Japan's Comments on

The Aquatic Commission Report of the March 2015 meeting

Japan would like to express its appreciation to the Aquatic Animal Health Standards Commission (AAHSC) for all the works they have done and for giving us the opportunity of offering comments on proposed revisions to the Aquatic Animal Health Codes texts.

1. Acute hepatopancreatic necrosis disease (new draft Chapter X.X.X.)

General comment

This manual is difficult to comprehend meanings of some articles and the whole manual should be reviewed. The following points should be addressed at least.

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_{AHPND}, 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~~ pVA1, 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).

(Text partly omitted)

Rationale

Two designations, "pVPA3-1" and "pVA1" are used to indicate the same plasmid.

2.2.5. Persistent infection with lifelong carriers

No data~~See Section 2.1.4 Life cycle~~.

Rationale
The description in 2.1.4. is “Not applicable.”
In addition, Article 2.1.4. is about the causative bacterium, and referring to 2.1.4. here seems irrelevant.

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_{AHPND} by the *per os* route if the coated feed contains 10^8 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 contains either ~~begins with~~ 10^8 CFU/ml ~~per gram~~ or more bacterium ~~of inoculum~~ (Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b).

Rationale
Rhetorical correction and clarification

2.4.4. Resistance breeding

A~~n AHPND~~ shrimp 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).

Rationale
What has some resistance to the disease is not pathogens but host species. The shrimp species should be specified.

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).

Rationale

Rhetorical correction

4.1.1. Gross signs

The clinical signs could be used for presumptive diagnosis. The gross clinical signs , 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 tThe onset of clinical signs and mortality may be observed starting as early as 10 days post-stocking (NACA, 2012; 2014).

Rationale

Rhetorical correction and clarification

4.2.2. Microscopic pathology

The An acute phase is characterised by an acute, massive and 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 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; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

Rationale
Rhetorical correction

4.2.5. Fixed sections (for ISH)~~:~~
In-situ hybridisation is a useful technique provided it is done properly. ~~The result of an~~ With ISH, the presence of the aetiological bacterium can be clearly observed against host tissues counterstained with an appropriate dye ~~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.~~

Rationale
Rhetorical correction and clarification

4.3.1.2. Agent isolation and identification

~~On marine or blood agar, the strains of VP_{AHPND} are capable of swarming (Han *et al.*, 2015). Hence, i~~It is possible to isolate $PirB^{vp}$ toxin-producing forms of *V. parahaemolyticus* on standard media used for isolation of bacteria from diseased shrimp or other samples, especially because $PirB^{vp}$ produces a more potent toxin than $PirA^{vp}$ (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.

Rationale
Why is the isolation of the VP_{AHPND} strains possible, if they are capable of swarming on agar plate? This article should be rewritten thoroughly so that readers can comprehend the meanings.

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

(Text partly omitted)

The AP3 method and five ~~four~~ other more recently published methods that target the AHPND *pirA*^{vp} gene (the Pir^{vp}*A* method ~~and,~~ the VpPirA-284 and TUMSAT-Vp3) and *pirB*^{vp} (Pir^{vp}*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 and manuscript in preparation). This method does not give rise to non-specific amplicons and has sensitivity to detect a minimum of ~~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.

Rationale

Rhetorical correction and clarification

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.

~~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).~~

Rationale

Although it seems that PCR with AP1 and AP2 primers may detect bacterial strains that do not cause AHPND (article 4.3.1.2.3.1.), this article is written as if the ability of the isolated bacterium to cause the disease could be confirmed with those primers.

4.3.1.2.3.1.4 PCR primers for one-step PCR detection of AHPNDbacteria

~~Five~~ Six one-step PCR methods called AP3, Pir^{vp}A, Pir^{vp}B, VpPirA ~~and,~~ VpPirB and TUMSAT-Vp3 have been developed (see above) for detection of VP_{AHPND}. The AP3, Pir^{vp}*A* ~~and,~~ VpPirA and TUMSAT-Vp3 methods target the *pir*A^{vp} gene while the Pir^{vp}*B* and VpPirB methods target the *pir*^{vp}*B* 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_{AHPND}

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	Sirikharin *et al.*, 2014 Sirikharin *et al.*, 2015
Pir^{vp}A	*Pir*^{vp}*A* F: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-G-3' *Pir*^{vp}*A* R: 5'-TTA-GTG-GTA-ATA-GAT-TGT-ACA-G-3'	*pir*A^{vp}	336	Lee *et al.*, 2015
Pir^{vp}*B*	*PirvpB* F: 5'-GAG-CCA-GAT-ATT-GAA-AAC-ATT-TGG-3' *PirvpB* R: 5'-CCA-CGC-AGC-GAG-TTC-TGT-AAT-GTA-3'	*pirB*^{vp}	438	Lee *et al.*, 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'	*pir*A^{vp}	284	KM067908 Han *et al.*, 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'	*pirB*^{vp}	392	KM067908 Han *et al.*, 2015
TUMSAT-Vp3	TUMSAT-Vp3F: 5'-GTG-TTG-CAT-AAT-TTT-GTGC-A-3' TUMSAT-Vp3R: 5'-TTG-TAC-AGA-AAC-CAC-GAC-TA-3'	*pirAvp*	360	Tinwongger *et al.*, 2014

Rationale

TUMSAT-Vp3 targets the AHPND *pir*A^{vp} gene and the specificity and sensitivity is as same as those of other five primers described in the table 4.1..

5. Rating of tests against purpose of use

Comment

Gross signs, histopathology, and transmission EM are rated as “a (the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity)” for confirmatory diagnosis. However, these methods are apparently inappropriate for the confirmatory diagnosis. PCR is also rated as “a” for confirmatory diagnosis, but some PCRs described in this manual are not suitable for confirmatory diagnosis. Therefore, PCRs that are recommended for confirmatory diagnosis should be specified. (e.g. as a footnote of the table)

2. Other comments

A comment on the chapter 2.2.6. “White spot disease”, article 2.2.7. “Known or suspected wild aquatic animal carriers”

The word “molluscs” should be removed from the following sentence: “Other marine molluscs, polychaete worms (Vijayan et al., 2005), as well as ….. can mechanically carry the virus without evidence of infection (Lo & Kou, 1998).”

This is because no such evidence supporting the presence of molluscan vectors has actually been obtained even in the two papers referred to in this sentence. Vijayan et al. (2005) only reports polychaete worms as vectors for the virus, and Lo & Kou (1998) describes crustaceans and some arthropods as reservoirs, but none of the authors mentions molluscs.