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. Although there are reports of the isolation of other *Vibrio* species from clinical cases of AHPND, only *Vp*AHPND has been demonstrated to cause AHPND.

**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 Pir*vp* 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: f1F1F[[1]](#footnote-1)leshy 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, stomach, and hepatopancreas.

**2.2.5. Aquatic animal reservoirs of infection**

In experimental challenges, *Macrobrachium rosenbergii* and *Cherax quadricarinatus* did not show clinical signs of the disease or histopathological changes induced by AHPND but tested positive by PCR assay. However, whether these species serve as reservoirs of infection or are resistant to AHPND needs further investigation (Powers *et al.,* 2021; Schofield *et al.,* 2020).

**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 clinical signs and mortality can start as early as 10 days post-stocking. Clinical signs include a pale-to-white hepatopancreas (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).

**2.3.3 Gross pathology**

AHPND has three infection phases. In the acute phase, there is massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of the HP tubule epithelial cells into the lumen of the tubule, the HP collecting ducts and the posterior stomach and the absence of bacterial cells. In the terminal phase, the HP shows intra-tubular haemocytic inflammation and develops massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells. Animals that survive an acute infection reach a chronic phase, in which they present with limited cellular changes in the hepatopancreas tubule and only a few tubules with epithelial necrosis accompanied by bacteria and inflammation. The chronic phase pathology resembles a septic hepatopancreatic necrosis (SHPN) (Aranguren *et al.,* 2020a; NACA, 2014; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 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 OIE WAHIS (<https://wahis.oieint/#/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. In the case of valuable broodstock, non-lethal faecal samples may be collected instead, however the utility of faecal samples compared with tissue samples has not been evaluated.

**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.

**3.5. Preservation of samples for submission**

Samples to be submitted are (i) fresh and chilled on ice for bacterial isolation, (ii) fixed in 90% ethanol for PCR detection and (iii) preserved in Davidson’s AFA fixative for histology (Joshi *et al.,* 2014a; 2014b; Lee *et al.,* 2015; Nunan *et al.,* 2104; Sirikharin *et al.,* 2015; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013).

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 DNAzol for PCR testing. If material cannot be fixed it may be frozen.

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

Tissue samples for histopathology, immunohistochemistry or in-situ hybridization 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.

OIE 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 OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

***Table 4.1.*** *OIE 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** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Histopathology** |  | + | + | NA |  | + | + | NA |  |  |  |  |
| **Cell culture** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Real-time PCR** | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |  |  |  |  |
| **Conventional PCR** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 |
| **Amplicon sequencing** |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Bioassay** |  |  |  |  | + | + | + | NA | + | + | + | NA |
| **LAMP** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ab-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ag-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other antigen detection methods3** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other methods3** |  |  |  |  |  |  |  |  |  |  |  |  |

LV = level of validation, refers to the stage of validation in the OIE 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.
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.
3Specify the test used. 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**

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 in the absence of bacterial cells (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, 2012; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

iii) 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. Cell culture for isolation**

**4.3.1. Enrichment of samples prior to DNA extraction**

Preliminary enrichment culture for detection of *Vp*AHPND from sub-clinical infections or environmental samples 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 purification**

*Vp*AHPND may be isolated in pure culture from diseased shrimp, sub-clinically infected shrimp, or environmental samples 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 methods have been developed that target the VpAHPND 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 100% positive and negative predictive value by testing 104 isolates of VpAHPND 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 VpAHPND 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 VpAHPND at low levels (e.g. sub-clinical infections) or in environmental samples such as sediments and biofilms. 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 VpAHPND 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 VpAHPND), allowing it to be used directly with tissue and environmental samples without an enrichment step.

In addition, real-time PCR methods, for example the VpAHPND-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 and environmental samples 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.

The following controls should be included in all *Vp*AHPND PCR assays: a) negative extraction control i.e. DNA template extracted at the same time from a known negative sample; b) DNA template from a known positive sample, such as *Vp*AHPND-affected shrimp tissue or DNA from an *Vp*AHPND-positive bacterial culture, or plasmid DNA that contains the target region of the specific set of primers; c) a non-template control. In addition, a further control is required to demonstrate that extracted nucleic acid is free from PCR inhibitors, for example, for shrimp tissues use of the decapod 18S rRNA PCR (Lo *et al*., 1996) or use the 16S rRNA PCR for bacteria (Weisburg *et al*., 1991).

**4.4.1. Real-time PCR**

This protocol is based on the method described by Han *et al*. (2015b).The TaqMan Fast Universal PCR Master Mix (Life Technologies) is used and extracted DNA is added to the real-time PCR mixture containing 0.3 µM of each primer and 0.1 µM probe to a final volume of 10 µl. Real-time PCR conditions consist of 20 seconds at 95°C, followed by 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. At the completion of the TaqMan real-time PCR assay, the presence of PirA DNA is demonstrated by the presence of specific amplicons, identified by software-generated characteristic amplification curves. No-template controls must have no evidence of specific amplicons.The primers and probe and target gene for the *Vp*AHPND-specific real-time PCR are listed in Table 4.4.1.1.

***Table 4.4.1.1.*** *Primers and probe for the real-time PCR method for detection of pirA toxin gene*

|  |  |  |  |
| --- | --- | --- | --- |
| Primer/ probe name | Sequence (5’–3’) | Target gene | Reference |
| VpPirA-F | TTG-GAC-TGT-CGA-ACC-AAA-CG | pirA | Han *et al.,* 2015b |
| VpPirA-R | GCA-CCC-CAT-TGG-TAT-TGA-ATG |
| VpPirA Probe | FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA |

**4.4.2. Conventional PCR**

**One-step PCR detection of pVA1 plasmid**

Two one-step PCR methods (AP1 and AP2) are described here for detection of the pVA1 plasmid in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.4.2.1.

***Table 4.4.2.1.*** *PCR primers for one-step PCR detection of pVA1 plasmid*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Method name | Primers (5’–3’) | Target gene | Expected amplicon size | Reference |
| AP1 | AP1F: 5CCT-TGG-GTG-TGC-TTA-GAG-GAT-GAP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-C | *pVA1* | 700bp | Flegel & Lo (2014) |
| AP2 | AP2F: TCA-CCC-GAA-TGC-TCG-CTT-GTG-GAP2R: CGT-CGC-TAC-TGT-CTA-GCT-GAA-G | *pVA1* | 700bp | Flegel & Lo (2014) |

**Protocol for the AP1 and AP2 PCR methods**

This protocol follows the method described by Flegel & Lo (2014). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM MgCl2, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP1/AP2F, 0.5 µl 10 µM AP1/AP2R, 0.2 µl Taq DNA polymerase and approximately 0.01–1 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 25–30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds with a final extension step at 72°C for 10 minutes and then the reaction mixture can be held at 4°C (<https://enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf>).

**One-step PCR detection of PirA/PirB toxin genes**

Four one-step PCR methods (AP3, TUMSAT-Vp3*,* VpPirA-284 and VpPirB-392)are described here for detection of Pir toxin genes in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.4.2.3.

***Table 4.4.2.2.*** *PCR primers for one-step PCR detection of PirA and PirB toxin genes*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Method name | Primers (5’–3’) | Target gene | Expected amplicon size | Reference |
| AP3 | AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-ACAP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA | *pir*A*vp* | 333bp | Sirikharin *et al*., 2015 |
| TUMSAT-Vp3 | TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CATUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA | *pir*A*vp* | 360bp | Tinwongger *et al*., 2014 |
| VpPirA-284 | VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TGVpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA | *pir*A*vp* | 284bp | Han *et al.,* 2015a |
| VpPirB-392 | VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TCVpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA | *pirBvp* | 392bp | Han *et al.,* 2015a |

**Protocol for the AP3 PCR method**

This protocol follows the method described by Sirikharin *et al*. (2015). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM MgCl2, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP3-F1, 0.5 µl 10 µM AP3-R1, 0.2 µl Taq DNA polymerase and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds with a final extension step at 72°C for 5 minutes and then the reaction mixture can be held at 4°C.

**Protocol for the VpPirA-284 and VpPirB-392 PCR methods**

This protocol follows the method described by Han *et al.* (2015a) and uses PuReTaq ready-to-go PCR beads (GE Healthcare). A 25 µl PCR reaction mixture is prepared with PuReTaq ready-to-go PCR beads. Each reaction contains 0.2 μM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, and 1 μl of extracted DNA. For PCR a 3-minute denaturation step at 94°C is followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

**Protocol for the TUMSAT-Vp3 PCR method**

This protocol follows the method described by Tinwongger *et al.* (2014). A 30 µl PCR mixture is prepared containing 1 µl DNA template, 10× PCR buffer, 0.25 mM dNTP mixture, 0.6 µM of each primer and 0.01 U Taq polymerase. PCR conditions consist of an initial preheating stage of 2 minutes at 95°C, followed by 30 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 56°C and 30 seconds extension at 72°C.

**AP4 nested PCR protocol for detection of VpAHPND**

This protocol follows the method described by Dangtip *et al.* (2015). The first PCR reaction mixture consists of 2.5 µl 10× PCR mix, 1.5 µl 50 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM AP4-F1, 0.5 µl 10 µM AP4-R1, 0.3 µl of Taq DNA pol (5 units µl–1) and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension step at 72°C for 2 minutes and hold at 4°C.

The nested PCR reaction mixture consists of 2.5 µl 10x PCR mix, 1.5 µl 50 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.375 µl 10 µM AP4-F2, 0.375 µl 10 µM AP4-R2, 0.3 µl Taq DNA pol (5 units µl–1) and 2 µl of the first PCR reaction in a total volume of 25 µl. The nested PCR protocol is 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds and hold at 4°C.

The nested PCR primers, designed using the China (People’s Rep. of) isolate of AHPND bacteria (Yang *et al.,* 2014), are shown in Table 4.4.2.7. The expected amplicon sizes are 1269 bp for the outer primers (AP4-F1 and AP4-R1) and 230 bp for the inner primers (AP4-F2 and AP4-R2). At high concentrations of target DNA, additional amplicons may occur as the product of residual primer AP4-F1 pairing with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

***Table 4.4.2.3.*** *Primers for the AP4, nested PCR method for detection of PirA and PirB toxin genes*

|  |  |  |  |
| --- | --- | --- | --- |
| Method name | Primers (5’–3’) | Expected amplicon size | Reference |
| AP4Step 1 | AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-ACAP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA | 1269 | Dangtip *et al.,* 2015 |
| AP4Step 2 | AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GGAP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC | 230 |

**Analysis of conventional PCR products by agarose gel electrophoresis**

After PCR, amplicons are visualised by agarose gel electrophoresis. Twenty µl of the PCR reaction mixture, with 6× loading dye added, is loaded onto a 1.5% agarose gel and electrophoresis is carried out at 90 volts for 40 minutes. Amplicons are visualised with SYBR Safe gel stain (Invitrogen, Cat. No. 33102) according to the manufacturer’s instructions. Amplicons of the expected size appropriate for the PCR methods used (Tables 4.4.2.1, 4.4.2.2 and 4.4.2.3) indicate a positive result.

**4.4.3. 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. A LAMP-based assay for AHPND detection developed by Koiwai *et al.* (2016) also shows high specificity and sensitivity.

**4.5. Amplicon sequencing**

The positive results obtained from conventional PCR described in 4.4.2 need to be confirmed by sequencing.

**4.6. *In-situ* hybridisation**

ISH is not currently available (December 2021).

**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 × 108 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 × 106 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 PirA*vp* and 0.008 ng µl–1 for PirB*vp*) 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) is 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. 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 OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

**6.1. Apparently healthy animals or animals of unknown health status**2F2F**[[2]](#footnote-2)**

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic 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 AHPND shall be suspected if at least one of the following criteria is met:

i) A positive result by any of the real-time or conventional PCR methods recommended in Table 4.1

ii) Histo- or cytopathological changes consistent with the presence of the pathogen or the disease

**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 the following criterion is met:

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

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 *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 as described in this chapter, with or without elevated mortality

ii) A positive result by real-time PCR

iii) A positive result by conventional PCR

**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 the following criterion is met:

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

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 *Vibrio parahaemolyticus* (*Vp*AHPND) are provided in Tables 6.3.1. and 6.3.2. 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 | 1001 | 100 | Bioassay | Tinwongger *et al*., 2014 |
| Real-time PCR | Diagnosis | Clinicallly diseased animals | Hepato-pancreas | *Penaeus vannamei* | 100 | NA | Bioassay and histopathology | Han *et al.* 2015b |

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

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**NB:** There are OIE Reference Laboratories for acute hepatopancreatic necrosis disease
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
<https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
Please contact the OIE Reference Laboratory for any further information on
acute hepatopancreatic necrosis disease

**NB**: First adopted in 2017; Most recent updates adopted in 2018.

1. [↑](#footnote-ref-1)
2. For example transboundary commodities. [↑](#footnote-ref-2)