

Japan's Comments on

The Aquatic Commission Report of the September 2016 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 and Manual of Diagnostic Tests for Aquatic Animals.

1. (Annex 23) INFECTION WITH INFECTIOUS MYONECROSIS VIRUS (Chapter 2.2.4. on Aquatic manual)

4.3.1.2.3. Molecular techniques

RT-PCR reaction mixture (SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Life Technologies Thermo Fisher Scientific):^{*1}

Reagent	Volume	Final concentration
<u>dH₂O</u> ^{*2}	<u>5.5 µl</u> ^{*2}	<u>—</u> ^{*2}
2× reaction mix	12.5 µl	1 ×
Forward/reverse primer (10 <u>mM</u> <u>µM</u> ^{*3} each)	1.0 µl	0.4 <u>µM</u> <u>µM</u> ^{*3}
RT/Taq enzyme mix	1.0 µl	
RNA template ¹	<u>5.0 1.0-10.5</u> µl ^{*5}	<u>1-50 ng 20 ng/µl</u> ^{*5}
<u>dH₂O</u> ^{*2}	<u>0-9.5</u> µl ^{*4}	

²dH₂O should be added so that the final volume of the reaction mixture will be 25 µl.^{*4}

RT-PCR thermal cycling conditions:

PCR Primers	Temperature (°C)	Time	No. cycles	Amplicon length
4587F/4914R	<u>60 55</u> ^{*6} , 95	30 minutes, 2 minutes	1	328 bp
	95, <u>62 60, 68</u> ^{*6}	<u>45 15</u> ^{*6} seconds, <u>45 30</u> ^{*6} seconds, <u>45 seconds</u> ^{*6}	39	
	<u>60 68</u> ^{*6}	<u>7 2</u> minutes ^{*6}	1	

Rationale

1. Revision of the RT-PCR reaction mixture:

*1: SuperScript III is manufactured by Thermo Fisher Scientific under the brand name of Invitrogen.

*2: Moved dH₂O to the last row of the table because dH₂O is used for only adjusting the volume of the reaction mixture.

*3: The concentration of primers specified in the table (10 mM) is apparently too high and this must be a typo. When 1.0 µl of a primer of 10 µM is diluted to 25 µl, the final concentration is 0.4 µM.

*4: The volume of dH₂O should be adjusted according to the volume of RNA template so that the final volume of the mixture would be 25 µl.

*5: In the newly suggested table, 500 ng (the maximal amount recommended by the manufacturer's protocol) of RNA template dissolved in 1.0-10.5 µl of solution is recommended, which translates into 20 ng/µl in the final reaction mixture. Good results are obtained with this recipe (Inada et al., 2016).

2. Revision of the RT-PCR thermal cycling conditions:

*6: Although SuperScript III is recommended in the draft, the RT-PCR program remains almost the same as that in the present manual. However, the sensitivity of the detection of IMN is much lower with the present RT-PCR program in the manual than that with the 3-step program suggested here, when SuperScript III is used (Inada et al., 2016). The newly suggested RT-PCR program is mostly in accordance with that recommended by the manufacturer of SuperScript III.

Reverse transcription temperature: Revised from "60°C" to "55°C".

Temperatures of PCR: Revised from "95°C, 62°C" to "95°C, 60°C, 68°C".

Time intervals for PCR: Revised from "45 seconds, 45 seconds" to "15 seconds, 30 seconds, 45 seconds".

Temperature of final extension: Revised from "60°C" to "68°C".

Number of seconds for final extension: Revised from "7 minutes" to "2 minutes".

Reference:

M. Inada et al., (2016) Modification of PCR Program for Detection of Infectious Myonecrosis Virus. *Fish Pathology*, 51(4), 1-3.

2. (Annex 20) Acute hepatopancreatic necrosis disease (Chapter 2.2.X. on Aquatic manual)

Aquatic Manual Chapter 2.2.X

2.2.2.Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the Aquatic Code include: fleshy prawn (*Penaeus chinensis*) and kuruma shrimp (*Marsupenaeus japonicus*).

Rationale

There is a new study which showed that kuruma shrimp (*Marsupenaeus japonicus*) was infected with AHPND in an immersion test (Tinwongger et al., 2016). The results of this experiment fulfil the criteria for the “Species with incomplete evidence for susceptibility” mentioned in “Criteria for listing species as susceptible to infection with a specific pathogen” in Chapter 1.5 of Aquatic Code. Therefore, we have designated *M. japonicus* “Species with incomplete evidence for susceptibility”.

The criteria described in Chapter 1.5 of Aquatic Code and the corresponding results of the experiment are as follows.

1) Transmission has been obtained naturally or by experimental procedures that mimic natural pathways for the infection in accordance with article 1.5.4.

The result of the experiment: In the experiment, the mortality of *M. japonicus* due to infection with AHPND was observed by the immersion test with a bacterial concentration of 1×10^5 CFU ml⁻¹ and 1×10^6 CFU ml⁻¹ in the tank containing 10 litres of artificial seawater 30 ppt at 28 °C with aeration. 1×10^5 CFU ml⁻¹ is not unnaturally high loads of pathogens. Additionally, 10 litres of artificial seawater 30 ppt at 28 °C is suit to *M. japonicas*. It follows that the result of experiment fulfils 1).

2) The identity of the pathogenic agent has been confirmed in accordance with Article 1.5.5.

The result of the experiment: In the experiment, *V. parahaemolyticus* AHPND strain D6 was used and the pathogenic agent had been already identified. It follows that the result of experiment fulfils 2).

3) There is evidence of infection with the pathogenic agent in the suspect host species in accordance with criteria A to D in Article 1.5.6. Evidence to support criterion A alone is sufficient to determine infection. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D would be required to determine infection.

A. The pathogenic agent is multiplying in the host, or developing stages of the pathogenic agent are present in or on the host;

B. Viable pathogenic agent is isolated from the proposed susceptible species, or infectivity is demonstrated by way of transmission to naïve individuals;

C. Clinical or pathological changes are associated with the infection;

D. The specific location of the pathogen corresponds with the expected target tissues.

The result of the experiment: In the experiment, the observed mortality of *M. japonicus* fulfils C. But A, B, D are not mentioned.

For the above mentioned reasons, the experiment fulfils criteria 1) and 2). Therefore, we designate *M. japonicus* to be a “Species with incomplete evidence for susceptibility”. We will submit comments once we have collected scientific evidence sufficient to fulfil all criteria.

Reference

Tinwongger S, Nochiri Y, Thawonsuwan J, Nozaki R, Kondo H, Awasthi SP, Hinenoya A, Yamasaki S, Hirono I. (2016) Virulence of Acute Hepatopancreatic Necrosis Disease PirAB-like Relies on Secreted Proteins Not on Gene Copy Number. J Appl Microbiol. doi: 10.1111/jam.13256.