CHAPTER 2.2.1.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (*V p* $_{AHPND}$) 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, agent strains

AHPND has a bacterial aetiology (Kondo et al., 2015; Tran et al., 2013a; Tran et al., 2013b). 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* $_{AHPND}$.

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 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).

2.1.3. Stability of the agent (effective inactivation methods)

Experimental studies have shown that *Vp* $_{AHPND}$ could not be transmitted via frozen infected shrimp (Tran et al., 2013a). Similarly, other strains of *V. parahaemolyticus* are known to be sensitive to freezing, refrigeration, heating and common disinfectants (Andrews et al., 2000; Muntada-Garriga et al., 1995; Su & Liu, 2007; Thomson & Thacker, 1973).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: 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* include: 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. Susceptible stages of the host

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; Leaño & Mohan, 2012; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b). de la Peña et al., 2015reported disease outbreaks in the Philippines occurring as late as 46–96 days after pond-stocking.

2.2.4. Species or subpopulation predilection (probability of detection)

Not applicable.

2.2.5. Target organs and infected tissue

Gut-associated tissues and organs.

2.2.6. Persistent infection

No data or not known.

2.2.7. Vectors

None 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. Transmission mechanisms

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., 2013b), simulating natural horizontal transmission via oral routes and co-habitation.

2.3.2. Prevalence

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

2.3.3. Geographical distribution

The disease has been reported from China (People's Rep. of) (2010), Vietnam (2010), Malaysia (2011), Thailand (2012) (Flegel, 2012; Lightner et al., 2012), Mexico (2013) (Nunan et al., 2014) and the Philippines (2014) (Dabu et al., 2017; de la Peña et al., 2015).

2.3.4. Mortality and morbidity

AHPND is characterised by sudden, mass mortalities (up to 100%) usually within 30–35 days of stocking grow-out ponds with PLs or juveniles (FAO, 2013; Hong et al., 2016; NACA, 2012). Older juveniles may also be affected (de la Peña et al., 2015).

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 and 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 (FAO, 2013; NACA, 2012).

2.4. Control and prevention

2.4.1. Vaccination

Not applicable.

2.4.2. Chemotherapy

None available.

2.4.3. Immunostimulation

None known to be effective.

2.4.4. Breeding for resistance

Not applicable.

2.4.5. Restocking with resistant species

None available.

2.4.6. Blocking agents

None available.

2.4.7. Disinfection of eggs and larvae

None known.

2.4.8. General husbandry practices

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 (NACA, 2012).

3. Sampling

3.1. Selection of individual specimens

Samples of moribund shrimp or shrimp that show clinical signs (see Section 4.1.1) 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., 2013b). Therefore, broodstock without clinical signs may also be selected for diagnostic testing.

3.2. 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 polymerase chain reaction (PCR) detection and (iii) preserved in Davidson's AFA fixative for histology (Joshi et al., 2014a; Joshi et al., 2014b; Leaño & Mohan, 2012; Lee et al., 2015; Nunan et al., 2014; Sirikharin et al., 2015; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

3.3. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, small life stages, especially PL or specimens up to 0.5 g, may need to be pooled to obtain enough material for molecular testing.

3.4. Best organs or tissues

Samples of gut-associated tissues and organs, such as hepatopancreas, stomach, the midgut and the hindgut are suitable. In addition, faecal (non-lethal) samples may be collected from valuable broodstock.

3.5. Samples/tissues that are not suitable

Samples other than gut-associated tissues and organs are not appropriate (FAO, 2013; NACA, 2012; NACA, 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

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, 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, 2012; NACA, 2014).

4.1.2. Behavioural changes

Not applicable.

4.2. Clinical methods

4.2.1. Clinical chemistry

None is known.

4.2.2. Microscopic pathology

The disease has two 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 (FAO, 2013; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; Tran et al., 2013b; Tran et al., 2014a; Tran et al., 2014b).

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 (FAO, 2013; Leaño & Mohan, 2012; NACA, 2012; NACA, 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; Tran et al., 2013b; Tran et al., 2014a; Tran et al., 2014b).

4.2.3. Wet mounts

Not applicable.

4.2.4. Smears

Not applicable.

4.2.5. Fixed sections (for *in-situ* hybridisation)

In-situ hybridisation is not currently available (October 2015).

4.2.6. Electron microscopy/cytopathology

Not applicable.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Not applicable.

4.3.1.1.2. Smears

Not applicable.

4.3.1.1.3. Fixed sections

See Section 4.2.5.

4.3.1.2. Agent isolation and identification

Vp_{AHPND} can be isolated on standard media used for isolation of bacteria from diseased shrimp (Lee et al., 2015; Soto-Rodriguez et al., 2015). Bacterial species identification may be carried out using 16S rRNA PCR (Weisburg et al., 1991) or toxR-targeted PCR (Kim et al., 1999) and sequencing. AHPND-specific PCR methods that target the Vp_{AHPND} toxin genes are described in section 4.3.1.2.3.1.

4.3.1.2.1. Cell culture/artificial media

See sections 4.3.1.2.3.1.1and 4.3.1.2.2.

4.3.1.2.2. Antibody-based antigen detection methods

None is available to date (October 2015).

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. PCR protocols for detection of AHPND-causing bacteria from cultures or infected shrimp

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 100% positive and negative predictive value by testing 104 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) or in environmental samples such as sediments and biofilms. For such samples, a preliminary enrichment step (see Section 4.3.1.2.3.1.1) 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 and environmental samples 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., 2016also have high sensitivity and can be used directly with tissue and environmental samples without an enrichment step.

4.3.1.2.3.1.1. 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.

4.3.1.2.3.1.2. Agent purification

Vp AHPND may be isolated in pure culture from diseased shrimp, subclinically infected shrimp, or environmental samples using standard microbiological media for isolation of *Vibrio* species from such sources (Lightner, 1996; Tran et al., 2013a; Tran et al., 2013b). Confirmation of identification of *Vp* AHPND may be undertaken by PCR analysis and bioassay.

4.3.1.2.3.1.3. DNA extraction

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 above). 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.

4.3.1.2.3.1.4. 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 4.1.

Table 4.1. *PCR primers for one-step PCR detection of AHPND-causing bacteria*

Method name	Primers	Target gene	Expected amplicon size	Reference
AP1	AP1F: 5'-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3' AP1R: 5'-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3'	*pVA1*	700 bp	Flegel & Lo, 2014
AP2	AP2F: 5'-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3' AP2R: 5'-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3'	*pVA1*	700 bp	Flegel & Lo, 2014

4.3.1.2.3.1.5. 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 $MgCl_2$, 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 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 (http://www.enaca.org/modules/library/publication.php?publication_id=1128).

4.3.1.2.3.1.6. 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 4.2.

Table 4.2. *PCR primers for one-step PCR detection of AHPND-causing bacteria*

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

4.3.1.2.3.1.7. 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 MgCl$_2$, 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.

4.3.1.2.3.1.8. Protocol for the VpPirA-284 and VpPirB-392 PCR methods

This protocol follows the method described by Han et al., 2015band 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.

4.3.1.2.3.1.9. 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.

4.3.1.2.3.1.10. AP4 nested PCR protocol for detection of Vp$_{AHPND}$

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 MgCl$_2$, 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 MgCl$_2$, 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 4.3.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 4.3. Primers for the AP4, nested PCR method for detection of VpAHPND

Method name	Primers	Expected amplicon size	Reference
AP4 Step 1	AP4-F1: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP4-R1: 5'-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'	1269 bp	Dangtip et al., 2015
AP4 Step 1	AP4-F2: 5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3' AP4-R2: 5'- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'	230 bp	Dangtip et al., 2015

4.3.1.2.3.1.11.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 4.2.and 4.3.) indicate a positive result. Positive results must be confirmed by sequence analysis.

4.3.1.2.3.1.12.Protocol for the AHPND-specific real-time PCR method

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.

Table 4.4. Primers and probe for the real-time PCR method for detection of VpAHPND

Primer/probe name	Sequence	Target gene	Reference
VpPirA-F	5'-TTG-GAC-TGT-CGA-ACC-AAA-CG-3'	pirA	Han et al., 2015b
VpPirA-R	5'-GCA-CCC-CAT-TGG-TAT-TGA-ATG-3'	pirA	Han et al., 2015b
VpPirA Probe	5'-6FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA-3'	pirA	Han et al., 2015b

4.3.1.2.3.1.13.Controls for all PCR methods

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 the 16S rRNA PCR for bacteria (Weisburg et al., 1991).

4.3.2. Serological methods

Not applicable.

4.3.3. Bioassay

Vp $_\text{AHPND}$ has been transmitted experimentally by immersion and reverse gavage (Joshi et al., 2014b; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b), 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* $_\text{AHPND}$ 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* $_\text{AHPND}$by PCR and sequencing.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of AHPND are listed in Table 5.1.The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PL	Juveniles	Adults		
Gross signs	d	d	d	d	c	d
Bioassay	d	d	d	d	d	a
Histopathology	d	c	a	c	a	b
Real-time PCR	d	a	a	a	a	b
Nested PCR and sequence	d	b	b	b	a	a
1-step PCR and sequence	d	c	c	c	a	a

PL = postlarvae; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from AHPND

As indicated in Table 5.1., real-time PCR is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

AHPND is suspected if at least one of the following criteria is met:

i)   Mortality and clinical signs consistent with AHPND;

ii)   Histopathology consistent with AHPND;

iii)   Detection of Pir toxin genes by PCR or real-time PCR.

7.2. Definition of confirmed case

AHPND is considered to be confirmed if two or more of the following criteria are met:

i)   Histopathology consistent with AHPND;

ii)   Detection of Pir toxin gene in the pVA1 plasmid in *Vibrio parahaemolyticus* by PCR and sequence analysis;

iii)   Positive results by bioassay (characteristic histological lesions and detection of *Vp* $_{AHPND}$ by PCR and sequencing).

8. References

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NB: There is an OIE Reference Laboratory for AHPND
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on AHPND.

NB: FIRST ADOPTED IN 2017; MOST RECENT UPDATES ADOPTED IN 2018.