pathogenic isolates of V. parahaemolyticus reported that the AP3 method produced the highest positive (90%) and negative (100%) predictive values of five PCR methods tested.

Single-step PCRs such as the AP3 method and others, e.g. VpPirA-284, VpPirB-392 (Han *et al*., 2015a) and TUMSAT-Vp3 (Tinwongger *et al*., 2014), have relatively low sensitivity when used for detection of *Vp*$_{AHPND}$ at

low levels (e.g. sub-clinical infections). For such samples, a preliminary enrichment step (see Section 4.3.1. Enrichment of samples prior to DNA extraction) is recommended.

Alternatively, a nested PCR method, AP4, has been developed with a 100% positive predictive value for *Vp*$_{AHPND}$ using the same 104 bacterial isolates used to validate AP3 above (Dangtip *et al*., 2015), and has greater sensitivity (1 fg of DNA extracted from *Vp*$_{AHPND}$), allowing it to be used directly with tissue without an enrichment step.

In addition, real-time PCR methods, for example the *Vp*$_{AHPND}$-specific TaqMan real-time PCR developed by Han *et al*. (2015b), and an isothermal loop-mediated amplification protocol (LAMP) method developed by Koiwai *et al*. (2016) also have high sensitivity and can be used directly with tissue without an enrichment step.

A general DNA extraction method may be used to extract DNA from the stomach or hepatopancreatic tissue of putatively infected shrimp, from cultures of purified bacterial isolates or from bacterial pellets from enrichment cultures (see Section 4.3). The amount of template DNA in a 25 µl PCR reaction volume should be in the range of 0.01–1 ng of DNA when extracted from bacterial isolates (i.e. directly from a purified culture) and in the range of 10–100 ng of total DNA when extracted from shrimp tissues or from a bacterial pellet derived from an enrichment culture.

4.4.1. Real-time PCR

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Han *et al.,* 2015b; GenBank Accession No.: KM067908			
pirA	Fwd VpPirA-F: TTG-GAC-TGT-CGA-ACC-AAA-CG Rev VpPirA-R: GCA-CCC-CAT-TGG-TAT-TGA-ATG VpPirA Probe: FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA	Fwd: 0.3 µM Rev: 0.3 µM probe: 0.1 µM	95°C/20 sec; 45 cycles 95°C/3 sec and 60°C/30 sec

4.4.2. Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1(AP3): Sirikharin *et al*., 2015; GenBank Accession No.: JALL01000066.1; amplicon size: 333 bp			
pirA^{vp}	Fwd AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA	0.2 µM each	94°C/5 min; 30 cycles of 94°C/30 sec, 53°C/30 sec, 72°C/40 sec; final elongation step at 72°C/7 min; Reaction mixture can be held at 4°C
Method 2 (TUMSAT-Vp3): Tinwongger *et al*., 2014; GenBank Accession No.: AB972427; amplicon size: 360 bp			
pVA1	Fwd TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CA Rev TUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA	0.6 µM each	95°C/2 min; 30 cycles of 95°C/30 sec, 56°C/30 sec, 72°C/30 sec
Method 3 (VpPirA-284): Han *et al.,* 2015a; GenBank Accession No.: KM067908; amplicon size: 284 bp			
pirA^{vp}	Fwd VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG Rev VpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 4 (VpPirB-392): Han *et al.,* 2015a; GenBank Accession No.: KM067908; amplicon size: 392 bp			
pirB^{vp}	Fwd VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC Rev VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 5 (AP4): Dangtip *et al.,* 2015; GenBank Accession No.: JPKS01000000; amplicon size: 1269 bp			
PirA and PirB toxin genes	Primary Fwd AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA Nested Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 µM each	Primary 94°C/2 min; 30 cycles of 94°C/30 sec, 55°C/30 sec, 72°C/90 sec; final extension step at 72°C/2 min; hold at 4°C Nested 94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C

4.4.3. Isothermal loop-mediated amplification protocol (LAMP)

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method: Koiwai *et al.,* 2017; GenBank Accession No.: AB972427.1			
Toxin PirAB-like	F3: TGA-TAA-TGC-ATT-CTA-TCA-TCA-GC B3: ATT-TGA-AAG-ACC-AAA-TGA-AAC-C FIP-F1c: GTG-AGC-ACC-TTC-TTA-GTG-GTA-ATA FIP-F2: GTT-GTA-ATT-AAC-AAT-GGC-GCT-AG BIP-B1c: TGA-CGG-AAT-TTA-ACC-CTA-ACA-ATG-C BIP-B2: GCT-TTG-AAA-GCA-TAG-TTA-GGA-TC	F3: 5.0 pmol B3: 5.0 pmol FIP: 40 pmol BIP: 40 pmol	65°C/60 min and 80°C/5 min

4.4.4. Other nucleic acid amplification methods

Cruz-Flores *et al*. (2019) developed a multiplex real-time PCR-based SYBR green assay for simultaneous detection of pirA, pirB, 16S rRNA and 18S rRNA, and a duplex real-time PCR-based Taqman probe assay showing high specificity and sensitivity – limit of detection was 10 copies for both pirA and pirB. A recombinase polymerase amplification assay was developed by Mai *et al*. (2021). This assay has a limit of detection of five copies of the pirAB gene and high specificity.

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.

4.6. *In-situ* hybridisation

Not available.

4.7. Immunohistochemistry

An immunohistochemistry assay to detect AHPND was developed by Kumar *et al*., (2019). However, the assay requires further validation.

4.8. Bioassay

Vp$_{AHPND}$ has been transmitted experimentally by immersion and by reverse gavage (Joshi *et al*., 2014b; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013), simulating natural horizontal transmission via oral routes and co-habitation. Thus, following isolation and purification of a bacterium that is suspected to cause AHPND, a bioassay can be performed to confirm the presence of the causative agent. The immersion procedure is carried out by immersing 15 shrimp for 15 minutes, with aeration, in a suspension (150 ml clean artificial seawater) of 2×10^8 cells of the cultured bacterium per ml. Following this initial 15-minute period, the shrimp and the inoculum are transferred to a larger tank with a volume of clean artificial seawater to make the final concentration of the bacterium 2×10^6 cells ml^{-1}. Shrimp are monitored at 6- to 8-hour intervals. Dead shrimp can be processed for *Vp*$_{AHPND}$ PCR and sequence analysis. Moribund or surviving shrimp are processed for histology, bacterial re-isolation, PCR and sequence analysis. A positive bioassay is indicated by the detection of characteristic histological lesions and *Vp*$_{AHPND}$ by PCR and amplicon sequence analysis.

4.9. Antibody- or antigen-based detection methods

An indirect enzyme-linked immunosorbent assay (I-ELISA) for AHPND detection developed by Mai *et al*. (2020) showed high sensitivity (the limit of detection was 0.008 ng µl^{-1} for PirAvp and 0.008 ng µl^{-1} for PirBvp) and specificity.

4.10. Other methods

None.

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

Real-time PCR (Han *et al*., 2015b) and conventional PCR (Dangtip *et al*., 2015) are recommended for demonstrating freedom from AHPND in an apparently healthy population.

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 suspect and confirmed cases 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. If a Competent Authority does not have the 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 status[1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical 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.

1 For example transboundary commodities.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with AHPND 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

iii) A positive result by LAMP

iv) Histopathology consistent with the disease

v) A positive result by Ag-ELISA

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Vibrio* parahaemolyticus (Vp_{AHPND}) is considered to be confirmed if at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA and conventional PCR followed by amplicon sequence analysis

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 *Vibrio* parahaemolyticus (Vp_{AHPND}) shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease

ii) A positive result by agent isolation

iii) A positive result by real-time PCR

iv) A positive result by conventional PCR

v) A positive result by bioassay

vi) A positive result by LAMP

vii) A positive result by Ag-ELISA

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *Vibrio* parahaemolyticus (Vp_{AHPND}) is considered to be confirmed if at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis.

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA and conventional PCR followed by amplicon sequence analysis

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *Vibrio* parahaemolyticus (Vp_{AHPND}) are provided in Tables 6.3.1. and 6.3.2 (no data are currently available). This information can be used for the design of surveys for infection with *Vibrio* parahaemolyticus (Vp_{AHPND}), 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
Conventional PCR	Diagnosis	Clinically diseased and apparently healthy shrimp	AHPND causing and non-causing bacterial isolates	*Penaeus vannamei*	100	100	Bioassay	Sirikharin *et al.,* 2015
Conventional PCR	Diagnosis	Clinically diseased and apparently healthy shrimp	AHPND causing and non-causing bacterial isolates	NA	100[1]	100	Bioassay	Tinwongger *et al*., 2014

DSe = diagnostic sensitivity, DSp = diagnostic specificity, NA= Not available, PCR: = polymerase chain reaction.
1100% sensitivity for TUMSAT-Vp3 primer set.

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, NA= Not available, PCR: = polymerase chain reaction.

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NB: There are WOAH Reference Laboratories for acute hepatopancreatic necrosis disease (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 Laboratory for any further information on acute hepatopancreatic necrosis disease

NB: First adopted in 2017. Most recent updates adopted in 2023.