

# USA Comments to the Aquatic Animal Health Standards Commission - February 2015 Report

## CHAPTER 2.2.X.

### ACUTE HEPATOPANCREATIC NECROSIS DISEASE

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#### 1. Scope

For the purpose of this chapter, acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), is considered to be infection with unique strains of *Vibrio parahaemolyticus*, namely AHPND-causing *V. parahaemolyticus* (VP<sub>AHPND</sub>).

The disease has two distinct phases:

- i) An acute phase characterised by acute progressive, massive degeneration of the hepatopancreas (HP) tubules from medial to dorsal, 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; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).
- ii) The terminal stage 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; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

#### 2. Disease information

##### 2.1. Agent factors

##### 2.1.1. Aetiological agent, agent isolates

AHPND has a bacterial aetiology (Tran, 2013a; 2013b; Zhang *et al.*, 2012). It is caused by specific virulent strains of *Vibrio parahaemolyticus*, namely VP<sub>AHPND</sub>, which contains one or more extrachromosomal plasmids, including a unique, previously unreported, large, plasmid with a size of ~70 kbp (Gomez-Gil *et al.*, 2014; Gomez-Jimenez *et al.*, 2014; Kondo *et al.*, 2014; Yang *et al.*, 2014). This plasmid has been designated pVPA3-1, and its size may vary slightly. Removal (or "curing") of pVA1 abolishes the AHPND-causing ability of the virulent strain of *V. parahaemolyticus*. A pVA1-cured strain fails to induce the massive sloughing of cells in the hepatopancreatic tubules that is a primary histopathological characteristic of disease (Lee *et al.*, 2015).

VP<sub>AHPND</sub> expresses a deadly plasmid-encoded toxin (Pir<sup>VP</sup>), which is homologous to the Pir (*Photobacterium* insect-related) binary toxin. The toxin is formed from two subunits, PirA<sup>VP</sup> and PirB<sup>VP</sup>, but unlike other Pir binary toxins, *V. parahaemolyticus* PirB (PirB<sup>VP</sup>, a 50.1 kDa protein) alone is capable of inducing AHPND histopathology in the hepatopancreatic tubules, while PirA<sup>VP</sup> (a 12.7 kDa protein) causes only minor histological changes (Han *et al.*, 2015; Lee *et al.*, 2015; Sirikharin *et al.*, 2015).

Within a population of AHPND-causing bacteria, natural deletion of the Pir<sup>VP</sup> region may occur in a few individuals (Tinwongger *et al.*, 2014). This deletion is due to the instability caused by the repeat sequences/transposase that flank the pir toxin operon, and although different strains exhibit different levels of stability, when the deletion occurs, it means that a virulent strain of *V. parahaemolyticus* 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 virulent bacteria.

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. So far, however, there have been no published reports that any bacteria other than *V. parahaemolyticus* carry pVA1. The pVA1 plasmid also carries the *pndA* gene, which is associated with a post-segregational killing (psk) system. For a bacterium that

harbours a plasmid with the psk system (PSK<sup>+</sup>), only progeny that inherit the PSK<sup>+</sup> plasmid will be viable. Progeny that do not inherit the PSK<sup>+</sup> plasmid will die because the stable *pndA* mRNA will be translated to PndA toxin that will kill the bacterium. The presence of a psk system on a plasmid thus ensures that the plasmid is inherited during bacterial replication. The pVA1 plasmid will therefore be passed on to subsequent generations of VP<sub>AHPND</sub> producing PirA<sup>VP</sup> and PirB<sup>VP</sup>. Hence, when PirA<sup>VP</sup> is present there is little or no histopathology. When PirB<sup>VP</sup> is present, its larger size of 50.1 kDa is adequate to produce an enzyme that denudes the hepatopancreatic tubules (Lee *et al.*, 2015).

**2.1.2. Survival outside the host (i.e. in the natural environment)**

Not known.

**2.1.3. Stability of the agent**

Not known.

**2.1.4. Life cycle**

Not applicable.

**2.2. Host factors**

**2.2.1. Susceptible host species (common and Latin names)**

*Penaeus vannamei* (white leg or Pacific white shrimp); *P. monodon* (black tiger prawn) and *P. chinensis* (fleshy prawn).

**2.2.2. Susceptible stages of the host**

In the acute phase, this disease is characterised by a massive acute progressive degeneration of the HP tubules from medial to dorsal, 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; 2013b; 2014a; 2014b).

In the terminal phase ~~of~~ ANDHP, 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; Leaña & Mohan, 2013; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

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**Rationale:** English Syntax

**2.2.3. Species or sub-population predilection (probability of detection)**

Not applicable.

**2.2.4. Target organs and infected tissue**

Gut-associated tissues and organs.

#### 2.2.5. Persistent infection with lifelong carriers

See Section 2.1.4 Life cycle.

#### 2.2.6. Vectors

None are known.

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

None are known (except in South-East Asia, some molluscs and certain polychaetes).

### 2.3. Disease pattern

#### 2.3.1. Transmission mechanisms

Mortalities are expected within 30 days of stocking shrimp ponds with postlarvae (PL) or juveniles (from 15 mg to ~1 g in weight) (Nunan *et al.*, 2014; Leaño & Mohan, 2013; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

With laboratory infections, mortality can be induced within 12 hours of exposure to strains of VP<sub>AHPND</sub> by the *per os* route if the coated feed contains 10<sup>8</sup> CFU (colony-forming units) per gram of inoculum (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

Alternatively, mortalities can be induced ~~is~~ with a bath challenge, provided that the challenge bath begins

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**Rationale:** English Syntax

with 10<sup>8</sup> CFU per gram of inoculum (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

#### 2.3.2. Prevalence

Nunan *et al.*, 2014, Soto-Rodriguez *et al.*, 2015, and Tran *et al.*, 2013b found a near 100% prevalence in pond-reared stocks in South-East Asia and in Mexico after 2013.

#### 2.3.3. Geographical distribution

The disease has been introduced into south-east China (People’s Rep. of), Vietnam, Malaysia, Thailand and Mexico. In other countries in the East and South-East Asia regions, and neighbouring Mexico, farms may have been exposed to the toxin-producing strains of *V. parahaemolyticus* (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b; Zhang *et al.*, 2012).

#### 2.3.4. Mortality and morbidity

Joshi *et al.*, 2014a; 2014b; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; and Tran *et al.*, 2013b found a near 100% mortality and morbidity prevalence in pond-reared stocks in South-East Asia and in Mexico.

#### 2.3.5. Environmental factors (e.g. temperature, salinity, season, etc.)

Water sources with low salinity (below ~5 to ~10 ppt) seem to reduce the prevalence of the disease. Although AHPND can be found year round in South-East Asia, the hot and dry season from April to July seems to be the peak. Bad feed management, algal bloom or crash are also factors that may lead to AHPND in endemic areas.

### 2.4. Control and prevention

#### 2.4.1. Vaccination

Not applicable.

#### **2.4.2. Chemotherapy**

Not useful.

#### **2.4.3. Immunostimulation**

Not useful.

#### **2.4.4. Resistance breeding**

An AHPND line with some resistance to the disease has been developed in Mexico and in Ecuador. This was accomplished through mass selection over 10 years for growth and survival, rather than for SPF (specific-pathogen free) stock development (Lightner, unpublished data).

#### **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**

None known.

### **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 detection. It is assumed that adults (broodstock) can carry toxin-bearing strains (especially PirB<sup>VP</sup>) of *V. parahaemolyticus* (Han *et al.*, 2015; 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 testing, but only for testing for the presence of PirB<sup>VP</sup> toxin.

#### **3.2. Preservation of samples for submission**

Carefully selected shrimp samples can be submitted to a variety of laboratories for diagnosis of AHPND. The samples can be submitted in 90% ethanol for polymerase chain reaction (PCR) detection, or preserved in Davison's AFA fixative for routine histopathology (Joshi *et al.*, 2014a; 2014b; Leño & Mohan, 2013; Lee *et al.*, 2015; Nunan *et al.*, 2014; Sirikharin *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

#### **3.3. Pooling of samples**

For molecular testing, samples of shrimp cephalothoracies can be selected (pooled when less than 0.5 g).

Samples, especially PL or specimens up to 0.5 g can be pooled. Larger shrimp should not be pooled and should be processed individually (Lightner, unpublished data).

#### **3.4. Best organs or tissues**

Gut-associated tissues and organs, such as hepatopancreas, stomach, the midgut, the hindgut, and faeces of selected shrimp for samples. Valuable broodstock may be worth saving, and from these only faeces should be collected.

#### **3.5. Samples/tissues that are not appropriate (i.e. when it is never possible to detect)**

Samples other than gut-associated tissues and organs are not appropriate (FAO, 2013; NACA, 2012; 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. Gross signs

The clinical signs could be used for presumptive diagnosis, which can be further confirmed by histopathology observed at the animal level include a pale to white HP due to pigment loss in the connective tissue capsule, significant atrophy of the HP, soft shells and guts with discontinuous contents or no contents, black spots or streaks sometimes visible within the HP, soft HP which does not squash easily between the thumb and forefinger, and the onset of clinical signs and mortality starting as early as 10 days post-stocking (NACA, 2012; 2014).

#### 4.1.2. Behavioural changes

Not applicable.

### 4.2. Clinical methods

#### 4.2.1. Clinical chemistry

None are known.

#### 4.2.2. Microscopic pathology

An acute phase characterised by an acute, massive progressive degeneration of the HP tubules from medial to dorsal, 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; 2013b; 2014a; 2014b).

The terminal ~~phaseis~~ 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, 2013; NACA, 2012; 2014; Nunan *et al.*, 2014;

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Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

#### 4.2.3. Wet mounts

Not applicable.

#### 4.2.4. Smears

Not applicable.

#### 4.2.5. Fixed sections (for ISH):

*In-situ* hybridisation is a useful technique provided it is done properly. The result of an ISH test will be apparent as a Bismarck Brown stained material will remain. This will be used to distinguish ANHPD tissues from those tissues which are not affected.

#### 4.2.6. Electron microscopy/cytopathology

None reported to date (February 2015).

### 4.3. Agent detection and identification methods

#### 4.3.1. Direct detection methods

#### 4.3.1.1. Microscopic methods

See Section 2.2.2.

##### 4.3.1.1.1. Wet mounts

Not applicable.

##### 4.3.1.1.2. Smears

Not applicable.

##### 4.3.1.1.3. Fixed sections

Not applicable.

#### 4.3.1.2. Agent isolation and identification

On marine or blood agar, the strains of VP<sub>AHPND</sub> are capable of swarming (Han *et al.*, 2015). Hence, it is possible to isolate PirB<sup>VP</sup> toxin-producing forms of *V. parahaemolyticus* on standard media used for isolation of bacteria from diseased shrimp or other samples, especially because PirB<sup>VP</sup> produces a more potent toxin than PirA<sup>VP</sup> (Lee *et al.*, 2015; Soto-Rodriguez *et al.*, 2015). The identity of the *V. parahaemolyticus* may be confirmed by use of a PCR method to detect lecithin dependent haemolysin gene (Taniguchi *et al.*, 1985) and their probable ability to cause AHPND by PCR methods described in section 4.3.1.2.3. This must be followed by bioassay to confirm ability to cause AHPND.

##### 4.3.1.2.1. Cell culture/artificial media

No methods are available.

##### 4.3.1.2.2. Antibody-based antigen detection methods

None is available to date (February 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

Although the AP1 and AP2 methods are not recommended for detection of VP<sub>AHPND</sub>, they are included here because they may find utility in studying the environmental prevalence and distribution of bacterial isolates carrying pVA1 plasmid variants that lack the PirA<sup>VP</sup> and PirB<sup>VP</sup> genes, would not cause AHPND and would give negative results with the methods recommended here for VP<sub>AHPND</sub> detection.

Methods for detection of isolates of VP<sub>AHPND</sub> by PCR have been developed. The most successful methods target the unique genes for AHPND toxin PirA<sup>VP</sup> (12.7 kDa) and/or PirB<sup>VP</sup> (50.1 kDa) that together cause sloughing of shrimp hepatopancreatic cells. These toxin genes have been found as episomal elements in all AHPND isolates so far sequenced (Gomez-Gil *et al.*, 2014; Kondo *et al.*, 2014; Yang *et al.*, 2014) and GenBank Accession number KM067908. Two earlier, preliminary PCR detection methods (AP1 and AP2) that targeted the plasmid carrying the toxin genes (Yang *et al.*, 2014) were announced on the internet at the website of the Network of Aquaculture Centres in Asia-Pacific (NACA) in December 2013 (Flegel & Lo, 2014) but were later abandoned because of 3% false positive results from non-AHPND bacteria that carried the plasmid without the toxin genes.

To overcome the problem of false positive results, methods have been developed that target the AHPND toxin genes. The first such method (AP3) was announced in June 2014 and targeted the 12.7 kDa *pirA<sup>VP</sup>* gene (Sirikharin *et al.*, 2014). It was validated for 100% positive and negative predictive value by testing with 104 AHPND-causing and non-pathogenic bacteria (including other *Vibrio* and non-*Vibrio* species) that had previously been confirmed by bioassay (Kwai *et al.*, 2014; Sirikharin *et al.*, 2015). A subsequent publication using 9 AHPND-causing and 11 non-pathogenic

isolates of *V. parahaemolyticus* from Mexico (Soto-Rodriguez *et al.*, 2015) reported that the AP3 method gave the highest positive (90%) and negative (100%) predictive values of five PCR methods tested, including one commercial method.

The AP3 method and four other more recently published methods that target the AHPND *pirA<sup>VP</sup>* gene (the *Pir<sup>VP</sup>A* method and the VpPirA-284) and *pirB<sup>VP</sup>* (*Pir<sup>VP</sup>B* method and the VpPirB-392) are one-step PCR methods of relatively low sensitivity when used for detection of AHPND-causing bacteria at carrier levels or in environmental samples such as sediments and biofilms. For such samples, a preliminary enrichment step is recommended since experience has shown that these PCR methods are not sensitive enough to detect low numbers of bacterial cells at carrier levels and that adaptation to a nested PCR protocol was not successful due to the occurrence of non-specific amplicons.

An additional two-tube nested PCR method called AP4 has been devised and found to give 100% positive predictive value for AHPND-causing bacteria using the same 104 bacterial isolates used to validate AP3 above (announcement at [www.enaca.org](http://www.enaca.org) and manuscript in preparation). This method does not give rise to non-specific amplicons and has a minimum sensitivity for 1 fg of DNA extracted from AHPND-causing bacteria, allowing it to 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 cultures for detection of AHPND-causing bacteria at carrier levels or in environmental samples may be carried out in any suitable medium (e.g. tryptic–soy broth) or alkaline peptone water containing 2.5% NaCl supplement) incubated for 4 hours at around 30°C with shaking. After this, let any debris settle, remove the cloudy supernatant for centrifugation to pellet the bacteria it contains and discard the supernatant solution. Extract DNA from the bacterial pellet.

#### *4.3.1.2.3.1.2 Agent purification*

The causative agent of AHPND may be isolated in pure culture from diseased shrimp, carrier shrimp, or environmental samples using standard microbiological media [used](#) for isolation of *Vibrio* species from such sources (Lightner, 1996; Tran *et al.*, 2013a; 2013b). Isolation of pure cultures must be followed by PCR analysis and/or bioassays to confirm the ability to cause AHPND.

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AP1 (AHPND Primer set 1)

AP1F: 5'-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3'

AP1R: 5'-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3'

AP1 Amplicon sequence 700 bp (Lee *et al.*, 2015).

AP2 (AHPND Primer set 2)

AP2F: 5'-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3'

AP2R: 5'-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3'

AP2 Amplicon sequence 700 bp (Lee *et al.*, 2015).

#### *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 a 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 from an enrichment culture.

#### *4.3.1.2.3.1.4 PCR primers for one-step PCR detection of AHPND bacteria*

Five one-step PCR methods called AP3, Pir<sup>VP</sup>A, Pir<sup>VP</sup>B, VpPirA and VpPirB have been developed (see above) for detection of VP<sub>AHPND</sub>. The AP3, Pir<sup>VP</sup>A and VpPirA methods target the *pirA<sup>VP</sup>* gene while the Pir<sup>VP</sup>B and VpPirB methods target the *pirB<sup>VP</sup>* gene. These primers are listed in Table 4.1 together with the size of their expected amplicons.

**Table 4.1.** PCR primers for one-step PCR detection of VP<sub>AHPND</sub>

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'	<i>pirA<sup>VP</sup></i>	333	Sirikharin <i>et al.</i> , 2014 Sirikharin <i>et al.</i> , 2015
Pir <sup>VP</sup> A	Pir <sup>VP</sup> A F: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-G-3' Pir <sup>VP</sup> A R: 5'-TTA-GTG-GTA-ATA-GAT-TGT-ACA-G-3'	<i>pirA<sup>VP</sup></i>	336	Lee <i>et al.</i> , 2015
Pir <sup>VP</sup> B	Pir <sup>VP</sup> B F: 5'-GAG-CCA-GAT-ATT-GAA-AAC-ATT-TGG-3' Pir <sup>VP</sup> B R: 5'-CCA-CGC-AGC-GAG-TTC-TGT-AAT-GTA-3'	<i>pirB<sup>VP</sup></i>	438	Lee <i>et al.</i> , 2015,
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'	<i>pirA<sup>VP</sup></i>	284	KM067908 Han <i>et al.</i> , 2015
VpPirB-392	VpPirB-392F: 5'-TGA-TGA-AGT-GAT-GGG-TGC-TC-3' VpPirB-392R: 5'-TGT-AAG-CGC-CGT-TTA-ACT-CA-3'	<i>pirB<sup>VP</sup></i>	392	KM067908 Han <i>et al.</i> , 2015

Note that the primer sequences and amplicons for the two methods that target the *pirA<sup>VP</sup>* gene differ slightly.

#### 4.3.1.2.3.1.5 PCR primers for nested PCR for detection of AHPND bacteria

A two-tube, nested PCR method called AP4 has been devised and found to give 100% positive predictive value for AHPND-causing bacteria using the same 104 bacterial isolates used to validate AP3 above ([www.enaca.org](http://www.enaca.org)) (Sritunyaluksana *et al.*, 2015). Nonspecific amplicons do not arise from this method and it has a minimum sensitivity for 1 fg of DNA extracted from AHPND-causing bacteria, allowing it to be used directly with tissue and environmental samples that may have low levels of AHPND bacteria. The target sequence consists of a chimeric DNA fragment comprising the full *pirA<sup>VP</sup>* gene sequence plus the 12 bp linker plus the full succeeding *pirB<sup>VP</sup>* gene sequence for a total of 1269 bp. The first-step and second-step PCR primers are listed in Table 4.2 below. The primers were designed from the China (People's Rep. of) isolate of AHPND bacteria (Yang *et al.*, 2014). The expected amplicons 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 bands for amplicons may occur as the product of residual primer AP4-F1 working with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

**Table 4.2.** Primers for the AP4, two-step PCR method for detection of AHPND-causing bacteria

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	Sirikharin <i>et al.</i> , 2015
AP4 Step 2	AP4-F2: 5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3' AP4-R2: 5'-GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'	230	

Note that the AP4-F1 primer sequence is equal to that of AP3-F.

#### 4.3.1.2.3.1.6 Protocol for the AP3, 1-step PCR method

This protocol follows the method described by Sirikharin *et al.* 2014. The PCR reaction mixture consists of 10x PCR mix (Invitrogen<sup>1</sup>) 2.5 µl, 50 mM MgCl<sub>2</sub>, 0.7 µl, 10 mM dNTPs, 0.4 µl, 10 µM

<sup>1</sup> Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

AP3-F1, 0.5 µl, 10 µM AP3-R1, 0.5 µl, 0.3 µl of Taq DNA pol (5 units µl<sup>-1</sup>, Invitrogen) and 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 5 minutes 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 hold at 4°C.

#### *4.3.1.2.3.1.7 Protocol for the VpPirA-284 1-step PCR method*

Perform PCR with PuReTaq ready-to-go PCR beads (GE Healthcare). This consists of a 3-minute step at 94°C to denature DNA prior to the primers binding and activation of the Taq DNA polymerase, 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.

Prepare a 25 µl PCR reaction with a PuReTaq ready-to-go PCR bead. Each reaction contains 0.2 µM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2.5 U of Taq DNA polymerase, and 1 µl of extracted DNA.

#### *4.3.1.2.3.1.8 Protocol for the Pir<sup>VPB</sup> 1-step PCR method*

PCR consists of an initial preheating stage of 5 minutes at 94°C, followed by 25–30 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 60 seconds extension at 72°C, and a final 10 minutes extension at 72°C. The amplified PCR products are analysed in 2% agarose gels, stained with ethidium bromide, and visualised under ultraviolet transillumination. AHPND positive samples give a positive band at 336 bp and 400 bp with the PirA<sup>VP</sup> and PirB<sup>VP</sup> primer sets, respectively. No band is produced by non-AHPND samples (Lee *et al.*, 2015).

#### *4.3.1.2.3.1.9 Protocol for the VpPirB-392 1-step PCR method*

The protocol for this method is the same as that for the VpPirA-284 method above

#### *4.3.1.2.3.1.10 Protocol for the AP4 nested PCR method*

This protocol follows the method described by Sritnyalucksana *et al.*, 2015. The first PCR reaction mixture consists of 10× PCR mix (Invitrogen) 2.5 µl, 50 mM MgCl<sub>2</sub>, 1.5 µl, 10 mM dNTPs, 0.5 µl, 10 µM AP4-F1, 0.5 µl, 10 µM AP4-R1, 0.5 µl, 0.3 µl of Taq DNA pol (5 units µl<sup>-1</sup>, Invitrogen) and 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 10× PCR mix (Invitrogen) 2.5 µl, 50 mM MgCl<sub>2</sub>, 1.5 µl, 10 mM dNTPs, 0.5 µl, 10 µM AP4-F2, 0.375 µl, 10 µM AP4-R2, 0.375 µl, 0.3 µl of Taq DNA pol (5 units µl<sup>-1</sup>, Invitrogen) 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.

#### *4.3.1.2.3.1.11 Controls for all PCR methods*

The following controls should be included in all AHPND PCR assays: a) DNA template extracted from a known negative sample, such as specific-pathogen-free shrimp tissues; b) DNA template from a known positive sample, including AHPND-affected shrimp tissue, or DNA from a VP<sub>AHPND</sub> bacteria culture, or plasmid DNA that contains the target region of the specific set of primers; c) a none-template control, i.e. adding nuclease-free water as the template.

#### *4.3.1.2.3.1.12 Analysis of PCR products by agarose gel electrophoresis*

After PCR, load 5–10 µl of the PCR reaction mix onto a 1.5% agarose gel (containing 0.5 µg ml<sup>-1</sup> ethidium bromide). Look for the expected amplicons appropriate for the PCR method used (Tables 4.1 and 4.2).

### **4.3.2. Serological methods**

Not applicable.

## **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	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PL	Juveniles	Adults		
Gross signs	d	d	b	c	b	a
Bioassay	d	d	b	d	b	b
Direct LM	d	d	d	c	c	c
Histopathology	d	c	a	c	a	a
Transmission EM	d	d	d	d	d	a
PCR	d	b	a	a	a	a
Sequence	d	d	a	a	a	a

PL = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

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

Two years of freedom from AHPND is adequate to declare freedom from the acute hepatopancreatic necrosis disease (VP<sub>AHPND</sub>) (Leaño & Mohan, 2013; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

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

Histopathology indicative of AHPND

or

Detection of VP<sub>AHPND</sub>

or

Mortality associated with clinical signs of AHPND.

### 7.2. Definition of confirmed case

AHPND is considered to be confirmed if the following criteria are met:

Detection of VP<sub>AHPND</sub>

and

Histopathology indicative of AHPND

or

Mortality associated with clinical signs of AHPND

or

Positive results by bioassay.

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