Report of the Meeting of WOAH Aquatic Animal Health Standards Commission

Original: English (EN)

14 to 21 September 2022
Hybrid

Introduction and Member contribution

The WOAH Aquatic Animal Health Standards Commission (hereinafter "the Aquatic Animals Commission") wished to thank the following Members for providing written comments on draft texts for the WOAH *Aquatic Animal Health Code* (Hereinafter "the *Aquatic Code*") and WOAH *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter "the *Aquatic Manual*") circulated in the Commission's February 2022 report: Australia, Canada, China (People's Republic of), Chinese Taipei, Japan, Norway, Switzerland, Thailand, the United States of America (USA), and the Member States of the European Union (EU). The Commission also wished to acknowledge the valuable advice and contributions from numerous experts of the WOAH scientific network.

The Aquatic Animals Commission reviewed all comments that were submitted on time and were supported by a rationale. Due to the large number of comments, the Commission was not able to provide a detailed explanation of the reasons for accepting or not each of the comments considered, and focused its explanations on significant issues. Where amendments were of an editorial nature, no explanatory text has been provided. The Commission wished to note that not all texts proposed by Members to improve clarity were accepted; in these cases, it considered the text clear as currently written. The Commission made amendments to draft texts in the usual manner by 'double underline' and 'strikethrough'. In relevant Annexes, amendments proposed at this meeting are highlighted in yellow to distinguish them from those made previously.

To note

The Aquatic Animals Commission informed Members that *ad hoc* Group reports would no longer be annexed to its report. Instead a hyperlink will be provided for *ad hoc* Group reports that will take the reader to the dedicated webpages on the WOAH website for all *ad hoc* Group reports:

https://www.woah.org/en/what-we-do/standards/standards-setting-process/ad-hoc-groups/

Status of annexes

Texts in Annexes 1 to 9 and 11 to 30 are presented for comments.

How to submit comments

The Aquatic Animals Commission strongly encourages Members and International Organisation's with a Cooperative Agreement with WOAH to participate in the development of WOAH International Standards by submitting comments on relevant annexes of this report.

Comments should be submitted as Word files rather than pdf files because pdf files are difficult to incorporate into the Commission's working documents.

Comments should be presented in the relevant annex, and include any amendments to the proposed text, supported by a structured rationale or by published scientific references. Proposed deletions should be indicated in '~~strikethrough~~' and proposed additions with 'double underline'. Members should not use the automatic 'track-changes' function provided by word processing software, as such changes may be lost in the process of collating submissions into working documents.

Deadline for comments

Comments on relevant texts in this report must reach the Headquarters by 6 January 2023 to be considered at the February 2023 meeting of the Aquatic Animals Commission.

Where to send comments

All comments should be sent to the Standards Department at: AAC.Secretariat@woah.org

Date of the next meeting

The Commission noted the dates for its next meeting: 15 to 22 February 2023.

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Table of Contents

1. Welcome 5

1.1. WOAH Deputy Director General, International Standards and Science 5

1.2. WOAH Director General 5

2. Adoption of the agenda 5

3. Cooperation with the Terrestrial Animal Health Standards Commission 5

4. Work plan and priorities 6

The WOAH Aquatic Animal Health Code 6

5. Items for Member comments 6

5.1. Chapter 1.3. Diseases listed by the OIE – Listing of infection with Megalocytivirus 7

5.2. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis) 7

5.3. Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus 8

5.4. Article 9.5.2 of Chapter 9.5. Infection with infectious myonecrosis virus 8

5.5. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus 8

5.6. New Chapter 10.X. Infection with tilapia lake virus 9

5.7. Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa* and Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae* 9

5.8. Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens* 10

5.9. Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters 11

5.10. Member questionnaire for revision of Chapter 4.3. Application of Compartmentalisation 12

6. Items for Member information 12

6.1. Glossary definitions ‘Competent Authority’, ‘Veterinary Authority’ and ‘Aquatic Animal Health Services’ 12

6.2. Emerging diseases 12

6.2.1. Infection with carp edema virus (CEV) 12

6.2.2. Infection with covert mortality nodavirus (CMNV) 13

6.3. E-Certification 14

The WOAH Manual of Diagnostic Tests for Aquatic Animals 14

7. Items for Member comments 14

7.1. Section 2.2. Disease of crustaceans 15

7.1.1. Chapter 2.2.0. General information (diseases of crustaceans) 15

7.1.2. Chapter 2.2.1. Acute hepatopancreatic necrosis disease 15

7.1.3. Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague) 17

7.1.4. Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis) 17

7.1.5. Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus 18

7.1.6. Chapter 2.2.5. Infection with infectious myonecrosis virus 19

7.1.7. Chapter 2.2.7. Infection with taura syndrome virus 20

7.1.8. Chapter 2.2.8. Infection with white spot syndrome virus 20

7.2. Section 2.3. Diseases of fish 21

7.2.1. Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome) 21

7.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus 22

7.2.3. Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus 23

7.3. Section 2.4. 'Diseases of molluscs' 23
7.3.1. Section 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa* and Section 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae* 23
7.3.2. Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens* 23
8. Items for Member information 23
8.1. Proposed table of PCR parameters to harmonise PCR protocols 23
8.2. Chapter 2.3.7. Infection with red sea bream iridoviral disease 24
8.3. Develop a mechanism to speed up the process of making updates to diagnostic methods in the *Aquatic Manual* available to Members 25
9. *Ad hoc* Groups 25
9.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases 25
9.2. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases 25
9.3. *Ad hoc* group on new draft chapters on emergency disease preparedness and disease outbreak management 25
10. WOAH Reference Centres or Change of experts 26
10.1. Evaluations of applications for WOAH Reference Centres for aquatic animal health issues or change of experts 26
10.2. Call for applications for candidates for WOAH Reference Laboratory status 26
11. Any other business 26
11.1. Registration of diagnostic test kits 26

List of Annexes

Annex 1. Item 2 – Adopted Agenda 27
Annex 2. Item 2 – List of Participants 29
Annex 3. Item 4. – Work plan and priorities 30
Annex 5. Item 5.1. – Chapter 1.3. Diseases listed by the OIE – Listing of infection with Megalocytivirus 36
Annex 6. Item 5.3. – Article 9.3.1. of Chapter 9.3. Infection with NHP 44
Annex 7. Item 5.4. – Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with IHHNV 45
Annex 8. Item 5.5. – Article 9.5.2. of Chapter 9.5. Infection with IMNV 46
Annex 9. Item 5.6. – Article 10.9.2. of Chapter 10.9. Infection with SVCV 47
Annex 10. Item 5.6. – Article 10.9.2. of Chapter 10.9. Infection with SVCV (for information) 48
Annex 11. Item 5.7. – Chapter 10.X. Infection with TiLV 51
Annex 12. Item 5.8. – Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa* 58
Annex 13. Item 5.8. – Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae* 59
Annex 14. Item 5.9. – Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens* 60
Annex 15. Item 5.10. – Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters 61
Annex 16. Item 5.11. – Member questionnaire for revision of Chapter 4.3. Application of Compartmentalisation 65
Annex 17. Item 7.1.1. – Chapter 2.2.0. General information (diseases of crustaceans) 66
Annex 18. Item 7.1.2. – Chapter 2.2.1. AHPND 74
Annex 19. Item 7.1.3. – Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague) 92
Annex 20. Item 7.1.4. – Chapter 2.2.3. Infection with NHP 108

Annex 21. Item 7.1.5. – Chapter 2.2.4. Infection with IHHNV 123
Annex 22. Item 7.1.6. – Chapter 2.2.5. Infection with IMNV........ 142
Annex 23. Item 7.1.7. – Chapter 2.2.7. Infection with TSV........ 155
Annex 24. Item 7.1.8. – Chapter 2.2.8. Infection with WSSV 171
Annex 25. Item 7.2.1. – Chapter 2.3.1. Infection with EUS 188
Annex 26. Item 7.2.2. – Chapter 2.3.2. Infection with EHNV........ 208
Annex 27. Item 7.2.3. – Section 2.2.1. of Chapter 2.3.9. Infection with SVCV........ 228
Annex 28. Item 7.3.1. – Sections 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*........ 229
Annex 29. Item 7.3.1. – Sections 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae* 230
Annex 30. Item 7.3.2. – Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens* 231

1. Welcome

1.1. WOAH Deputy Director General, International Standards and Science

Dr Montserrat Arroyo, the WOAH Deputy Director General, International Standards and Science, welcomed members of the Aquatic Animals Commission and thanked them for their ongoing contributions to the work of WOAH. Dr Arroyo commended the Commission for its ambitious agenda and extended her appreciation to the members' employing institutions and national governments.

Dr Arroyo briefed the Commission on the intent to host the 90th General Session as a physical meeting with a focus on reconnecting after the previous virtual and hybrid General Sessions. She encouraged Commission members to present highlights of its September 2022 report in regional webinars as this was proving to be an excellent mechanism to strengthen engagement of Members. She also informed the Commission that the new WOAH acronym will be introduced progressively in the *Aquatic Code* and *Aquatic Manual*. Dr Arroyo provided a summary of ongoing WOAH initiatives for digitalisation, including the development and planning for new digital tools. Dr Arroyo updated the Commission on the new WOAH Research Coordination Network. Dr Arroyo gave a brief update on the implementation of the WOAH Aquatic Animal Health Strategy while acknowledging that the Commission would receive additional presentations on Strategy activities during its meeting. The members of the Aquatic Animals Commission thanked Dr Arroyo for the excellent support provided by the WOAH Secretariat.

1.2. WOAH Director General

Dr Monique Eloit, the WOAH Director General, met the Aquatic Animals Commission on 21 September and thanked its members for their support and commitment to achieving WOAH objectives. Dr Eloit informed the Commission that implementation of the WOAH Aquatic Animal Health Strategy has resulted in positive initiatives but noted that there needs to be a balance between activities supporting Objective 1. Standards, and the other three Objectives to ensure improvement of aquatic animal health and welfare in all areas. She informed the Commission that supporting and strengthening regional activities and capacity building will be increasingly important moving forward. Dr Eloit updated the Commission on the review of the WOAH Science System currently underway and emphasised that the science system needs to align with current best practices and be an agile, responsive system. Dr Eloit discussed WOAH's role in the prevention of disease, specifically in the context of wildlife and explained some of WOAHs work under the Wildlife Health Framework. The Aquatic Animals Commission thanked Dr Eloit for these updates.

2. Adoption of the agenda

The draft agenda was adopted by the Commission. The agenda and the list of participants are attached as Annexes 1 and 2 respectively.

3. Cooperation with Terrestrial Animal Health Standards Commission

The Bureaus (i.e. the President and the two Vice-Presidents) of the Terrestrial Animal Health Standards Commission and the Aquatic Animals Commission held a short meeting on 19 September 2022, chaired by WOAH Deputy Director General, International Standards and Science. The purpose of the meeting was to share information and ensure a harmonised approach for revisions of horizontal chapters, as relevant. Both Commissions committed to continue to convene Bureau meetings at least annually to ensure enhanced coordination. The Bureaus discussed issues of mutual interest in the *Aquatic Code* and the *Terrestrial Code* notably:

- The approach taken by both Commissions in the development of their respective work programmes and criteria for prioritisation of items;

- The approach that will be taken to review the use of Glossary definitions (Competent Authority, Veterinary Authority, Veterinary Services and Aquatic Animal Health Services) in the Codes, as a consequence of the adoption of revised definitions in May 2022. The Bureaus agreed to coordinate the review of the usage to ensure a harmonised approach and circulate for comments in February 2023 (See item 6.1.);

- Proposed new work on electronic certification. The Bureaus agreed to add the revision of Chapter 5.2. of the *Aquatic Code* and *Terrestrial Code* to their respective work programmes (See item 6.3.);

- Progress on each Commission's respective work to revise Section 4. The Bureaus agreed to keep each other informed of relevant work;

- Work to revise Chapters 5.4. to 5.7. in the *Terrestrial Code*. The Code Commission Bureau agreed to share the Terms of Reference (ToR) with the Aquatic Animals Commission and to continue to share working documents as developed;

- Revision of Chapter 6.10. Responsible and Prudent Use of Antimicrobial Agents in Veterinary Medicine, in the *Terrestrial Code*. The Code Commission Bureau agreed to share the AMR working group report, draft chapter and to continue to share working documents as comments are received.

4. Work plan and priorities

Comments were received from Australia, Canada, Norway and the EU.

The Commission reviewed comments received.

The Commission agreed with a comment that mechanisms to promote the generation of data for inactivation time/temperatures for safe commodities needs to be promoted amongst Members. The Commission highlighted two WOAH mechanisms that will help to identify research needs and encourage research, the new WOAH Research Coordination Program and a new Reference Laboratory network that are being developed through the Aquatic Animal Health Strategy as means to encourage research in this area. The Commission also amended the annual report template for Reference Centres to request identification of research gaps and will provide this information to the WOAH Research Coordination Program. This amendment will assist in informing these two new processes. The Commission noted that a consultancy has been established to update the Safe Commodity Assessments, which had been published in 2016. The outcome of the consultancy will be considered at the Commission's February 2023 meeting and used to update the relevant Articles X.X.3. of the *Aquatic Code*.

The Commission agreed with a comment that any proposed amendments of Chapter 4.2. Zoning and Compartmentalisation, is interlinked with the amendments of Chapter 4.3. Application of Compartmentalisation. The Commission wished to remind Members that Chapter 4.2. will be amended to only address zoning and that information currently in the chapter on compartmentalisation will be addressed following the adoption of amendments to Chapter 4.3. Application of Compartmentalisation. The Commission's plan for updating Section 4 is continuing and will be dependent on available resources.

The Commission wished to thank a Member for their offer of support for the development of a new chapter for trade in genetic material and noted that this would be discussed further when planning the new chapter.

The Commission discussed plans for the development of Chapter 5.X. Trade of ornamental aquatic animals, Chapter 5.Y. Trade of genetic materials and amendment of Chapter 4.3. Application of Compartmentalisation. The Commission added these chapters to its workplan, discussed a project plan for each chapter to establish how each chapter would be progressed, the milestones for completion, and timing for circulation for comments.

The Commission wished to thank a Member for providing new scientific evidence for a susceptible species for infection with spring viraemia of carp virus (See item 5.5.). The Commission noted that for all previously evaluated diseases, as new scientific evidence becomes available, assessments of new or reassessments of existing susceptible species will need to be undertaken, and would be added to its work plan. The Commission encouraged Members to provide any new scientific evidence on susceptibility to the Commission for assessment.

The Commission discussed with the WOAH Status Department the process for self-declaration of freedom of disease and amending the current procedure for the publication of a self-declaration of animal health status in order to ensure alignment with the newly adopted Chapter 1.4. Aquatic animal disease surveillance. The Commission agreed to add an item to its workplan to develop additional guidance in the form of a self-declaration template in order to support Members in their submission of self-declarations.

The Commission reviewed the status of ongoing items on its work plan and agreed on the anticipated milestones for their completion.

The Commission reviewed the prioritisation of new work items, taking into account a number of criteria including expected improvement to the standards and its impact, the benefit to Members, Member comments, relevance to activities of the WOAH Aquatic Animal Health Strategy, WOAH Headquarters' comments, and progress of ongoing workplan items.

The Commission noted that the progression of work plan items that were contingent on the convening of *ad hoc* Groups were anticipated to progress as planned for 2022. The list of current and planned *ad hoc* Groups for 2022 are available on the WOAH website.

The updated work plan is attached as Annex 3 for Member comments.

The WOAH Aquatic Animal Health Code

5. Items for Member comments

5.1. Chapter 1.3. Diseases listed by the OIE – Listing of infection with Megalocytivirus

Background

At its February 2022 meeting, the Aquatic Animals Commission informed Members that other viruses in the Genus *Megalocytivirus* may also cause disease in fish however, they are not within the scope of Chapter 2.3.7. infection with red sea bream iridovirus (RSIV) of the *Aquatic Manual*. The Commission noted that if ISKNV, TRBIV or other megalocytiviruses were to be listed, the viruses would need to be assessed against the listing criteria in Chapter 1.2. of the *Aquatic Code*. If they were found to fulfil the listing criteria, they could be proposed for listing to the WOAH Assembly.

The *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases met in April 2022 to continue its work to apply the criteria in Chapter 1.5. Criteria for listing species as susceptible to infection with a specific pathogen. At this meeting, the *ad hoc* Group conducted preliminary assessments for susceptibility of fish species to Infection with red sea bream iridovirus and presented an interim report to the Commission for consideration.

Previous Commission reports where this item was discussed:

February 2022 (Part B, Item 3.1.2.3, page 13).

September 2022

The Commission reviewed the report of the *ad hoc* Group on susceptibility of fish species to infection with OIE listed diseases for infection with red sea bream iridovirus (RSIV). The Commission also noted that distinguishing susceptible species for infection with RSIV requires nucleic acid sequence and/or phylogenetic tree analyses to determine whether the pathogenic agent is RSIV or another genogroup. Therefore, evidence is sometimes lacking for the *ad hoc* Group to identify susceptible species to the level of genotype. As a result, the *ad hoc* Group completed preliminary assessments for susceptibility of fish species to infection with red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) and in its interim report recommended the Commission to consider listing the virus at the level of *Megalocytivirus*, including ISKNV, RSIV and TRBIV (but not the more distantly related scale drop disease virus, SDDV).

The Commission agreed that the complexity associated with the different genogroups of *Megalocytivirus* warranted an assessment against the criteria in Article 1.2.2. of Chapter 1.2. Criteria for listing aquatic animal diseases. The Commission agreed to assess the virus species infectious spleen and kidney necrosis virus (ISKNV), including its three genogroups RSIV, ISKNV and TRBIV. The Commission agreed that the RSIV genogroup (currently listed in the *Aquatic Code),* as well as the two genogroups ISKNV and TRBIV meet the listing criteria 1, 2, 3, and 4b.

The Commission noted that the three genogroups (RSIV, ISKNV, and TRBIV) have overlapping susceptible species, similar epidemiology, and similar diagnostic methods. As a result, the Commission agreed that the proposed listed disease should be named “infection with infectious spleen and kidney necrosis virus (ISKNV)”. Infection with ISKNV would be defined to include the three genogroups of the species ISKNV (i.e. ISKNV, RSIV and TRBIV) but would exclude the other recognized species of *Megalocytivirus*, SDDV.

The assessment of infection with infectious spleen and kidney necrosis virus for listing in the WOAH *Aquatic Code* is presented as Annex 5 for comments.

The revised Article 1.3.1. of Chapter 1.3. Diseases listed by the OIE is presented as Annex 4 for comments.

5.2. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)

Comments were received from China, Norway, Switzerland and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission agreed to amend Article 9.3.1. to ensure consistency with Chapter 1.3. Diseases listed by the OIE.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 2.1.2., page 6).

September 2022 meeting

The Commission agreed with a comment to modify the taxonomic description in Article 9.3.1. and in accordance with the convention used in the *Aquatic Code* and *Aquatic Manual*, added the level of Family and amended the Order to 'Rickettsiales' to reflect the correct taxonomic relationships. The Commission also amended Section 1. of Chapter 2.2.3. of the *Aquatic Manual*, to ensure alignment (see Item 7.1.4.).

The revised Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis), is presented as Annex 6 for comments.

5.3. Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus

Comments were received from Norway, Switzerland and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission agreed to amend Article 9.4.1. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) to reflect an update in the taxonomic classification of IHHNV and to ensure consistency with other disease-specific chapters.

In Article 9.4.2., the Commission agreed to amend the list of susceptible species in line with the convention used in Article X.X.2. of the *Aquatic Code*, i.e. to list susceptible species alphabetically according to the common name. The Commission also amended Section 2.2.2. of Chapter 2.2.4. of the *Aquatic Manual* to ensure alignment.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 2.1.3., page 6).

September 2022 meeting

The Commission reviewed comments received and did not propose any additional amendments noting that Members were supportive of the proposed changes.

The revised Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus, is presented as Annex 7 for comments.

5.4. Article 9.5.2. of Chapter 9.5. Infection with infectious myonecrosis virus

Background

In an on-going effort to ensure alignment between the *Aquatic Code* and *Aquatic Manual*, as the new template for the disease-specific chapters of the *Aquatic Manual* is progressively applied, the relevant articles of the disease-specific chapters of the *Aquatic Code* are also updated if necessary.

September 2022 meeting

In Article 9.5.2., the Commission agreed to amend the list of susceptible species in line with the convention used in Article X.X.2. of the *Aquatic Code*, i.e. to list susceptible species alphabetically according to the common name. The Commission also amended Section 2.2.2. of Chapter 2.2.5. Infection with infectious myonecrosis virus of the *Aquatic Manual* to ensure alignment (see Item 7.1.6.).

The revised Articles 9.5.2. of Chapter 9.5. Infection with infectious myonecrosis virus, is presented as Annex 8 for comments.

5.5. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus

Background

At its February 2022 meeting, the Aquatic Animals Commission agreed that as new scientific evidence becomes available on susceptibility of aquatic animal species to OIE listed diseases, assessments of new or reassessments of existing susceptible species will need to be undertaken. The Commission added an on-going item in its workplan to address this need. The Commission has encouraged Members to provide any new scientific evidence on susceptibility to the Commission for assessment.

September 2022 meeting

In response to a member providing scientific evidence for susceptibility of a new species, the Commission requested the *ad hoc* Group on Susceptibility of fish species to OIE listed diseases assess the susceptibility of Jinsha bass carp (*Percocypris pingi)* to infection with spring viraemia of carp virus.

The *ad hoc* Group applied the criteria outlined in their November 2017 report for the susceptibility of fish species to infection with spring viremia of carp virus for the assessment of Jinsha bass carp (*Percocypris pingi)*.

The Commission considered the assessment of the *ad hoc* Group and agreed to include Jinsha bass carp (*Percocypris pingi)* in the list of susceptible species in Article 10.9.2.

The *ad hoc* Group's assessment of Jinsha bass carp (*Percocypris pingi)* is presented as Annex 10 for information.

The revised Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus, is presented as Annex 9 for comments.

5.6. New Chapter 10.X. Infection with tilapia lake virus

Background

Following the adoption of 'infection with tilapia lake virus' (TiLV) in Article 1.3.1. of Chapter 1.3. Diseases listed by the OIE, in May 2022, the Aquatic Animals Commission agreed to develop a new draft chapter for infection with TiLV based on the article structure of other disease-specific chapters in Section 10.

September 2022 meeting

The Commission reviewed the draft Chapter 10.X. Infection with TiLV, developed by a Commission member.

The Commission wished to remind Members that '(under study)' is used in the draft Chapter 10.X. Infection with TiLV and as explained in point B.2. of the *Aquatic Code* User's Guide means that 'this part of the text has not been adopted by the World Assembly of WOAH Delegates and the particular provisions are thus not part of the *Aquatic Code"*.

The Commission agreed that the susceptible species in Article 10.X.2. would be placed 'under study' pending assessment against Chapter 1.5. Criteria for listing species as susceptible to infection with a specific pathogen. The Commission also agreed to place the aquatic animal products listed in points 1-2. of Articles 10.X.3. and point 1a. of Article 10.X.14. 'under study' pending assessments against Chapter 5.4. Criteria to assess the safety of aquatic animal commodities. The Commission agreed that the physical and chemical processes that are applied in the production of fish oil and fish skin leather would be sufficient to inactivate the presence of any TiLV and therefore meet the criteria in point 2. of Article 5.4.1. The Commission agreed to include these aquatic animal products in Article 10.X.3. and not be placed 'under study'.

The Commission agreed that the default periods for basic biosecurity conditions and targeted surveillance presented in Chapter 1.4. Aquatic Animal Disease Surveillance, be applied for infection with TiLV until an assessment of the default periods is completed. The Commission noted that it has requested that expert advice be sought on an assessment of these default periods for all listed diseases, including infection with TiLV. When this advice has been reviewed by the Commission, changes may be proposed to the disease-specific chapters, where applicable.

The new draft Chapter 10.X. Infection with TiLV, is presented as Annex 11 for comments.

5.7. Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa* and Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*

Background

In the *ad hoc* Group's June 2022 report on Susceptibility of mollusc species to infection with OIE listed diseases for infection with *Bonamia exitiosa* and infection with *Bonamia ostreae*, it was recognised that *Magallana gigas* is the accepted name for the Pacific cupped oyster by the World Registry of Marine Species (WoRMS). The *ad hoc* Group had previously maintained the name as *Crassostrea gigas* as the evidence was not considered sufficiently robust to support this proposed taxonomic change. At the *ad hoc* Group's May-June 2022 meeting to assess the susceptible species for infection with *Marteilia refringens*, new data and peer reviewed publications on the new name of *Magallana gigas* were considered. The *ad hoc* Group recommended a change to the scientific name of the Pacific cupped oyster for consideration by the Aquatic Animals Commission.

The *ad hoc* Group's June 2022 report is available on the WOAH Website.

September 2022 meeting

The Aquatic Animals Commission reviewed the report of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases for infection with *Marteilia refringens* and noted that the *ad hoc* Group's recommendations for the new nomenclature for the Pacific cupped oyster had implications for Articles 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa* and Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae.*

The Commission agreed to amend the scientific name for the Suminoe oyster to '*Magallana* (Syn. *Crassostrea*) *ariakensis*' and the Pacific cupped oyster to '*Magallana* (Syn. *Crassostrea*) *gigas*' where they are used in the disease-specific chapters of the *Aquatic Code* and *Aquatic Manual.* The Commission agreed to make these amendments progressively and to amend Articles 11.2.2. and 11.3.2. as the susceptible species have been assessed by the *ad hoc* Group. Amendments for other Articles 11.X.2. will be amended as the *ad hoc* Group progresses with their assessments of the remaining mollusc diseases. For the associated changes to Sections 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa* and Chapter 2.4.3. Infection with *Bonamia ostreae* of the *Aquatic Manual* (see item 7.3.1.).

The amended Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*, is presented as Annex 12 for comments.

The amended Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*, is presented as Annex 13 for comments.

5.8. Article 11.4.1. and Article 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*

Background

The *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases met in November-December 2021 and May-June 2022 to continue its work to apply the criteria in Chapter 1.5. Criteria for listing species as susceptible to infection with a specific pathogen. At these meetings the *ad hoc* Group conducted the assessments for susceptibility of mollusc species to Infection with *Marteilia refringens*.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 4.1., page 17).

September 2022 meeting

The Aquatic Animals Commission considered the *ad hoc* Group report on Susceptibility of mollusc species to infection with OIE listed diseases and commended its members for their comprehensive work.

The Commission amended Article 11.4.1. to ensure consistency with the approach taken in other mollusc disease-specific chapters.

The Commission agreed to amend the list of susceptible species in Article 11.4.2. in line with recommendations of the *ad hoc* Group, i.e.

- three species currently listed in Article 11.4.2., blue mussel (*Mytilus edulis*), European flat oyster (*Ostrea edulis*) and the Mediterranean mussel (*Mytilus galloprovincialis*), were assessed to meet the criteria for listing as susceptible to infection with *M. refringens* and are therefore proposed to remain in Article 11.4.2.
- five new susceptible species, the dwarf oyster (*Ostrea stentina*), European razor clam (*Solen marginatus*), golden mussel *(Xenostrobus securis*) and the striped venus (*Chamelea gallina*) were assessed to meet the criteria for listing as susceptible to infection with *M. refringens* and are therefore proposed to be added to Article 11.4.2.
- three species currently listed in Article 11.4.2., the Australian mud oyster (*Ostrea angasi*), Argentinean oyster (*Ostrea puelchana*) and the Chilean flat oyster (*Ostrea chilensis*), were assessed and did not meet the criteria for listing as a susceptible species to infection with *M. refringens* and are therefore proposed to be deleted from Article 11.4.2.

The Commission did not agree with the *ad hoc* Group's recommendation to add a copepod (*Paracartia grani*) to Article 11.4.2. despite that it meets the criteria for listing as susceptible to infection with *M. refringens*. The Commission did not consider that this species was relevant to trade in molluscs or mollusc products. The Commission

did agree, however, that the copepod (*Paracartia grani*) should be included in Section 2.2.1. of Chapter 2.4.4. Infection with *Marteilia refringens* in the *Aquatic Manual* to ensure Members are aware that it is a susceptible species and it may be relevant for control by Members in some circumstances to prevent spread of *Marteilia refringens*.

Relevant sections of Chapter 2.4.4. Infection with *Marteilia refringens*, in the *Aquatic Manual* were also amended in line with the recommendations of the *ad hoc* Group (see Item 7.3.2.).

The Commission encouraged Members to refer to the *ad hoc* Group's June 2022 report available on the WOAH Website for details of the assessments conducted by the *ad hoc* Group.

The revised Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*, are presented as Annex 14 for comments.

5.9. Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters

Background

At its February 2018 meeting, the Aquatic Animals Commission agreed to apply model Articles X.X.8., X.X.9., X.X.10. and X.X.11. to all disease-specific chapters in Sections 8, 9 and 10 of the *Aquatic Code*. The Commission agreed to apply the changes addressed in these model articles to Section 11 for the mollusc disease-specific chapters when amending these chapters concurrently with the amendments resulting from the work undertaken by the *ad hoc* Group for Susceptibility of mollusc species to OIE listed diseases.

Previous Commission reports where this item was discussed

February 2018 report (Item 1.11., page 13).

September 2022 meeting

The Commission noted that the amendments that had been applied to Sections 8, 9 and 10 had not been systematically applied to Section 11 chapters as had previously been agreed.

The Commission amended the text in Article 11.X.9. to 'Articles 11.X.5, 11.X.6. or 11.X.7. (as applicable) and 11.X.8.', to align with the newly adopted Articles X.X.5.-X.X.8. Given the proposed amendment, the Commission agreed that the text be amended in all disease-specific chapters once the proposed amendments for 11.X.9. to 11.X.14. are adopted.

The Commission noted that Article 11.X.13. is a new article to align with other disease-specific chapters within the *Aquatic Code* that were originally adopted in 2018. The Commission also noted that Article 11.X.14. includes disease-specific information on commodities that have been assessed and comply with Article 5.4.2. and are published in the 2022 version of the *Aquatic Code*. The assessments for these commodities can be found on the WOAH website in the 'Safe commodities assessments for OIE listed aquatic animal diseases'. The Commission informed members that there is no change to this disease-specific information and as a result this change is being presented as a model article with disease-specific information in point 1 of the article shown as […].The relevant information from the current disease-specific chapters is included in the table below for Member information and will be included within the disease-specific chapters upon adoption and publication.

Commodities that comply with Article 5.4.2. Criteria to assess the safety of aquatic animal products imported (or transited) for retail trade for human consumption regardless of the disease status of the exporting country, zone or compartment

Chapter	Pathogenic agent	Commodities
11.1.	Abalone herpes virus	a. off the shell and eviscerated abalone meat (chilled or frozen)
11.2.	*Bonamia exitiosa*	a. chilled oyster meat; and b. chilled half-shell oysters.
11.3.	*Bonamia ostreae*	a. chilled oyster meat; and b. chilled half-shell oysters.
11.4.	*Marteilia refringens*	a. mollusc meat (chilled or frozen); and b. half-shell oysters (chilled or frozen).
11.5.	*Perkinsus marinus*	a. mollusc meat (chilled or frozen); and

		b. half-shell oysters (chilled or frozen).
11.6	*Perkinsus olseni*	a. mollusc meat (chilled and frozen); and b. half-shell molluscs (chilled and frozen).
11.7.	*Xenohaliotis californiensis*	a. off the shell, eviscerated abalones (chilled or frozen)

The revised model Articles 11.X.9. to 11.X.14. are presented as Annex 15 for comments.

5.10. Member questionnaire for revision of Chapter 4.3. Application of Compartmentalisation

Background

At its February 2022 meeting, the Commission identified the revision of Chapter 4.3. Application of Compartmentalisation as a first priority as part of the progressive revision of Section 4 of the *Aquatic Code*. The Commission agreed to redevelop Chapter 4.3. to focus solely on compartmentalisation, improve the guidance to Members and to align with other new and revised chapters such as Chapter 4.1. Biosecurity in aquaculture establishments. The Commission noted that the revised Chapter 1.4. and associated model Articles X.X.4. - X.X.8. in disease-specific chapters on declaration of freedom, include specific reference to the requirements for demonstrating and maintaining freedom at the compartment level. The Commission agreed that the revision of Chapter 4.3. Application of Compartmentalisation was the appropriate next step.

September 2022 meeting

The Commission agreed that Member experiences using and applying compartmentalisation standards would be useful information to inform the revision of Chapter 4.3. Consequently, the Commission developed a short questionnaire (five questions) and wished to invite Members to answer these questions. Member response will be considered by the Commission at its February 2023 meeting as it continues its work to revise Chapter 4.3. to meet the needs of Members.

The Member engagement questionnaire is presented as Annex 16 for comments.

6. Items for Member information

6.1. Glossary definitions 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services'

Background

In May 2022, revised Glossary definitions for 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services' were adopted. The Aquatic Animals Commission agreed that once these revised Glossary definitions were adopted it would undertake a comprehensive review of their usage throughout the *Aquatic Code*. Previous Commission reports where this item was discussed.

Previous Commission reports where this item was discussed

September 2020 (Item 4.5.3., page 9); September 2021 (Item 5.1.2.2., page 7); February 2022 (Part A: Item 4.1.2.2., page 8).

September 2022 meeting

During the Bureau meeting of the Aquatic Animals Commission and the Code Commission, it was agreed that the two Commissions would coordinate their respective work to review and amend usage of the revised definitions in the two Codes, as relevant.

The two Commissions agreed to share their respective proposals prior to its February 2023 meetings and to circulate the proposed respective amendments in their February 2023 reports.

6.2. Emerging diseases

6.2.1. Infection with carp edema virus (CEV)

Comments were received from Japan.

Background

At its February 2020 meeting, the Aquatic Animals Commission reviewed the scientific information for infection with CEV and agreed it met the OIE definition of an 'emerging disease' and, as such, Members should report it in accordance with Article 1.1.4. of Chapter 1.1. Notification of diseases, and provision of epidemiological information, of the *Aquatic Code*.

At its February and September 2021 meetings, the Commission reviewed Member comments and new scientific evidence on infection with CEV and noted that infection with CEV continues to be reported to cause mortality events in wild and farmed populations but that the severity of the impact on production is not clear.

At its February 2022 meeting, the Commission reviewed new scientific evidence and highlighted that mortalities caused by infection with CEV is reported to be of concern among scientists and ornamental fish breeders with more reports of detection and articles in scientific literature published every year. The Commission also noted that the genome of CEV published in 2021 will assist in the promotion of epidemiological studies, phylogenetic analysis of CEV and development of new diagnostic assays for infection with CEV in the future. The Commission reiterated that new detections of infection with CEV should be reported to WOAH as an emerging disease, in accordance with Article 1.1.4. of the *Aquatic Code*.

Previous Commission reports where this item was discussed

February 2020 (Item 7.3.3., page 17); September 2020 (Item 6.3., page 17); February 2021 (Part B: Item 2.2., page 11); September 2021 (Item 5.2.1.1., page 27); February 2022 report (Part B: Item 2.2.1.1., page 6).

September 2022 meeting

The Commission reviewed new scientific evidence for infection with CEV and noted that since its February 2022 meeting there have been several more outbreaks reported in the Asia Pacific region. The Commission recognised that there is still uncertainty as to the impact associated with infection with CEV and the extent of the spread globally, particularly within Europe. The Commission agreed that this uncertainty emphasises the importance of Members reporting new detections of infection with CEV as an emerging disease, to ensure the collection of epidemiological information and maintain awareness of the spread of this pathogen.

The Commission agreed that infection with CEV still met the WOAH definition of an 'emerging disease'. Once again, the Commission requested Members to provide any relevant information on infection with CEV to inform the Commission's consideration whether the criteria for listing (Chapter 1.2.) should be applied or if it should no longer be considered as an emerging disease.

The Commission reminded Members that a technical disease card has been developed and is available on the WOAH website at: https://www.woah.org/en/what-we-do/animal-health-and-welfare/animal-diseases/

6.2.2. Infection with covert mortality nodavirus (CMNV)

Background

As part of its workplan, the Aquatic Animals Commission reviews scientific information on new or emerging diseases to determine whether any action by the Commission is warranted. Review may be initiated by the Commission, or by requests from WOAH headquarters, WOAH reference centres, WOAH regional representation, *ad hoc* Groups or Members. Review of new or emerging diseases occurs at every Commission meeting.

September 2022 meeting

The Commission considered available information on covert mortality nodavirus (CMNV) to determine if it meets the WOAH definition of an 'emerging disease'. The Commission noted the wide range of susceptible species and that CMNV can infect and cause disease in crustaceans (e.g. white leg shrimp [*Penaeus vannamei*] and giant river prawns [*Macrobrachium rosenbergii*]) and finfish (e.g. yellow croaker [*Larimichthys crocea*], Japanese flounder [*Paralichthys olivaceus*] and zebrafish [*Danio rerio*]).

The Commission noted that reported occurrences of CMNV indicated the presence of CMNV in Asia. The Commission noted that CMNV (in crustaceans) has been listed in the Asia-Pacific Quarterly Aquatic Animal Disease reporting program as "viral covert mortality disease" of crustaceans since 2017.

Mortality events and production impacts have been reported in association with CMNV infection. The presence of viral particles in gonads indicate the possibility of vertical transmission.

The Commission agreed that infection with CMNV meets the definition of an emerging disease and should be reported to WOAH in accordance with Article 1.1.4. of the *Aquatic Code*. The Commission agreed to develop a technical disease card for infection with CMNV that will be published on the WOAH website in the coming months.

6.3. E-Certification

The Secretariat informed the Aquatic Animals Commission that although Member's adoption of e-certification is limited, use of electronic systems is increasing. The Secretariat also updated the Commission on the activities that WOAH had implemented, including a WTO Standards and Trade Development Facility project on Electronic Veterinary Certification. The aim of this project was to gain a better understanding of practices implemented by some WOAH Members, both developed and under development, as well as other relevant work in other international organisations on e-certification and Single Window.

Given the close alignment between the work of Codex and WOAH (regarding food of animal origin) and that in practice a single export certificate may contain information relevant to food safety and aquatic animal health, and that in 2021 Codex adopted the revised Guidelines for design, production, issuance and use of generic official certificates (CXG 38-2011), specifically related to transitioning to paperless certification, WOAH considered that it should explore the development of similar guidance to that of Codex.

The Commission was informed that WOAH would develop Reference Data Models (electronic versions) of the WOAH model certificates for international trade, i.e. Chapter 5.11. of the *Aquatic Code*, which are similar to the reference data model of the generic model official certificate that is included in the Codex Guidelines.

Noting that introduction of electronic certification could contribute to facilitating international trade, minimising the risk of trade fraud, and that the disruptions caused by the COVID-19 pandemic have highlighted the advantages of the approach, the Commission agreed to include the update of Chapter 5.2. in its workplan. The Commission emphasised the importance of working closely with the Code Commission as the *Terrestrial Code* has a similar chapter.

The WOAH Manual of Diagnostic Tests for Aquatic Animals

7. Items for Member comments

The Aquatic Animals Commission has commenced the process of progressively reformatting the disease-specific chapters of the *Aquatic Manual* into a new template. As the reformatted and updated chapters have substantial changes, at its meeting in September 2019, the Commission agreed that only clean versions of the revised chapters would be provided in its report. Subsequent changes made to these initial revisions following Member comments would be indicated in the usual style (i.e. ~~strikethrough for deletions~~ and double underline for additions).

A software-generated document that compares the adopted version of a chapter and the proposed new text can be created. This comparison document is not included in the Commission's report, but will be available upon request from the WOAH Standards Department (AAC.Secretariat@WOAH.org).

In reviewing Member comments and newly updated chapters, the Commission noted that some amendments applied to all the chapters. The Commission therefore agreed to amend the template and apply the following changes to all the chapters under review:

1. In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals:* to clarify that for confirmatory diagnosis, amplicon sequencing always follows conventional polymerase chain reaction (PCR), "Conventional PCR followed by" was added before "amplicon sequencing" in the relevant row. And to clarify that conventional PCR is never to be used without sequencing to confirm a case, Section C *Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis* was shaded grey for the row "conventional PCR".

2. In Section 6. *Corroborative diagnostic criteria*, to add the following standard text to the end of the second paragraph:

   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 WOAH 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 WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

As a consequence of this addition, the following paragraph will be deleted from Sections 6.1.2. *Definition of confirmed case in apparently healthy animals* and 6.2.2. *Definition of confirmed case in clinically affected animals:*

~~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.~~

3. In Section 6.1. *Apparently healthy animals or animals of unknown health status*, to replace the word “Geographical” with the word “Hydrographical” in the second sentence of the first paragraph: Hydrographical ~~Geographical~~ proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link.

The Commission reviewed the text in Part 2. *Recommendations applicable to specific diseases*, *General introduction* of the *Aquatic Manual*, which had been adopted in 2012 and covers an overall approach to aquatic animal health management, surveillance and sampling. The Commission noted that some of the text is out of date or conflicts with more recently adopted chapters, for example Chapter 1.1.1. *Quality management in veterinary testing laboratories*. The Commission agreed to recommend removal of this chapter from the *Aquatic Manual* as it is no longer relevant or fit for purpose.

The Commission invited Member comments on removal of the chapter “Part 2. *Recommendations applicable to specific diseases*, *General introduction* of the *Aquatic Manual”* from the *Aquatic Manual*. No annex is provided as the chapter would be removed entirely.

7.1. Section 2.2. Disease of crustaceans

7.1.1. Chapter 2.2.0. General information (diseases of crustaceans)

September 2022 meeting

Chapter 2.2.0. *General Information* (*diseases of crustaceans*) had been updated in consultation with the crustacean disease Reference Laboratory experts.

The main amendments include:

- deleted the two paragraphs in Section A.1. *Assessing the health status of the epidemiological unit* as the information was not useful and there was no similar text in Chapter 2.3.0. *General Information* (*diseases of fish*);
- re-ordered the sections in Section A Sampling to align with Chapter 2.3.0.;
- for clarity, updated the text on selection of animal samples in Section A.1.2. *Specifications according to crustacean populations*;
- thoroughly updated the text in Sections B.5.5. *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis*, B.5.5.3. *Nucleic acid extraction* and B.5.5.4. *Preparation of slides for* in-situ *hybridisation;*
- expanded Section B.6. *Additional information to be collected* to include text on the history of the specimens;
- updated the references.

The revised Chapter 2.2.0. General information (diseases of crustaceans), is presented as Annex 17 for comments.

7.1.2. Chapter 2.2.1. Acute hepatopancreatic necrosis disease

Comments were received from Australia, Canada, China (People’s Rep. of), Chinese Taipei, Norway, Switzerland, the USA and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.2.1. Acute hepatopancreatic necrosis disease, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was presented for Member comments in the Commission’s February 2022 Part B report.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.1., page 9).

September 2022 meeting

The current chapter states that 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. One Member requested that the scope of the chapter be extended to take account of reports of AHPND caused by other *Vibrio* species. The Commission, in consultation with the two WOAH Reference Laboratories, will review the published information on non-*Vibrio parahaemolyticus* species that have been associated with AHPND and present their conclusions at the next meeting in February 2023.

The Commission agreed to delete the text in Section 2.2.5 *Aquatic animal reservoirs of infection* and replace it with "none known" as the text referred to experimental studies and not confirmed reservoirs of infection (subclinically infected animals that can transmit the disease).

In response to a request for clarity on the statement in Section 2.3.1 *Mortality, morbidity and prevalence* that "In regions where AHPND is enzootic in farmed shrimp, evidence indicates a near 100% prevalence (Tran *et al*., 2014)", the Commission confirmed that prevalence here refers to species of *Vibrio parahaemolyticus* carrying the AHPND PirA and Pir B genes.

The Commission accepted a proposal to move text on gross pathology from Section 2.3.2. *Clinical signs, including behavioural changes* to Section 2.3.3. *Gross pathology,* and to remove text on histo- and cytopathology from Section 2.3.3. as it is repeated in Section 4.2. *Histopathology and cytopathology*.

In Section 3.2. *Selection of organs or tissues*, the Commission agreed to delete text on faecal samples as it is repeated in Section 3.4. *Non-lethal sampling*. The Commission also agreed to delete text from Section 3.5. *Preservation of samples for submission* as it is repeated in Sections 3.5.1. *Samples for pathogen isolation*, 3.5.2. *Preservation of samples for molecular detection* and 3.5.3. *Samples for histopathology, immunohistochemistry or in-situ hybridisation.*

In Section 3.5.2. *Preservation of samples for molecular detection* clarified that if material cannot be fixed it may be frozen "but repeated freezing and thawing of samples should be avoided".

In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals,* purpose 'C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis', the Commission agreed to rate the real-time PCR '++' for all life stages with the level of validation of 1 to correspond with the case definition in Section 6 *Corroborative diagnostic criteria*, and to raise the level of validation for conventional PCR followed by amplicon sequencing to 2 as it is included in the table in Section 6.3.1. *For presumptive diagnosis of clinically affected animals*. Following consultation with the Reference Laboratory experts, the Commission agreed to add the loop-mediated isothermal amplification (LAMP) method to the Table for purpose A. Surveillance of apparently healthy animals, and the antigen ELISA for all three purposes. The changes to Table 4.1. described in agenda item 7 above were also made.

The Commission did not agree to a request to delete Section 4.2 *Histopathology and cytopathology* as the information is essential and is not presented in other sections of the chapter.

In response for clarification regarding a statement in Section 4.4. *Nucleic acid amplification*, on the number of isolates in the validation study for the AP3 PCR method, the Commission agreed to remove that part of the sentence as it caused confusion; details of the study can be found in the reference. Also in Section 4.4., a Member noted that sensitive nested-PCR and LAMP methods exist for the detection of AHPND and asked to either include these methods in Table 4.1. or to provide reasons why they are not currently recommended. The Member also noted that Section 4.9. *Antibody- or antigen-based detection methods*, refers to a sensitive and specific antigen enzyme-linked immunosorbent assay (ELISA) also not included in Table 4.1. Following consultation with the Reference Laboratories, both LAMP and the Ag-ELISA were added to Table 4.1.

In Section 4.4. *Nucleic acid amplification*, the Commission added the standard text on PCR controls and extraction of nucleic acids, along with the tables giving the primers, probes and cycling parameters for the real-time and conventional PCR for detection of the VpAHPND toxin genes, as detailed in agenda item 7.4. below. The inclusion of the standard tables allowed some details of the PCR protocols to be deleted.

In Section 4.4.2. *Conventional PCR*, *Protocol for the AP1 and AP2 PCR methods*, the Commission added to the listed reference the updated hyperlink for the protocol.

In Section 5. *Test*(*s*) *recommended for surveillance to demonstrate freedom in apparently healthy populations* a Member noted that both real-time PCR and conventional PCR methods have the same rating for use and validation level and proposed that conventional PCR be mentioned in Section 5. Following consultation with the Reference Laboratories, conventional PCR was included in Section 5.

The Commission amended the case definitions in Section 6. *Corroborative diagnostic criteria* to comply with the amendments made to Table 4.1. The Commission also made the generic changes described above in agenda item 7 to Section 6.

The revised Chapter 2.2.1. Acute hepatopancreatic necrosis disease*,* is presented as Annex 18 for comments.

7.1.3. Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague)

September 2022 meeting

The Aquatic Animals Commission reviewed Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague), which had been updated by the WOAH Reference Laboratory expert and reformatted using the new disease chapter template.

The main amendments include:

- updated information on the aetiological agent; as the *ad hoc* Group on Susceptibility of crustacean species to WOAH listed diseases has not assessed susceptible species, the current adopted text in Sections 2.2.1. *Susceptible host species* and 2.2.2 *Species with incomplete evidence for susceptibility* remains unchanged;
- updated sections on disease pattern, biosecurity and disease control strategies, and on specimen selection, sample collection, transportation and handling;
- updated Section 4. *Diagnostic methods*, including making the changes described in agenda item 7 and completing Table 4.1 *OIE Recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*;
- removed the detailed description in Section 4.3. *Culture for isolation*, as it is not a routine diagnostic method but rather used for research or maintaining cultures;
- updated Section 4.4. *Nucleic acid amplification* including adding the standard text, described in agenda item 7 above, on PCR controls and extraction of nucleic acids and the standard tables giving the primers, probes and cycling parameters; inclusion of the standard tables allowed some details of the PCR protocols to be deleted;
- updated Section 4.5. *Amplicon sequencing* by adding the standard text (see agenda item 7); and
- revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals.

The revised Chapter 2.2.2. *Infection with* Aphanomyces astaci (*crayfish plague*), is presented as Annex 19 for comments**.**

7.1.4. Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)

Background

At its February 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.2.3. *Infection with* Hepatobacter penaei (*necrotising hepatopancreatitis*), which had been updated by the WOAH Reference Laboratory expert and reformatted using the new disease chapter template. The revised chapter was presented for Member comments in the Commission's February 2022 Part B report.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.2., page 10).

September 2022 meeting

The Commission updated the taxonomy in Section 1. *Scope*, to align with *Aquatic Code* Chapter 9.3. *Infection with* Hepatobacter penaei (*necrotising hepatopancreatitis*) and included Family Holosporaceae and Order Rickettsiales to reflect the correct taxonomic relationships.

The Commission agreed to move the text on mortality from Section 2.3.3. *Gross pathology* to Section 2.3.1. *Mortality, morbidity and prevalence.* In response to a comment requesting details on NHP-affected broodstock experiencing mortality, the Commission noted that the information requested is in the reference provided and agreed not to repeat it in the chapter.

The Commission agreed to delete text from Sections 2.3.2. *Clinical signs, including behavioural changes* and 2.3.3. *Gross pathology*, that was not relevant to the topic of each section.

In Section 2.4. *Biosecurity and disease control strategies*, the Commission agreed to delete the last sentence on the sensitivity of *H. penaei* that was based on a reference published in 1994.

In Section 3.2. *Selection of organs or tissues*, the Commission clarified that hepatopancreas, the principal target organ, should be selected preferentially.

The Commission updated the Section 3.4. *Non-lethal sampling* to update the contents and improve the text.

In Section 3.5.2. *Preservation of samples for molecular detection* clarified that if material cannot be fixed it may be frozen "but repeated freezing and thawing of samples should be avoided".

In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals,* the Commission changed the rating of the conventional PCR from "+++" to "++" for the purpose A; *Surveillance of apparently healthy animals* because it is not a suitable test for surveillance. The changes to Table 4.1. described in agenda item 7 above were also made.

In Section 4.4. *Nucleic acid amplification*, the Commission added the standard text on PCR controls and extraction of nucleic acids, along with the tables giving the primers, probes and cycling parameters for the real-time and conventional PCR for detection of the *H. penaei*, as detailed in agenda item 7.4. below. The inclusion of the standard tables allowed some details of the PCR protocols to be deleted. The changes to Table 4.1. described in agenda item 7 above were also made.

In Section 4.5. *Amplicon sequencing* the standards text on amplicon sequencing was added to replace the existing text.

The Commission did not agree to include conventional PCR alongside real-time PCR in Section 5. *Test*(*s*) *recommended for surveillance to demonstrate freedom in apparently healthy populations*, because conventional PCR is not ideal for surveillance. The Commission had corrected the rating of conventional PCR in Table 4.1 as mentioned above.

The Commission made the generic changes described above in agenda item 7 to Section 6 *Corroborative diagnostic criteria*.

The revised Chapter 2.2.3. Infection with *Hepatobacter penae*i (necrotising hepatopancreatitis), is presented as Annex 20 for comments.

7.1.5. Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus

Comments were received from Australia, China (People's Rep. of), Norway, Switzerland, the USA and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was presented for Member comments in the Commission's February 2022 Part B report.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.3., page 10).

September 2022 meeting

Updated the taxonomy in Section 1. *Scope* to align with *Aquatic Code* Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus.

In Section 2.1.1. *Aetiological agent*, the Commission added a reference verifying that two distinct genotypes of IHHNV (Type 1 and Type 2) were shown to be infectious to *Penaeus vannamei* and *P. monodon*. The Commission also corrected the statement that Type 1 has been identified in South-East Asia rather than East Asia.

In Section 2.2.2. *Species with incomplete evidence for susceptibility*, the Commission agreed to the delete the sentence "~~Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection~~": it had been left over from a previous version of the chapter and no longer conforms with the template. Also in Section 2.2.2., the Commission did not agree to delete the introductory text to the second paragraph explaining that pathogen-specific positive PCR results had been reported in the following organisms, but an active infection has not been demonstrated. The Commission believes that this is important explanatory text reflecting the work of the *ad hoc* Group on Susceptibility of crustacean species to infection with WOAH listed diseases, and is included in the template.

The Commission agreed to a proposal to move some of the text in Section 2.3.3. *Gross pathology* to Section 2.3.1. *Mortality, morbidity and prevalence*, and to move the third paragraph from Section 2.3.1. to Section 2.3.2. as the texts better fit within those Sections. In Section 2.3.1., the Commission added new text and a reference on the lack of clinical signs and mortality in animals experimentally challenged with IHHNV genotypes found to be within a separate lineage of IHHNV type II genotypes circulating within Ecuador and Peru. Finally, the Commission added new text to Section 2.3.2. to clarify some clinical signs and behavioural changes caused by infection with IHHNV.

In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals,* the Commission deleted the ratings for histopathology for the purpose "C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis" because it is not sufficiently specific for confirmation of a case. The changes to Table 4.1. described in agenda item 7 above were also made.

In Section 4.4. *Nucleic acid amplification*, the Commission added the standard text on PCR controls and extraction of nucleic acids, along with the tables giving the primers, probes and cycling parameters for the real-time and conventional PCR for detection of IHHNV, as detailed in agenda item 7.4. below. The inclusion of the standard tables allowed some details of the PCR protocols to be deleted.

In Section 4.5. the standard text on amplicon sequencing was added to replace the existing text (see agenda item 7.4. below).

The Commission made the generic changes described above in agenda item 7 to Section 6 *Corroborative diagnostic criteria*.

The Commission harmonised the text in Sections 6.1.2. *Definition of confirmed case in apparently healthy animals*, and 6.2.2. *Definition of confirmed case in clinically affected animals* to align with the other chapters, and removed histopathology it is not sufficiently specific for confirmation of a case.

The revised Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis, is presented as Annex 21 for comments.

7.1.6. Chapter 2.2.5. Infection with infectious myonecrosis virus

September 2022 meeting

The Aquatic Animals Commission reviewed Chapter 2.2.5. *Infection with infectious myonecrosis virus*; as there is currently no WOAH Reference Laboratory for this disease, the chapter was reformatted using the new disease chapter template and reviewed by the Commission.

The main amendments include:

- updated information on the taxonomy in the scope of the chapter and on the aetiological agent;

- updated sections on vectors, mortality, morbidity and prevalence, geographical distribution, and chemotherapy including blocking agents;
- updated the section on diagnostic methods: completed Table 4.1., and revised the section on cell culture for isolation, on nucleic acid amplification and molecular tests, including adding the standard text on PCR controls, extraction of nucleic acids and amplicon sequencing and replacing the test protocols with the new tables of primer and probe sequences and cycling parameters;
- revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals; and
- completed the table in the section on diagnostic sensitivity and specificity for diagnostic tests for presumptive diagnosis of clinically affected animals.

The revised Chapter 2.2.5. Infection with infectious myonecrosis virus, is presented as Annex 22 for comments.

7.1.7. Chapter 2.2.7. Infection with Taura syndrome virus

September 2022 meeting

The Aquatic Animals Commission reviewed Chapter 2.2.7. *Infection with Taura syndrome virus*, which had been updated by the WOAH Reference Laboratory expert and reformatted using the new disease chapter template.

The main amendments include:

- updated information on distribution of the pathogen in the host, geographical distribution, breeding resistant strains, non-lethal sampling;
- updated the section on diagnostic methods: completed Table 4.1., and revised the section on the bioassay, on nucleic acid amplification and molecular tests, including adding the standard text on PCR controls, extraction of nucleic acids and amplicon sequencing and replacing the test protocols with the new tables of primer and probe sequences and cycling parameters;
- revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals; and
- updated the references.

The revised Chapter 2.2.7. Infection with Taura syndrome virus, is presented as Annex 23 for comments.

7.1.8. Chapter 2.2.8. Infection with white spot syndrome virus

September 2022 meeting

The Aquatic Animals Commission reviewed Chapter 2.2.8. *Infection with white spot syndrome virus*, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include:

- updated information on the aetiological agent;
- updated sections on Survival and stability in processed or stored samples, aquatic animal reservoirs of infection, vectors, geographical distribution, inactivation methods;
- updated the section on diagnostic methods: completed Table 4.1., and revised the section on nucleic acid amplification and molecular tests, including adding the standard text on PCR controls, extraction of nucleic acids and amplicon sequencing and replacing the test protocols with the new tables of primer and probe sequences and cycling parameters;
- revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals;
- completed the tables in the section on diagnostic sensitivity and specificity for diagnostic tests; and
- updated the references.

The Commission noted that the assessments completed by the *ad hoc* Group on susceptibility of crustacean species to OIE listed diseases for infection with white spot syndrome virus in June 2016 had not been

previously applied by the Commission as Article 1.5.9 of Chapter 1.5 Criteria for listing species as susceptible to infection with a specific pathogen had not yet been adopted. These assessments will be further reviewed by the Commission for application of Article 1.5.9. The Commission noted that the current adopted text will remain in Section 2.2.1. until this review is completed.

The Commission encouraged Members to refer to the June 2016 report available on the WOAH Website for details of the assessments conducted by the *ad hoc* Group.

The revised Chapter 2.2.8. Infection with white spot syndrome virus, is presented as Annex 24 for comments.

7.2. Section 2.3. Diseases of fish

7.2.1. Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)

Comments were received from China (People's Rep. of), Thailand, the USA and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was presented for Member comments in the Commission's February 2022 Part B report.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.2.1., page 10).

September 2022 meeting

The Commission agreed to delete the word "fungus" after "oomycete" in Section 1. Scope as Oomyceta have been delimited from fungi and incorporated into the protozoa Kingdom.

The Commission put a comment regarding the common names listed in the Tables in Section 2.2.1 Susceptible host species as this Section is still under review by the *ad hoc* Group on Susceptibility of fish species to infection with WOAH listed diseases. Once the list has been finalised by the *ad hoc* Group, the common names will be checked against the FAO database.

In Section 3.6. *Pooling of samples*, the Commission agreed to amend the text by adding a sentence stating that the effect of pooling on diagnostic specificity has not been evaluated and recommending that larger animals be processed and tested individually; the text now aligns with the template and thus is consistent with other chapters.

In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals,* the Commission added a new row for clinical signs and rated it for all three purposes. The Commission also added ratings for squash mounts for purpose "C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis". The changes to Table 4.1. described in agenda item 7 above were also made.

In Section 4.4. *Nucleic acid amplification*, the Commission added the standard text on PCR controls and extraction of nucleic acids, along with the tables giving the primers, probes and cycling parameters for the real-time and conventional PCR for detection of *A. invadans* in fish tissues, as detailed in agenda item 7.4. below. The inclusion of the standard tables allowed some details of the PCR protocols to be deleted.

In Section 6. *Corroborative diagnostic criteria*, the Commission did not agree to include the standard text on referring suspect samples to the WOAH Reference Laboratory as there is currently no such designated laboratory. Should a WOAH Reference Laboratory be designated for EUS in the future, the text will be reinstated.

The Commission did not agree to delete clinical signs consistent with infection with *A. invadans* from Section 6.1.1. *Definition of suspect case in apparently healthy populations* as the detection of clinical signs is a recommended test for surveillance of healthy populations as stated in Section 5. *Test*(*s*) *recommended for surveillance to demonstrate freedom in apparently healthy populations*. Furthermore, the clinical signs observed may not be pathognomonic and hence meet the criteria for a suspect case. The Commission added a footnote to this criterion clarifying that surveillance of apparently healthy populations for EUS is based on examination of target populations for clinical signs of infection with *A. invadans.*

In Section 6.2.1. *Definition of suspect case in clinically affected animals*, the Commission agreed to clarify that the visual observation is of hyphae characteristic of *A. invadans* and that culture and isolation is of *A. invadans*-type colonies in criteria iv and v.

The revised Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), is presented as Annex 25 for comments.

7.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus

Comments were received from Norway, Switzerland, the USA and the EU.

Background

At its September 2021 meeting, the Aquatic Animals Commission reviewed Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was also presented for Member comments in the Commission's February 2022 Part B report.

Previous Commission reports where this item was discussed

September 2021 report (Item 6.1.3., page 31), February 2022 report (Part B: Item 3.1.2.2., page 11).

September 2022 meeting

In Section 3.6. *Pooling of samples*, the Commission did not agree to delete two sentences on the pooling procedures to be followed in cases where the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated. The Commission noted that both the sentences are part of the standard text for this section as given in the new template and are intended to support evidence-based recommendations. The Commission did however agree to delete the last sentence recommending that a maximum of five fish be used to pool organs, as it is left over from an older version of the chapter and is no longer recommended.

In Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals,* the Commission clarified that it is essential to indicate the ratings for cell culture, real-time PCR and conventional PCR even though amplicon sequencing is required for confirmatory diagnosis. Furthermore, the Commission reduced the level of validation from "2" to "1" for cell culture for all three purposes and the real-time PCR for purposes A. *Surveillance of apparently healthy animals* and C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis, as diagnostic sensitivity and specificity has not been published for either clinically diseased or apparently healthy animals. The changes to Table 4.1. described in agenda item 7 above were also made.

In Section 4.3.2. *Cell culture*, the Commission, in consultation with the Reference Laboratory expert, agreed to delete immunostaining, ELISA, and immuno-electron microscopy from the last sentence and revised the text to clarify that the identity of viruses in cell culture is determined by "PCR and amplicon sequencing".

In Section 4.4. *Nucleic acid amplification*, the Commission, in consultation with the Reference Laboratory expert, amended the first sentence to state that although several conventional PCR or quantitative real-time PCR methods have been described for the detection of ranaviruses, EHNV can only be detected when these methods are combined with methods that specifically detect EHNV. The Commission also agreed to delete a sentence referring to a PCR method that is not described in the chapter. Finally, the Commission added the standard text on PCR controls and extraction of nucleic acids, along with the tables giving the primers, probes and cycling parameters for the real-time and conventional PCR for the detection of EHNV, as detailed in agenda item 7.4. below. The inclusion of the standard tables allowed some details of the PCR protocols to be deleted.

In Section 4.5. *Amplicon sequencing* the standard text was added to replace the existing text (see agenda item 7.4. below).

In Section 5. *Test(s) recommended for surveillance to demonstrate freedom inapparently healthy populations*, the Commission agreed to clarify that real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish "EHNV from other" ranaviruses.

The Commission made the generic changes described above in agenda item 7 to Section 6. *Corroborative diagnostic criteria*.

In Section 6.1.1. *Definition of suspect case in apparently healthy animals*, the Commission replaced the first criteria 'Positive result for EHNV based on virus isolation in cell cultures' with 'EHNV-typical CPE in cell culture' which is more appropriate to align with Table 4.1.

The revised Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, is presented as Annex 26 for comments.

7.2.3. Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus

September 2022 meeting

The Aquatic Animals Commission agreed to include Jinsha bass carp (*Percocypris pingi*) in the list of susceptible species in Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus (See Item 5.5.).

The *ad hoc* Group's assessment of Jinsha bass carp (*Percocypris pingi)* is presented as Annex 10 for Member information.

The revised Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus, is presented as Annex 27 for comments.

7.3. Section 2.4. 'Diseases of molluscs'

7.3.1. Sections 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa* and Sections 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae*

September 2022 meeting

The Aquatic Animals Commission amended Sections 2.2.1. and 2.2.2 of Chapter 2.4.2, Infection with *Bonamia exitiosa* and Chapter 2.4.3. Infection with *Bonamia ostreae*, with respect to the taxonomy of the Suminoe oyster and Pacific cupped oyster (see Item 5.8.)

The amended Sections 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*, are presented as Annex 28 for comments.

The amended Sections 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae*, are presented as Annex 29 for comments.

7.3.2. Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*

September 2022 meeting

The Aquatic Animals Commission amended Sections 2.2.1 and 2.2.2 of Chapter 2.4.4, Infection with *Marteilia refringens*, in line with the recommendations of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases (see Item 5.8.).

The Commission did not agree with the *ad hoc* Group's recommendation to add a copepod (*Paracartia grani*) in Article 11.4.2. of the *Aquatic Code* (See item 5.8.). However, the Commission agreed to add a new paragraph 'Additionally, a copepod species (*Paracartia grani*) has been found to meet the criteria for listing as susceptible to infection with *Marteilia refringens* and is considered an intermediate host' in Section 2.2.1. to reflect the unique situation with the risk associated with an intermediate host. The Commission considered that the susceptibility of *Paracartia grani* may be relevant for Members in some circumstances to prevent spread of *Marteilia refringens*.

The amended Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*, are presented as Annex 30 for comments.

8. Items for Member information

8.1. Proposed table of PCR parameters to harmonise PCR protocols

Background

At its February 2022 meeting, the Aquatic Animals Commission noted that the process of reviewing the updated and reformatted chapters had brought to light the wide variation among the chapters in the level of detail given in the description of the PCR methods in Section 4.4. *Nucleic acid amplification*, and how the information is presented. The Commission decided to address this issue by developing a template for the description of PCR methods, which will include concise, uniform and generic text on nucleic acid extraction methods, and the controls used in the tests, while giving all the information necessary on the primer and probe sequences and cycling parameters in table format.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1. page 9).

September 2022 meeting

The Commission reviewed and approved the proposed table of primer and probe sequences and cycling parameters, and the text on controls, extraction of nucleic acid and amplicon sequencing. The table and text, given below, will be added to the template and to all the chapters under review so that critical information on PCR methods is presented in a uniform way in all the chapters of the *Aquatic Manual*. The table will replace the existing texts on PCR protocols.

Primers, probes (sequence) and cycling parameters (examples only)

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Reference, GenBank Accession No., Product size [bp]*)			
X	Fwd: TGC-GTC-CTG-CGT-ATG-GCA-CC Rev: GGC-TGG-CAT-GCC-CGA-ATA-GCA Probe: GGC-TGG-CAT-GCC-CGA-ATA-GCA	400 nM 300 nM	50 cycles of: 95°C/15 sec and 58°C/60 sec

*For conventional PCR only.

1. Section 4.4. *Nucleic acid amplification*

PCR assays should always be run with the controls specified in Section X.X *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.X.0. *General information* (diseases of crustaceans or fish, as appropriate) [Section 2.6. *Molecular methods* of Chapter 2.4.0. *General information* (diseases of molluscs)]. Each sample should be tested in duplicate.

2. Section 4.4. *Nucleic acid amplification*

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

3. Section 4.5. *Amplicon sequencing*

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published sequences.

8.2. Chapter 2.3.7. Infection with red sea bream iridoviral disease

Comments were received from China, Chinese Taipei, Norway, Switzerland, the USA and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.3.7. Infection with red sea bream iridoviral disease, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was presented for Member comments in the Commission's February 2022 Part B report.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.2.3. page 13).

September 2022 meeting

The Commission reviewed the report of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases for infection with red sea bream iridovirus (RSIV) and agreed that an assessment for listing of infection with spleen and kidney necrosis virus, including its three genogroups, red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) should be completed (See item 5.1.). The Commission agreed to delay review of comments received on the proposed Chapter 2.3.7. until comments were received on the proposed listing of infection with infectious spleen and kidney necrosis virus could be reviewed.

8.3. Develop a mechanism to speed up the process of making updates to diagnostic methods in the *Aquatic Manual* available to Members

The Commission identified two situations relating to the timely dissemination of important new information on diagnostic tests in the *Aquatic Manual*. The first is what to do when issues arise about the performance of a test that has been adopted and is included in the *Aquatic Manual*. The Commission agreed that in such situations a footnote can be added to the chapter detailing the nature of the problem and providing instructions on how to manage it. As the footnote would not replace or change any adopted text, it could be added immediately to the relevant chapter. This has occurred previously when specificity issues have arisen. The Commission would also request that advice be provided to Reference Laboratories and Aquatic Focal Points.

The second issue is that of including new diagnostic tests in the *Aquatic Manual*. At present, tests must be published in a peer-reviewed journal and should preferably be validated to at least level 2 of the WOAH validation pathway. The Commission was informed of the work of the Biological Standards Commission to develop a template of the validation data that would be requested of applicants wishing to have their tests included in the *Terrestrial Manual* (see agenda item 5.2.2 of the report of the meeting of the Biological Standards Commission, September 2022). A member of the Aquatic Animals Commission was identified to review the template and report back at the February 2023 meeting on its suitability and applicability to the *Aquatic Manual*. The requirement for tests to be published in the scientific literature remains, but if a time-sensitive situation arose, test developers could submit data in the validation template as an interim measure, possibly allowing the test to be included in the *Aquatic Manual* before publication in a peer-reviewed journal.

9. *Ad hoc* Groups

9.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases

The *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases met during June 2022 to complete the assessments for susceptibility of mollusc species to infection with *Marteilia refringens* (see Item 5.8. and 7.3.2.).

The Commission was informed that the *ad hoc* Group is planning to meet in November 2022 to progress its work assessing species susceptible to infection with *Perkinsus marinus*.

The report of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases (June 2022) can be found on the WOAH Website.

9.2. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases

The *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases met during April 2022 to conduct assessments for susceptibility of fish species to infection with red sea bream iridovirus.

The Aquatic Animals Commission was informed that the *ad hoc* Group did not complete their assessments of red sea bream iridovirus due to the complexity associated with the pathogenic agent. The Commission reviewed and provided feedback on the *ad hoc* Group's interim report which outlined the work completed to date. The *ad hoc* Group is planning to meet again in November 2022 to finalise the assessments of species susceptible to red sea bream iridovirus.

9.3. *Ad hoc* Group on new draft chapters on emergency disease preparedness and disease outbreak management

The Aquatic Animals Commission discussed the work of the *ad hoc* Group on Emergency disease preparedness and Disease outbreak management. The Commission thanked the members for their work on the draft Chapters and considered that sufficient work had been completed for the Commission to progress the two chapters. The Commission will continue the work on the draft Chapter 4.X. Emergency disease preparedness and Chapter 4.Y. Disease Outbreak management for further consideration at its February 2023 meeting.

10. WOAH Reference Centres or Change of experts

10.1. Evaluations of applications for WOAH Reference Centres for aquatic animal health issues or change of experts

The Aquatic Animals Commission reviewed applications for changes of expert and recommended acceptance of the following:

Infection with viral haemorrhagic septicaemia

Dr Britt Bang Jensen to replace Dr Niels Jørgen Olesen, who has retired from the Technical University of Denmark National Institute for Aquatic Resources, Lyngby, Denmark.

10.2. Call for applications for candidates for WOAH Reference Laboratory status

The Aquatic Animals Commission noted the need to designate WOAH Reference Laboratories for the following listed diseases:

Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)

Infection with *Batrachochytrium dendrobatidis*

Infection with *Batrachochytrium salamandrivorans*

Infection with infectious myonecrosis virus

Infection with *Perkinsus marinus*

Infection with *Perkinsus olseni*

Infection with tilapia lake virus

Infection with *Xenohaliotis californiensis*.

The Commission invites applications from Members with appropriate expertise in these diseases.

11. Any other business

11.1. Registration of diagnostic test kits

The Aquatic Animals Commission reviewed the current status of the WOAH Register of diagnostic kits with the Secretariat for Registration of Diagnostic Kits (WOAH SRDK). At present, there are 14 diagnostic test kits on the WOAH Register of Diagnostic Kits.

The Commission discussed the two active Aquatic Diagnostic Kit applications and the two Aquatic Diagnostic Kit applications that require five-year renewal of WOAH registration.

During the discussion, the Commission requested clarification on how registered tests align with the diagnostic test methods listed in the WOAH *Aquatic* and *Terrestrial Manuals*. This request will be discussed further internally within AMR & VMP department.

…/Annexes

Annex 1. Item 2 – Adopted Agenda

MEETING OF THE WOAH AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Hybrid, 14 to 21 September 2022

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1. **Welcome from the Deputy Director General**
2. **Adoption of the agenda**
3. **Meeting with the Director General**
4. **Cooperation with Terrestrial Code Commission**
5. **Work plan of the aquatic Animals Commission (AAC)**
   - 5.1. Approach for first priority items
     - 5.1.1. Chapter 4.3. Application of compartmentalisation
     - 5.1.2. New Chapter 5.X. Ornamental aquatic animals
     - 5.1.3. New Chapter 5.Y. Trade of genetic materials
6. **Aquatic Animal Health Strategy**
   - 6.1. Status report on the implementation of the Aquatic Animal Health Strategy
     - 6.1.1. Implementation update
     - 6.1.2. WOAH Observatory – Results of the Survey
     - 6.1.3. Reference Centre network - Science Department
     - 6.1.4. Update on AMR Workplan

THE *AQUATIC CODE*

7. **Items for Member comment**
   - 7.1. Glossary definitions: 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services'- review usage in *Aquatic Code*
   - 7.2. Safe commodities – Articles X.X.3. for disease-specific chapters
   - 7.3. Revised Articles 8.X.3. for amphibian disease-specific chapters
   - 7.4. Revised Articles 11.X.3. for mollusc disease-specific chapters
   - 7.5. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)
   - 7.6. Article 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus
   - 7.7. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus
   - 7.8. New Chapter 10.X. Infection with tilapia lake virus
   - *7.9.* Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*
   - 7.10. Harmonisation of Mollusc disease-specific chapters; Articles 11.X.8. - 11.X.12.
8. **Items for AAC discussion**
   - 8.1. Assessment of default periods in Articles X.X.4.-X.X.8. for disease-specific chapters
   - 8.2. Update on the status of safe commodities work
   - 8.3. Consideration of emerging diseases
     - 8.3.1. Infection with carp edema virus (CEV)
     - 8.3.2. Covert mortality nodavirus (CMNV) in zebrafish
     - 8.3.3. *Coxiella burnetii* in shrimp (Raised by WAHIAD)
     - 8.3.4. Review draft SOPs for emerging diseases
   - 8.4. E-Certification discussion
   - 8.5. Wildlife Strategy Consultancies

THE *AQUATIC MANUAL*

9. **Items for Member comment**
   - 9.1. Section 2.2. Diseases of crustaceans

9.1.1. Chapter 2.2.0. General information: diseases of crustaceans
9.1.2. Chapter 2.2.1. Acute hepatopancreatic necrosis disease
9.1.3. Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague)
9.1.4. Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)
9.1.5. Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus
9.1.6. Chapter 2.2.5. Infection with infectious myonecrosis virus
9.1.7. Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)
9.1.8. Chapter 2.2.7. Infection with taura syndrome virus
9.1.9. Chapter 2.2.8. Infection with white spot syndrome virus
9.1.10. Chapter 2.2.9. Infection with yellow head virus genotype 1

9.2. Section 2.3. Diseases of fish

9.2.1. Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)
9.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus
9.2.3. Chapter 2.3.7. Infection with red sea bream iridoviral disease
9.2.4. Section 2.2.2. of Chapter 2.3.9. Infection with spring viraemia of carp virus

9.3. Section 2.4. Diseases of molluscs

9.3.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*

9.4. Proposed table of PCR parameters to harmonise PCR protocols

10. Items for AAC Discussion

10.1. Develop a mechanism to speed up the process of making updates to diagnostic methods in the *Aquatic Manual* available to Members quicker

11. *Ad hoc Groups*

11.1. Report of the a*d hoc G*roup on Susceptibility of mollusc species to infection with OIE listed diseases
11.2. Interim report of the a*d hoc* Group on Susceptibility of fish species to infection with OIE listed diseases
11.3. Interim report of the a*d hoc* Group on new draft chapters on emergency disease preparedness and disease outbreak management

12. Reference Centres or change of experts

12.1. Evaluation of applications for Reference Centres for aquatic animal health issues or change of experts

13. Other issues

13.1. For Discussion

13.1.1. Registration of Diagnostic Kits

13.1.1.1. IQ PlusTM WSSV Kit
13.1.1.2. IQ 2000TM WSSV
13.1.1.3. WSSV LFT
13.1.1.4. Genic Shrimp Multipath testing package

13.1.2. Self declarations of freedom procedures
13.1.3. Feb Report – Part A/B

13.2. For Information

13.2.1. OIE Research Coordination
13.2.2. Global Burden of Animal Diseases (GBADs)

14. Meeting review

15. Next meeting : 15–22 February 2023

Annex 2. Item 2 – List of Participants

MEETING OF THE WOAH AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Hybrid, 14 to 21 September 2022

MEMBERS OF THE COMMISSION

Dr Ingo Ernst
(President)
Director Aquatic Pest and Health Policy,
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Dr Prof. Hong Liu
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Deputy Director,
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Legislative Officer,
European Commission,
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Professor in Virology,
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Ås,
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OTHER PARTICIPANTS

Dr Mark Crane
CSIRO Honorary Fellow,
Research Group Leader | AAHL Fish Diseases Laboratory
Australian Centre for Disease Preparedness (ACDP) I CSIRO,
Geelong,
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WOAH HEADQUARTERS

Dr Gillian Mylrea
Head
Standards Department

Dr Stian Johnsen
Chargé de mission
Standards Department

Ms Sara Linnane
Scientific Officer – International Standards
Science Department

Dr Bernita Giffin
Scientific Coordinator for Aquatic Animal Health
Standards Department

Dr Gounalan Pavade
Scientific Coordinator
Science Department

Annex 3. Item 4. – Work plan and priorities

WORK PLAN FOR THE AQUATIC ANIMALS COMMISSION

On-going work for adoption in 2023 or later

Aquatic Code			
Chapter/Subject	**Status**		
	September 2022	**February 2023**	**May GS 2023**
Monitor emerging diseases and consider any required actions	On-going		
Glossary definitions: 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services'		Review usage in the *Aquatic Code* and present amendments for comments	
Chapter 1.3. Diseases listed by OIE – Listing of infection with infectious spleen and kidney necrosis virus	Draft assessment for listing and present for comments.	Review Member comments	
SOP for Self Declaration of freedom		Draft template for Member guidance for submission of a self-declaration of freedom	
Chapter 4.3. Application of Compartmentalisation	Member questionnaire	Review Member responses to inform approach for amendments to Chapter 4.3.	
Chapter 4.X. New draft chapter on Emergency disease preparedness		Review draft Chapter 4.X.	
Chapter 4.Y. New draft chapter on Disease outbreak management		Review draft Chapter 4.Y.	
Chapter 5.2. Certification Procedures		Discuss plan for amendments	
Chapters 5.6. – 5.9.		Review TCC *ad hoc* Group report	
Chapter 5.X. Trade in ornamental aquatic animals	Develop a plan for drafting the new chapter	Review draft outline for chapter	
Chapter 5.Y. Trade in genetic materials	Develop a plan for drafting the new chapter	Review draft outline for chapter	
Safe commodities – disease specific chapters –Articles X.X.3.		Review updated safe commodities assessments and amended articles. Present amended articles for comment.	

Assessment of default periods in Articles X.X.4.-X.X.8. for disease-specific chapters	Establish approach for assessment of default periods	Present assessment of default periods for comments	
Susceptible Species – Crustacean diseases – Articles 9.X.1. and 9.X.2. for: – infection with decapod iridescent virus – infection with *Aphanomyces astaci* (Crayfish plague)		Re-convene AHG	
Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)	Review Comments (1st round)	Review Comments (2nd round)	Propose for adoption
Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)	Review Comments (1st round)	Review Comments (2nd round)	Propose for adoption
Article 9.4.2. of Chapter 9.5. Infection with infectious myonecrosis virus (IMNV)	Review amended article and present for comment	Review Comments (1st round)	Propose for adoption
Susceptible Species – Fish diseases – Articles 10.X.1. and 10.X.2. for: – Infection with Red seabream iridovirus – Infection with Tilapia lake virus – Infection with *Aphanomyces invadans* (Epizootic ulcerative syndrome	Review interim *ad hoc* Group report: Next *ad hoc* Group meeting planned for November 2022	Review amended articles for RSIV	
Articles 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus	Review *ad hoc* Group assessment, amended article and present for comment	Review Comments (1st round)	Propose for adoption
Susceptible Species Assessment of new species/evidence for previously assessed diseases as necessary	On-going		
Chapter 10.X. Infection with tilapia lake virus	Review draft chapter and present for comment	Review Comments (1st round)	Propose for adoption

Susceptible species – Mollusc diseases – Articles 11.X.1. and 11.X.2. for: – Infection with *Marteilia refringens* – Infection with *Perkinsus marinus* – Infection with *Xenohaliotis californiensis* – Infection with *Perkinsus olseni*	*Marteilia refringens* : Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption
	Next *ad hoc* Group meeting planned for November 2022	*Perkinsus marinus* : Review amended articles and present for comments	
Susceptible species – Articles 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*	Review amended article and present for comments	Review Comments (1st round)	Propose for adoption
Susceptible species – Articles 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*	Review amended article and present for comments	Review Comments (1st round)	Propose for adoption
Model articles 11.X.9.-11.X.14.: Harmonisation with other disease-specific chapters	Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption
Aquatic Manual			
Chapter/Subject	**Status**		
	September 2022	**February 2023**	**GS May 2023**
Section 2.2. General provisions – Crustaceans	Review amended Chapter and present for comments	Review comments (1st round)	
Chapter 2.2.1. Acute hepatopancreatic necrosis disease	Review comments (1st round)	Review Comments (2nd round)	Propose for adoption
Chapter 2.2.2. Infection with *Aphanomyces astaci* (Crayfish plague)	Review further updated draft and present for comments	Review comments (1st round)	
Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)	Review comments (1st round)	Review Comments (2nd round)	Propose for adoption
Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus	Review comments (1st round)	Review Comments (2nd round)	Propose for adoption
Chapter 2.2.5. Infection with infectious myonecrosis virus	Updated, reformatted and presented for comments	Review comments (1st round)	
Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)	Review updated draft	Review further updated draft and present for Member comments	
Chapter 2.2.7. Infection with taura syndrome virus	Updated, reformatted and presented for comments	Review comments (1st round)	

Chapter 2.2.8. Infection with white spot syndrome virus	Updated, reformatted and presented for comments	Review comments (1st round)	
Chapter 2.2.9. Infection with yellow head virus genotype 1	Updated, reformatted and reviewed	Review further updated draft and present for Member comments	
Chapter 2.2.X. Infection with decapod iridescent virus 1		Develop draft chapter for review	
Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)	Review comments (1st round)	Review Comments (2nd round)	Propose for adoption
Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus	Review comments (2nd round)	Review comments (3rd round)	Propose for adoption
Chapter 2.3.7. Infection with Red sea bream iridoviral disease		Review *ad hoc* Group report and revise in accordance with approach to listing	
Sections 2.2.2. of Chapter 2.3.9. Infection with spring viraemia of carp virus	Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption
Chapter 2.3.X. Infection with tilapia lake virus		Develop draft chapter for review	
Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*	Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption
Section 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*	Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption
Section 2.2.2. of Chapter 2.4.2. Infection with *Bonamia ostreae*	Review amended articles and present for comments	Review Comments (1st round)	Propose for adoption

Other prioritised items to commence before May 2024

Aquatic Code				
Chapter/Subject	**Status**	**First Priority**	**Second Priority**	**Next steps**
Chapter 1.3. Diseases listed by the OIE	Review any new diseases for listing or de-listing as necessary	On going		
Chapter 4.2. Zoning and Compartmentalisation	Re-develop chapter to focus solely on zoning		✓	
Aquatic Manual				
Chapter/Subject	**Status**	**First Priority**	**Second Priority**	**Next steps**
Section 2.4. General provisions – Molluscs	Review and update the introductory chapter on mollusc diseases		✓	

Chapters 2.4.X. Mollusc disease-specific chapters	Update and reformat chapters using the new template (all diseases)		✓	

Annex 4. Item 5.1. – Chapter 1.3. Diseases listed by the OIE – Listing of infection with Megalocytivirus

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

[…]

Article 1.3.1.

The following *diseases* of fish are listed by the OIE:

– Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)

– Infection with epizootic haematopoietic necrosis virus

– Infection with *Gyrodactylus salaris*

– Infection with HPR-deleted or HPR0 infectious salmon anaemia virus

– Infection with infectious haematopoietic necrosis virus

– Infection with *Infectious spleen and kidney necrosis virus*

– Infection with koi herpesvirus

– ~~Infection with red sea bream iridovirus~~

– Infection with salmonid alphavirus

– Infection with spring viraemia of carp virus

– Infection with tilapia lake virus

– Infection with viral haemorrhagic septicaemia virus.

[…]

Annex 5. Item 5.1. – Chapter 1.3. Diseases listed by the OIE – Listing of infection with Megalocytivirus

ASSESSMENT OF INFECTION WITH INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS (ISKNV) FOR LISTING IN THE WOAH *AQUATIC ANIMAL HEALTH CODE*

Assessment summary

1. The Aquatic Animal Health Standards Commission assessed the virus species *Infectious spleen and kidney necrosis virus*, including its three genogroups red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV), and turbot reddish body iridovirus (TRBIV) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code.*

2. The Aquatic Animals Commission agreed that the RSIV genogroup (currently listed in the *Aquatic Code*), as well as the two genogroups ISKNV and TRBIV meet the listing criteria 1, 2, 3, and 4b (see Table 1 below).

3. The Aquatic Animals Commission noted that the three genogroups have overlapping susceptible species, similar epidemiology, and similar diagnostic methods. The Commission agreed that the proposed listed disease should be named "infection with ISKNV". Infection with ISKNV would be defined to include the genogroups ISKNV, RSIV and TRBIV but would exclude the other recognized species of *Megalocytivirus*, *Scale drop disease virus*.

	Listing criteria						Conclusion
	1	2	3	4a	4b	4c	
Infection with ISKNV	+	+	+	NA	+	-	The disease meets the criteria for listing.

NA = not applicable.

Listing Criteria (Chapter 1.2. of the *Aquatic Code*)

The criteria for the inclusion of a disease in the OIE list are as follows:

1. International spread of the pathogenic agent (via aquatic animals, aquatic animal products, vectors or fomites) is likely.

AND

2. At least one country may demonstrate country or zone freedom from the disease in susceptible aquatic animals, based on provisions of Chapter 1.4.

AND

3. A precise case definition is available and a reliable means of detection and diagnosis exists.

AND

4a. Natural transmission to humans has been proven, and human infection is associated with severe consequences.

OR

4b. The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level.

OR

4c. The disease has been shown to, or scientific evidence indicates that it would affect the health of wild resulting in significant consequences e.g. morbidity or mortality at a population level, reduced productivity or ecological impacts.

Background

Megalocytivirus is one of seven genera of the family *Iridoviridae* and is classified within the subfamily Alphairidovirinae together with the genera *Ranavirus* and *Lymphocystivirus* (Chinchar ***et al***., 2017; Chinchar ***et al***., 2020). Megalocytiviruses are distinguished from ranaviruses and lymphocystiviruses by their ability to trigger marked cell enlargement in infected tissues and by sequence analysis of key viral genes (Chinchar ***et al***., 2017). Megalocytiviruses are the aetiological agents of severe disease associated with high mortality in a range of marine and freshwater finfish species (Kurita & Nakajima, 2012).

The ICTV recognises two species of *Megalocytivirus*: *Infectious spleen and kidney necrosis virus* (ISKNV) and *Scale drop disease virus* (SDDV) (Chinchar ***et al***., 2017). SDDV is genetically and epidemiologically distinct from the species ISKNV and is not considered further in this assessment.

Within the species ISKNV, three genogroups have been recognised: ISKNV, RSIV and TRBIV (Song ***et al***., 2008). However, it remains to be resolved whether these genogroups represent distinct species, or strains of a single species (Chinchar ***et al***., 2017). Megalocytiviruses have been given numerous unique names based on the species that they were detected in; however, all variants of the species ISKNV that have had their genomes analysed are placed within the three genogroups (ISKNV, RSIV and TRBIV) (Chinchar ***et al***., 2017).

The name ISKNV is used for one of two recognised species of *Megalocytivirus* and also for one of the three genogroups within that species. When used within this document, "ISKNV" refers to the genogroup ISKNV. "The species ISKNV" is used whenever referring to the species.

Red sea bream iridovirus (RSIV) was first listed by WOAH in the 2003 *Aquatic Animal Health Code*[1] and remains listed in the 2022 *Aquatic Code*. Disease caused by RSIV was first detected in cultured red sea bream (*Pagrus major*) in Japan in 1990 (Inouye ***et al***., 1992). RSIV has been detected principally from marine fish. Species currently listed as susceptible to infection with RSIV in the WOAH *Aquatic Code* include[2]: red sea bream (*Pagrus major*), yellowtail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), sea bass (*Lateolabrax* sp.), Asian sea bass (*Lates calcarifer*), albacore (*Thunnus thynnus*), Japanese parrotfish (*Oplegnathus fasciatus*), striped jack (*Caranx delicatissimus*), mandarin fish (*Siniperca chuatsi*), red drum (*Sciaenops ocellatus*), mullet (*Mugil cephalus*) and groupers (*Epinephelus spp.*).

The genogroup ISKNV is not currently listed in the WOAH *Aquatic Code*. Virions morphologically consistent with iridoviruses and presenting enlarged cells with inclusion bodies consistent with megalocytiviruses have been reported in species of freshwater fish since the late 1980s and 1990s (e.g. Armstrong & Ferguson, 1989; Anderson ***et al.***, 1993). ISKNV has been detected in archival ornamental fish samples from as early as 1996 (Go ***et al***., 2006; Go ***et al***., 2016). Infectious spleen and kidney necrosis disease was described from mandarin fish (*Siniperca chuatsi*; He ***et al***., 2000; He ***et al***., 2002) and in 2001 the genome of ISKNV was analysed and found to be genetically similar to RSIV (He ***et al***., 2001). ISKNV has been detected from numerous freshwater fish species, including many associated with ornamental fish trade (see review by Johan & Zainathan, 2020). This genotype has been reported from numerous species of ornamental fish that have been traded internationally. ISKNV has also been reported as a cause of mass mortality in species important for human consumption (e.g. Subramaniam ***et al.***, 2016; Ramírez-Paredes ***et al.***, 2020).

The genogroup turbot reddish body iridovirus (TRBIV) is not currently listed in the WOAH *Aquatic Code*. TRBIV was first described as causing disease in turbot, *Scophthalmus maximus* (Shi ***et al.,*** 2004). TRBIV has been known to principally cause disease in flatfishes in China and Korea (e.g. Shi ***et al.,*** 2004; Do ***et al.,*** 2005) but it has also been detected in other species including in the ornamental fish trade (Go ***et al***., 2016; Koda ***et al***., 2018). TRBIV has also caused disease in other economically important farmed fish species such as Asian sea perch (*Lates calcarifer*) (Tsai ***et al.***, 2020) and barred knifejaw (*Oplegnathus fasciatus*) (Huang ***et al.***, 2011).

The Aquatic Animals Commission previously proposed an approach to differentiating pathogen strains (refer to the Commission's February and October 2011 meeting reports). Three main criteria were considered for the applicability of pathogen strain differentiation in the standards of the *Aquatic Code* and *Aquatic Manual*: 1) variants of the pathogen are clearly recognized in the scientific literature and have different disease characteristics; 2) there are robust methods for consistently differentiating the variants; and 3) there is, or there is potential for, different management of variants within or between countries. In the case of the species ISKNV, RSIV was listed prior to research that defined the 3 genogroups within the species ISKNV, and the genetic and epidemiological relationships among them. Given the precedent of infection with RSIV having been listed, but not ISKNV and TRBIV, this assessment presents information for each of these three genogroups, despite the three genogroups, being proposed for listing collectively as the species ISKNV.

Assessment against listing criteria

[1] RSIV was included in the *Aquatic Code* prior to 2003 as an "other disease of significance".
[2] Note that an assessment of species listed as susceptible to infection with RSIV in accordance with Chapter 1.5. of the *Aquatic Code* has not yet been completed.

Criterion No. 1. International spread of the pathogenic agent (via aquatic animals, aquatic animal products, vectors or fomites) is likely.

Assessment

The species ISKNV can be transmitted horizontally via water and is known to remain viable in frozen host tissues. The likelihood of transmission is expected to be greater for trade in live fish but is also possible in aquatic animal products, particularly if not eviscerated.

Numerous marine and freshwater species are susceptible to the species ISKNV and are traded internationally, either as live aquatic animals (for human consumption, aquaculture or for ornamental purposes) or as aquatic animal products.

RSIV has been detected in several countries in Asia where it has been associated with disease in species of farmed marine fish (Kurita & Nakajima, 2012). Some susceptible species are traded live for human consumption (e.g. red sea bream, groupers), others are traded as aquatic animal products.

ISKNV has been detected in numerous species traded as ornamental fish and the ornamental fish trade has been implicated in disease spread and outbreaks (e.g. Jeong *et al.*, 2008; Johan & Zainathan, 2020). Infected ornamental fish may not present clinical signs (e.g. Subramaniam *et al.*, 2014) and as such may act as carriers of the virus. ISKNV has also been detected in important farmed species for human consumption that are traded internationally, such as tilapia (Ramírez-Paredes *et al.*, 2020). ISKNV has also been detected in unprocessed fish used for aquaculture feed (Lajimin *et al.*, 2015) suggesting that fish traded for aquaculture feed or bait may present a pathway. Transmission from freshwater finfish species to marine finfish species has been demonstrated by direct inoculation and cohabitation (Jeong *et al.*, 2008b; Go & Whittington, 2019).

TRBIV is known to occur in several species that are important for international trade (e.g. turbot, flounder, Asian sea bass), including live trade or as aquatic animal products. Phylogenetic analysis indicates that there has been recent international spread of TRBIV (Tsai *et al.*, 2020).

Variants of the species ISKNV have been detected in numerous species of marine and freshwater species that are traded internationally. Each of the three genogroups has been detected in traded commodities and there is evidence of international spread associated with trade.

Conclusion

The criterion is met.

Criterion No. 2. At least one country may demonstrate country or zone freedom from the disease in susceptible aquatic animals, based on provisions of Chapter 1.4.

Assessment

Infection with RSIV has been notifiable to the WOAH since 2003. Several countries continue to report that RSIV has never been reported from their territory (refer to WOAH World Animal Health Information System) and it is likely that some of these countries could demonstrate country freedom.

ISKNV has been reported from numerous fish species traded through the ornamental fish trade and it is likely that this genogroup is widespread through ornamental fish supply chains. However, some countries maintain *basic biosecurity measures*[3] for ISKNV and may be able to demonstrate freedom. Further, PCR assays used in surveillance for RSIV would also detect ISKNV, providing evidence of freedom from ISKNV.

TRBIV has been primarily detected in farmed flatfish from China and Korea but has also been detected in ornamental fish and in farmed Asian sea bass. PCR assays recommended in the WOAH Aquatic Manual chapter for RSIV may not be inclusive of TRBIV resulting in a lower confidence in the distribution of TRBIV. However, given TRBIV has demonstrated pathogenicity in farmed populations of several species, it is likely that TRBIV would be detected in those species if it had occurred. Although there is less certainty regarding the distribution of TRBIV, it seems likely that at least one country could claim freedom at the level of country or zone.

[3] Basic Biosecurity conditions are defined in article 1.4.6. of the Aquatic Code and include requirements for an early detection system (as described in Article 1.4.7.) and measures to prevent the introduction of the pathogenic agent.

Conclusion

The criterion is met.

Criterion No. 3. A precise case definition is available and a reliable means of detection and diagnosis exists

Assessment

Case definitions for suspicion and confirmation of infection with RSIV are available in the WOAH *Aquatic Manual*. As most PCR assays for RSIV (and some other methods, e.g. histopathology), are inclusive of ISKNV, the case definitions could be easily adapted to include ISKNV. Kawato *et al.* (2021) compared the analytical performance of four real-time PCR methods for the detection of megalocytiviruses (excluding SDDV) and found that three of the four assays detected ISKNV, RSIV, and TRBIV targets. Kim *et al.* (2022) reported on the performance of a real-time PCR assay with inclusivity for RSIV, ISKNV and TRBIV. There are sufficient diagnostic tools available to detect the species ISKNV and to construct case definitions inclusive of the three genogroups.

Conclusion

Criterion is met.

Criterion No. 4a Natural transmission to humans has been proven, and human infection is associated with severe consequences.

Assessment

There is no evidence of transmission to humans.

Conclusion

Criterion not applicable.

Criterion No. 4b The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level.

Assessment

RSIV has caused mass mortalities in cultured fish populations. The disease was first detected in red sea bream in Japan with affected fish becoming lethargic, exhibiting severe anaemia, petechiae of the gills, and enlargement of the spleen (Inouye *et al*., 1992; Jung *et al*., 1997; Nakajima & Maeno, 1998). RSIV has been reported to cause production losses, morbidity and mortality in many other species (e.g. Chao *et al*., 2004; Chen *et al*., 2003; Girisha *et al*., 2020; Ni *et al*., 2021; Sumithra *et al*., 2022).

ISKNV has been associated with numerous cases of disease in ornamental fish (see review by Johan & Zainathan*,* 2020). ISKNV has also been associated with high mortalities in important species farmed for human consumption; for example, in Asian sea bass (Dong *et al.,* 2017; Kerddee *et al*., 2021), Tilapia (e.g. Figueiredo *et al*., 2021; Ramírez-Paredes *et al*., 2021) and groupers (e.g. Chao *et al*., 2004; Huang *et al*., 2020).

TRBIV has caused disease and high mortality in turbot aquaculture in China (e.g. Shi *et al*., 2010). Mortalities of up to 90% have occurred in Asian sea bass farms in Taiwan (Tsai *et al.,* 2020).

Conclusion

Criterion is met.

Criterion No. 4c The disease has been shown to, or scientific evidence indicates that it would affect the health of wild resulting in significant consequences e.g. morbidity or mortality at a population level, reduced productivity or ecological impacts.

Assessment

There is limited information on the occurrence of RSIV, ISKNV or TRBIV in wild fish populations and their consequences such as morbidity, mortality or ecological impacts. ISKNV has been reported as the cause of a mass mortality event in a

population of wild cichlids in India (Swaminathan *et al*., 2022), but has also been detected in many apparently healthy wild fish from a diverse range of fish species (Wang *et al*., 2007).

Conclusion

Criterion is not met.

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CHAPTER 9.3.

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

Article 9.3.1.

For the purposes of the *Aquatic Code*, infection with *Hepatobacter penaei* (necrotising hepatopancreatitis) means *infection* with the *pathogenic agent Candidatus* ~~*Hepatobacter penaei*~~ Hepatobacter penaei, an obligate intracellular bacterium of the Family Holosporaceae of the Order Rickettsiales~~alpha-Proteobacteria~~. ~~The *disease* is commonly known as necrotising hepatopancreatitis.~~

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

[…]

Annex 7. Item 5.3. – Article 9.4.1. and 9.4.2. of Chapter 9.4. Infection with IHHNV

CHAPTER 9.4.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

Article 9.4.1.

For the purposes of the *Aquatic Code*, infection with infectious hypodermal and haematopoietic necrosis virus means *infection* with the *pathogenic agent Decapod ~~penstyldensovirus~~penstylhamaparvovirus* 1, ~~commonly known as infectious hypodermal and haematopoietic necrosis virus (IHHNV),~~ of the Genus ~~*Penstyldensovirus*~~*Penstylhamaparvovirus* and Family *Parvoviridae*.

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

Article 9.4.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: blue shrimp (*Penaeus stylirostris*), giant tiger prawn (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), yellowleg shrimp (*Penaeus californiensis*), ~~giant tiger prawn (*Penaeus monodon*)~~, ~~northern white shrimp (*Penaeus setiferus*)~~, ~~blue shrimp(*Penaeus stylirostris*)~~ and whiteleg shrimp (*Penaeus vannamei*).

[…]

Annex 8. Item 5.4. – Article 9.5.2. of Chapter 9.5. Infection with IMNV

CHAPTER 9.5.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

[…]

Article 9.5.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: ~~brown tiger prawn (*Penaeus esculentus*),~~ banana prawn (*Penaeus merguiensis*), brown tiger prawn (*Penaeus esculentus*) and whiteleg shrimp (*Penaeus vannamei*).

[…]

Annex 9. Item 5.5. – Article 10.9.2. of Chapter 10.9. Infection with SVCV

CHAPTER 10.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[…]

Article 10.9.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.:

Family	Scientific name	Common name
Cyprinidae	*Abramis brama*	Bream
	Aristichthys nobilis	Bighead carp
	Carassius auratus	Goldfish
	Ctenopharyngodon idella	Grass carp
	Cyprinus carpio	Common carp (all varieties and subspecies)
	Danio rerio	Zebrafish
	Notemigonus crysoleucas	Golden shiner
	Pimephales promelas	Fathead minnow
	Percocypris pingi	Jinsha bass carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	Roach
Siluridae	Silurus glanis	Wels catfish

[…]

ASSESSMENT OF JINSHA BASS CARP (*PERCOCYPRIS PINGI)* AS SUSCEPTIBLE SPECIES TO INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

Background

In response to a comment requesting an assessment of Jinsha bass carp (*Percocypris pingi*) as a susceptible species for infection with spring viraemia of carp virus, the Aquatic Animals Commission requested that the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases (the *ad hoc* Group) review the scientific evidence and provide a recommendation to the Commission for decision.

Methodology

- The *ad hoc* Group applied criteria, as outlined in Article 1.5.3 of the *Aquatic Code*, to assess Jinsha bass carp (*Percocypris pingi*) in order to determine susceptibility to infection with spring viraemia of carp virus (SVCV). The same methodology and considerations outlined in the *ad hoc* Group report (https://www.woah.org/app/uploads/2021/10/a-ahg-susceptibility-of-fish-november-2017.pdf) was applied to these assessments.

Assessments of host susceptibility to infection with spring viraemia of carp virus

Results

The *ad hoc* Group agreed that Jinsha bass carp (*Percocypris pingi*) met the criteria for listing as susceptible to infection with spring viraemia of carp virus in accordance with Chapter 1.5. of the *Aquatic Code* and was proposed to be added to Article 10.9.2.

Table 1. Assessment for Jinsha bass carp (*Percocypris pingi*) for susceptibility to infection with spring viraemia of carp virus.

Family	Scientific name	Common name	Stages 1: Route of infection	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Individual Outcome	References
					A	B	C	D		
Overall Score 1										
Cyprinidae	*Percocypris pingi*	Jinsha bass carp	N	Culture + subsequent sequencing	Y	Y	Y	Y	1	ZHENG *et al.*, 2018

Family	Scientific name	Common name	Stages 1: Route of infection	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Individual Outcome	References
					A	B	C	D		
Cyprinidae	*Percocypris pingi*	Jinsha bass carp	E/I						ND	ZHENG *et al*., 2018

Assessment Table Key

N: Natural infection
E: Experimental (non-invasive)
EI: Experimental (invasive)
YES: Demonstrates criterion is met
NO: Criterion is not met.
ND: Not determined.

***Ad hoc* Group comments**

- The *ad hoc* Group agreed that despite the fact that there was only one paper to assess, the evidence provided by Zheng *et al*. (2018), a single strong study (natural outbreak with pathology, virus isolation and virus identification by sequence analysis) with an outcome of '1', was sufficient to conclude susceptibility in the absence of conflicting evidence.

- The *ad hoc* Group also considered that Jinsha bass carp (*Percocypris pingi*) belongs to the Cyprinidae family which contains other susceptible species. Sequence analysis indicates that the virus belongs to the Ia genogroup which contains other Chinese isolates of SVCV.

- Zheng *et al.* (2018) concerned a natural outbreak. The experimental infection was only part of the study and was not included in the assessment due to it being 'invasive'. The assessment of the invasive experimental procedure was not progressed past Stage 1 (i.e. Article 1.5.4.).

References:

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CHAPTER 10.X.

INFECTION WITH TILAPIA LAKE VIRUS

Article 10.X.1.

For the purposes of the *Aquatic Code*, infection with tilapia lake virus (TiLV) means *infection* with the *pathogenic agent Tilapia tilapinevirus*, of the Genus *Tilapinevirus* and the Family *Amnoonviridae*.

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

Article 10.X.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: [blue tilapia (*Oreochromis aureus*), Malaysian red hybrid tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*), Mango tilapia (*Sarotherodon galilaeus)*, Mozambique tilapia (*Oreochromis mossambicus*), Nile tilapia (*Oreochromis niloticus*), redbelly tilapia (*Tilapia zilli*), tinfoil barb (*Barbonymus schwanenfeldii*), Tvarnun simon (*Tristramella simonis*) and blue-nile tilapia hybrid (*Oreochromis niloticus* X *Oreochromis aureus*)] (under study).

Article 10.X.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with TiLV status of the exporting country, zone or compartment

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to TiLV, regardless of the infection with TILV status of the *exporting country*, *zone* or *compartment*:

1) [*aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 56°C for at least five minutes, or a time/temperature equivalent that inactivates TiLV;

2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 56°C for at least five minutes, or a time/temperature equivalent that inactivates TiLV] (under study)

3) fish oil;

4) fish skin leather.

Article 10.X.4.

Requirements for self-declaration of freedom from infection with TiLV

A Member Country may make a self-declaration of freedom from infection with TILV for the entire country, a *zone* or a *compartment* in accordance with the provisions of Articles 10.X.5. to 10.X.8., as relevant. The self-declaration of freedom must be made in accordance with other relevant requirements of the *Aquatic Code,* including that the Member Country meet the following conditions:

1) complies with the provisions of Chapter 3.1.; and

2) uses appropriate methods of *diagnosis*, as recommended in the *Aquatic Manual*; and

3) meets all requirements of Chapter 1.4. that are relevant to the self-declaration of freedom.

Article 10.X.5.

Country free from infection with TILV

If a country shares water bodies with other countries, it can only make a self-declaration of freedom from infection with TILV if all shared water bodies are within countries or *zones* declared free from infection with TILV (see Article 10.X.6.).

As described in Article 1.4.4., a Member Country may make a self-declaration of freedom from infection with TILV for its entire *territory* if it can demonstrate that:

1) none of the *susceptible species* referred to in Article 10.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [six] months;

OR

2) there has been no occurrence of infection with TILV for at least the last [ten] years, and:

   a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with TILV, as described in the corresponding chapter of the *Aquatic Manual*; and

   b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for at least the last [ten] years;

OR

3) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of TILV, and *basic biosecurity conditions* have been continuously met for at least [one] year prior to commencement of *targeted surveillance*;

OR

4) it previously made a self-declaration of freedom from infection with TILV and subsequently lost its free status due to the detection of TILV but the following conditions have been met:

   a) on detection of TILV, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of TILV, and the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed followed by fallowing as described in Chapter 4.7.; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with TILV; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for:

      i) at least the last [two] years in wild or farmed *susceptible species* without detection of TILV; or

      ii) at least the last [one] year without detection of TILV if affected *aquaculture establishments* were not epidemiologically connected to wild populations of *susceptible species*.

   In the meantime, part or all of the country, apart from the *infected* and *protection zones,* may be declared a free *zone* provided that such a part meets the conditions in point 2 of Article 10.X.6.

Article 10.X.6.

Zone free from infection with TILV

If a *zone* extends over the *territory* of more than one country, it can only be declared a *zone* free from infection with TILV if all of the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.X., a Member Country may make a self-declaration of freedom from infection with TILV for a *zone* within its *territory* if it can demonstrate that:

1) none of the *susceptible species* referred to in Article 10.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [six] months;

OR

2) there has been no occurrence of infection with TILV for at least the last [ten] years, and:

   a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with TILV, as described in Article 1.4.8. of Chapter 1.4.; and

   b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for the *zone* for at least the last [ten] years;

OR

3) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *zone* for at least the last [two] years without detection of TILV, and *basic biosecurity conditions* have been continuously met for at least [one] year prior to commencement of *targeted surveillance*;

OR

4) it previously made a self-declaration of freedom for a *zone* from infection with TILV and subsequently lost its free status due to the detection of TILV in the *zone* but the following conditions have been met:

   a) on detection of TILV, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of TILV, and the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed followed by fallowing as described in Chapter 4.7.; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with TILV; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of TILV.

Article 10.X.7.

Compartment free from infection with TILV

As described in Article 1.4.4., a Member Country may make a self-declaration of freedom from infection with TILV for a *compartment* within its *territory* if it can demonstrate that:

1) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *compartment* for at least the last [one] year without detection of TILV, and *basic biosecurity conditions* have been continuously met for at least [one] year prior to commencement of *targeted surveillance*;

OR

2) it previously made a self-declaration of freedom for a *compartment* from infection with TILV and subsequently lost its free status due to the detection of TILV in the *compartment* but the following conditions have been met:

   a) all *aquatic animals* within the *compartment* have been killed and disposed of by means that minimise the likelihood of further transmission of TILV, the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed, and the *compartment* has been fallowed as described in Chapter 4.7.; and

   b) previously existing *basic biosecurity conditions*, including the *compartment biosecurity plan*, have been reviewed and modified as necessary and have continuously been in place from the time of restocking with *aquatic animals* from an approved pathogen free source in accordance with the requirements of Articles 10.X.9. and 10.X.10. as appropriate; and

   c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [one] year without detection of TILV.

Article 10.X.8.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TILV following the provisions of Articles 10.X.4. to 10.X.7. (as relevant) may maintain its status as free from infection with TILV provided that the requirements described in Article 1.4.15. are continuously maintained.

Article 10.X.9.

Importation of aquatic animals or aquatic animal products from a country, zone or compartment declared free from infection with TILV

When importing *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with TILV, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country*. The *international aquatic animal health certificate* should state that, on the basis of the procedures described in Articles 10.X.5., 10.X.6. or 10.X.7. (as applicable) and 10.X.8., the place of production of the *aquatic animals* or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with TILV.

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal products* listed in Article 10.X.3.

Article 10.X.10.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with TILV

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving *quarantine* (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal*

products referred to in Article 10.X.3. or other products authorised by the *Competent Authority*; and

c) the treatment of all transport water, equipment, effluent and waste materials to inactivate TILV in accordance with Chapters 4.4., 4.8. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

a) In the *exporting country*:

i) identify potential source populations and evaluate their *aquatic animal* health records;

ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with TILV.

b) In the *importing country*:

i) import the F-0 population into a *quarantine* facility;

ii) test the F-0 population for TILV in accordance with Chapter 1.4. to determine their suitability as broodstock;

iii) produce a first generation (F-1) population in *quarantine*;

iv) culture the F-1 population in *quarantine* for a duration sufficient for, and under conditions that are conducive to, the clinical expression of infection with TILV, and sample and test for TILV in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter X.X.6. of the *Aquatic Manual*;

v) if TILV is not detected in the F-1 population, it may be defined as free from infection with TILV and may be released from *quarantine*;

vi) if TILV is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.8.

Article 10.X.11.

Importation of aquatic animals or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with TILV

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing into one of the products referred to in Article 10.X.3. or in point 1 of Article 10.X.14., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

For these *aquatic animals* or *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product* being used for any purpose other than for human consumption.

Article 10.X.12.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption, including animal feed and agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with TILV

When importing *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, intended for uses other than human consumption, including animal *feed* and agricultural, industrial, research or pharmaceutical use, from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in Article 10.X.3. or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

Article 10.X.13.

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with TILV

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of TILV or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.; and

4) the carcasses are disposed of in accordance with Chapter 4.8.

Article 10.X.14.

Importation (or transit) of aquatic animal products for retail trade for human consumption regardless of the infection with TILV status of the exporting country, zone or compartment

1) [*Competent Authorities* should not require any conditions related to TILV, regardless of the infection with TILV status of the *exporting country*, *zone* or *compartment*, when authorising the importation (or transit) of the following commodities that have been prepared and packaged for retail trade and comply with Article 5.4.2.:

   a) fish fillets or steaks (chilled)] (under study).

   Certain assumptions have been made in assessing the safety of the *aquatic animal products* mentioned above. Member Countries should refer to these assumptions at Article 5.4.2. and consider whether the assumptions apply to their conditions.

   For these *aquatic animal products* Member Countries may wish to consider introducing internal measures

to address the *risks* associated with the *aquatic animal product* being used for any purpose other than for human consumption.

2) When importing *aquatic animal products*, other than those referred to in point 1 above, derived from a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

CHAPTER 11.2.

INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: Argentinean flat oyster (*Ostrea puelchana*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*), Olympia oyster (*Ostrea lurida*) and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*).

[…]

CHAPTER 11.3.

INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: European flat oyster (*Ostrea edulis*), Chilean flat oyster (*Ostrea chilensis*) and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*).

[…]

CHAPTER 11.4.

INFECTION WITH *MARTEILIA REFRINGENS*

Article 11.4.1.

For the purposes of the *Aquatic Code*, infection with *Marteilia refringens* means *infection* with the *pathogenic agent* *M. refringens* of the Family Marteiliidae.

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

Article 11.4.2.

Scope

The recommendations in this chapter apply to: blue mussel (*Mytilus edulis*), dwarf oyster (*Ostrea stentina*), European flat oyster (*Ostrea edulis*), European razor clam (*Solen marginatus*), golden mussel (*Xenostrobus securis*), ~~Australian mud oyster (*Ostrea angasi*), Argentinean oyster (*Ostrea puelchana*), Chilean flat oyster (*Ostrea chilensis*), blue mussel (*Mytilus edulis*) and~~ Mediterranean mussel (*Mytilus galloprovincialis*) and striped venus (*Chamelea gallina*). ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

[…]

Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters

CHAPTER 11.X.

INFECTION WITH [PATHOGEN X]

[…]

Article 11.X.9.

Importation of aquatic animals orand aquatic animal products from a country, zone or compartment declared free from infection with [Pathogen X]

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 11.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country*. The *international aquatic animal health certificate* should state that, or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 11.X.45., or 11.X.56. or 11.X.7. (as applicable) and 11.X.68., the place of production of the *aquatic animals* orand *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with [Pathogen X].

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal productscommodities* referred to in point 1 of Article 11.X.3.

Article 11.X.10.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving *quarantine* (either in the original facility or following biosecure transport to another *quarantine* facility), the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1 of Article 11.X.3. or other products authorised by the *Competent Authority*; and

   c) the treatment of all transport water, equipment, effluent and waste materials to inactive [Pathogen X] in accordance with Chapters 4.4., 4.8. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

a) In the *exporting country*:

i) identify potential source populations and evaluate their *aquatic animal* health records;

ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with [Pathogen X].

b) In the *importing country*:

i) import the F-0 population into a *quarantine* facility;

ii) test the F-0 population for [Pathogen X] in accordance with Chapter 1.4. to determine their suitability as broodstock;

iii) produce a first generation (F-1) population in *quarantine*;

iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with [Pathogen X] (as described in Chapter 2.4.X. of the *Aquatic Manual*) and test for [Pathogen X] in accordance with Chapter1.4.;

v) if [Pathogen X] is not detected in the F-1 population, it may be defined as free from infection with [Pathogen X] and may be released from *quarantine*;

vi) if [Pathogen X] is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner.

Article 11.X.11.

Importation of aquatic animals or~~and~~ aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for processing for human consumption, *aquatic animals* of species referred to in Article 11.X.2. or *aquatic animal products* derived thereof, ~~of species referred to in Article 11.X.2.~~from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 11.X.3., or ~~products described~~ in point 1 of Article 11.X.12., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that~~ to ensure~~s~~ inactivation of [Pathogen X] or ~~is~~ disposed of in a biosecure manner ~~that prevents contact of waste with *susceptible species*~~ in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

For these *aquatic animals* or *aquatic animal products* ~~*commodities*~~ Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product*~~*commodity*~~ being used for any purpose other than for human consumption.

Article 11.X.12.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption, including ~~in~~ animal feed, and ~~or for~~ agricultural, industrial, research or pharmaceutical use, from a country, zone

or compartment not declared free from infection with [Pathogen X]

When importing *aquatic animals* of a species referred to in Article 11.X.2., or *aquatic animal products* derived thereof, intended for uses other than human consumption, including in animal *feed* or for and agricultural, industrial, research or pharmaceutical use, *aquatic animals* of species referred to in Article 11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* facilities for slaughter and processing into products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers,* and packaging material used in transport and all effluent and waste materials from the processing are treated in a manner to that ensures inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

This article does not apply to *commodities* referred to in point 1 of Article 11.X.3.

Article 11.X.13.

[**Note**: this is a new article to align with other disease-specific chapters within the *Aquatic Code*.]

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 10.2.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.; and

4) the carcasses are disposed of in accordance with Chapter 4.8.

Article 11.X.1314.

Importation (or transit) of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from infection with [Pathogen X]

1) *Competent Authorities* should not require any conditions related to infection with [Pathogen X], regardless of the infection with [Pathogen X] status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of the following *commodities* which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

   a) [...]

   Certain assumptions have been made in assessing the safety of the *aquatic animal products* mentioned above. Member Countries should refer to these assumptions at Article 5.4.2. and consider whether the

assumptions apply to their conditions.

For these *aquatic animal products* ~~*commodities*~~ Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal products* ~~*commodity*~~ being used for any purpose other than for human consumption.

2) When importing ~~*aquatic animals* or~~ *aquatic animal products*, other than those referred to in point 1 above, derived from a ~~of~~ species referred to in Article 11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Annex 16. Item 5.10. – Member questionnaire for revision of Chapter 4.3. Application of Compartmentalisation

MEMBER QUESTIONNAIRE:

REVISION OF CHAPTER 4.3. APPLICATION OF COMPARTMENTALISATION

A questionnaire developed by the WOAH Aquatic Animal Health Standards Commission for Member comment.

Background

The need to revise Chapter 4.3. Application of Compartmentalisation has been included in the workplan of the Aquatic Animals Commission for some time as part of the progressive revision of Section 4 of the *Aquatic Code*. It was recognized as a need at the 2019 OIE Global Conference on Aquatic Animal Health and is included as an activity in the OIE Aquatic Animal Health Strategy (2021-2025).

At its February 2022 meeting, the Aquatic Animals Commission identified the revision of Chapter 4.3. Application of Compartmentalisation as a priority in its immediate workplan. The chapter would be redeveloped to focus solely on compartmentalization, improve the guidance to Members and to align with other new and revised chapters, such as Chapter 4.1. Biosecurity in aquaculture establishments. Of further relevance is the recently adopted Chapter 1.4. on surveillance and the associated model Articles X.X.4.-X.X.8. in disease-specific chapters on declaration of freedom, both of which have specific reference to the requirements for demonstrating and maintaining freedom at the Compartment level. Revision of Chapter 4.3. Application of Compartmentalisation is an appropriate next step.

The current Chapter 4.2. Zoning and Compartmentalisation, was adopted in 1995 and most recently revised in 2010 while Chapter 4.3. Application of Compartmentalisation, was adopted in 2010 and most recently revised in 2016. The experiences of Members in implementing these standards and with developing Compartments will be helpful to the Commission to inform the revision of the chapter. Members are invited to comment on their experiences with the standards on Compartmentalisation in the *Aquatic Code*. For convenience, several questions have been provided as a basis for Member responses.

Questions for Members

1) Have the relevant Competent Authority and Aquatic Animal Health Services in your country established Compartments? If so, what is the purpose of these compartments (e.g. hatchery production; aquatic animals or their products for human consumption; domestic or international trade).

2) What is the experience of your country for establishing compartments with respect to the following:

   a) Positive experiences (e.g. benefits for aquatic animal health management or trade);

   b) Any impediments to establishing compartments;

   c) Utility of the *Aquatic Code* standards on compartmentalisation (Chapters 4.2. and 4.3.) (e.g. useful information, gaps, particular emphasis or guidance that is required);

   d) Acceptance by international trade partners of the established Compartments.

3) Have you developed national compartmentalisation policies or procedures? If yes, would you be willing to share those policies or procedures with the Aquatic Animals Commission for consideration when revising Chapter 4.3.?

4) Have you consulted your industry about their interest in developing compartments? If yes, is there interest in establishing compartments? Which industry sectors have expressed interest? What is the intended purpose of the proposed compartments?

SECTION 2.2.

DISEASES OF CRUSTACEANS

CHAPTER 2.2.0.

GENERAL INFORMATION

A. SAMPLING

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material and the number of samples to be collected depend on the specific disease or pathogen, the size of animals and the objective of testing (i.e. diagnosis of overt disease, detection of subclinical infection in apparently healthy animals or sampling for targeted surveillance to demonstrate freedom from infection with a specified pathogen). See the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to crustacean populations

For details of animals to sample for a specific listed disease, see the relevant disease chapter in the *Aquatic Manual*. The design of a surveillance system for demonstrating disease-free status for a country, zone or compartment should be in accordance with the recommendations of the OIE *Aquatic Code* Chapter 1.4.

Animals to be sampled are selected as follows:

i) Susceptible species should be sampled proportionately or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. replacement with stocks of unknown disease status).

ii) If more than one water source is used for production, animals from all water sources should be included in the sample.

iii) For the study of presumptively diseased crustaceans select those animals that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal.

iv) When sampling is aimed at assessing disease occurrence (e.g. estimation of disease prevalence), the preferred selection method is probability sampling.

1.3. Specifications according to clinical status

In clinical disease episodes, carefully selected quality specimens with representative lesions should be obtained from live or moribund crustaceans. Collection of dead specimens during disease outbreaks should be avoided when possible, but recently dead samples may be suitable for some diagnostic assays. When cultured or wild crustacean stocks are presenting clinical signs of an active disease that are consistent with, or suggestive of, any one of the OIE-listed crustacean diseases, care should be taken to ensure that the samples collected are preserved appropriately for the anticipated diagnostic tests (see sample preservation section for recommended methods). In situations other than when clinical disease episodes are

investigated, for the OIE-listed diseases it is highly recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. Disease-specific recommendations are provided in Section 3 *Sample selection, sample collection, transportation and handling* of the individual chapters.

Recently dead crustaceans may be suitable (depending on their condition) for certain diagnostic assays such as nucleic acid detection techniques.

1.4. Specifications according to crustacean size

See the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

2. General processing of samples

2.1. Macroscopic examination

See disease-specific chapters in this *Aquatic Manual*.

2.2. Virological examination

2.2.1. Transportation and antibiotic treatment of samples

Culture systems for crustacean viruses are not available; antibiotic treatment of samples is not required. For transportation of samples see Section 3 of disease-specific chapters in this *Aquatic Manual*.

2.2.2. Virus isolation

For processing of tissues see Section 3 of disease-specific chapters in this *Aquatic Manual*.

2.2.3. Treatment to neutralise enzootic viruses

Not applicable.

2.3. Bacteriological examination

The strains of *Vibrio parahaemolyticus* (Vp_{AHPND}) that cause acute hepatopancreatic necrosis disease (AHPND) can be isolated on standard bacteriological media. *Hepatobacter penaei*, the causative agent of necrotising hepatopancreatitis (NHP) has not been cultured and, because of its very small size, bacteriological examination may be limited to Gram staining. See disease-specific chapters in this *Aquatic Manual* for identification methods.

2.4. Parasitic examination

Not applicable for currently listed diseases.

2.5. Fungal and other protists examination

See Chapter 2.2.2 *Infection with* Aphanomyces astaci (*Crayfish plague*).

B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF CRUSTACEAN PATHOGENS

1. Crustacean viruses

1.1. Crustacean cell lines

Not applicable. There are currently no confirmed or documented crustacean cell lines.

1.2. Culture media

Not applicable.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus nomenclature

In general, the virus nomenclature used in the disease-specific chapters follows the most recent taxonomy for viruses as given in the Report of the Committee on Taxonomy of Viruses (see: ICTV [ictvonline.org] for latest information). Also provided in the disease-specific chapters are the disease and virus names that are in common use by the shrimp/prawn farming industries, as well as the more common synonyms that have been used or are in current use.

1.3.2. Virus production

As no cell lines (crustacean, arthropod, or vertebrate) are known that can be used to produce crustacean viruses, infection of known susceptible host species (which are free of infection by the agent in question) is the preferred method for virus production for experimental purposes.

1.3.3. Virus preservation and storage

Infectivity of all of the OIE-listed crustacean viruses can be preserved by freezing infected whole crustaceans or infected target tissues at −20°C for short-term storage, or at −80°C or lower for long-term storage.

2. Crustacean bacteria

2.1. Culture media

See Chapter 2.2.1. Acute hepatopancreatic necrosis disease for details.

2.2. Storage of cultures

Lyophilisation or storage at −70°C is recommended for long-term storage of bacterial cultures.

3. Crustacean parasites

3.1. Culture media

Not applicable for currently listed diseases.

3.2. Storage of cultures

Not applicable for currently listed diseases.

4. Crustacean fungi and protists

4.1. Culture media

See chapter 2.2.2.

4.2. Storage of cultures

See chapter 2.2.2.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the OIE-listed crustacean diseases or detection of their aetiological agents are based on:

i) Gross and clinical signs.

ii) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands.

iii) Histology of fixed specimens.

iv) Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen.

v) Antibody-based tests for pathogen detection using specific antisera, polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs).

vi) Molecular methods (including sequencing):

DNA probes or RNA probes for *in-situ* hybridisation (ISH) assays with histological sections of fixed tissues;

Conventional and real-time PCR/RT-PCR and LAMP for direct assay with fresh tissue samples or with extracted DNA or RNA.

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. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger crustaceans should be processed and tested individually. However, for eggs, larvae and postlarvae pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50 to 150 postlarvae depending on their size/age) may be necessary to obtain sufficient sample material to run a diagnostic assay.

5.1. Antibody-based tests

See disease-specific chapters in this *Aquatic Manual*.

5.2. Direct microscopy

Samples for direct microscopic examination should be examined as soon as possible after collection. Use live specimens whenever possible, or use fresh, chilled, or fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

5.3. Histological techniques

Only live or moribund specimens with clinical signs should be sampled for histology. Collect crustaceans by whatever means are available with a minimum of handling stress. Hold animals in a container appropriate for maintaining suitable water quality and supply adequate aeration to the container if the crustaceans are to be held for a short period of time before actual fixation.

5.3.1. Fixation

A general rule is that a minimum of ten volumes of fixative should be used for one volume of tissue sample (i.e. a 10 g sample of crustacean would require 100 ml of fixative).

i) Davidson's AFA (alcohol, formalin, acetic acid) fixative

Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in decapod crustaceans (i.e. especially in crustaceans in tropical and subtropical regions), and its acidic content decalcifies the cuticle. The formulation for Davidson's AFA is (for 1 litre):

330 ml 95% ethyl alcohol
220 ml 100% freshly made formalin (a saturated 37–39% aqueous solution of formaldehyde gas)

4. 115 ml glacial acetic acid
335 ml tap water (for marine crustaceans, seawater may be substituted)
Store the fixative in glass or plastic bottles with secure caps at room temperature.

ii) Fixation procedures with Davidson's AFA

For larvae and postlarvae that are too small to be easily injected with fixative using a tuberculin syringe: Using a fine mesh screen or a Pasteur pipette, select and collect specimens. Immerse crustaceans selected for sampling directly in the fixative. Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

For juveniles that are too small to be injected: Select and collect specimens. Use a needle or fine-pointed forceps to incise the cuticle and immediately immerse crustaceans selected for sampling directly into the fixative. Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

For large juveniles and adults: to ensure proper fixation, kill the crustacean using a humane method, then immediately inject fixative (use 5–10% volume:weight). Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

The hepatopancreas (HP) should be injected first and at two or more sites, with a volume of fixative sufficient to change the HP to a white-to-orange colour (when Davidson's AFA is used); then inject fixative into adjacent regions of the cephalothorax, into the anterior abdominal region, and into the posterior abdominal region.

The fixative should be divided between the different regions, with the cephalothoracic region, specifically the HP, receiving a larger share than the abdominal region.

Immediately following injection, slit the cuticle with dissecting scissors, from the sixth abdominal segment to the base of the rostrum, being particularly careful not to cut deeply into the underlying tissue. The incision in the cephalothoracic region should be just lateral to the dorsal midline, while that in the abdominal region should be approximately mid-lateral.

For crustaceans larger than ~12 g: After injection of fixative, the body should then be transversely bisected, at least once, just posterior to the abdomen/cephalothorax junction, and (optional) again mid-abdominally.

For very large crustaceans (e.g. lobsters, crabs, adult penaeids, adult Macrobrachium rosenbergii, some species and life stages of crabs, crayfish, etc.): The organs of interest may be excised after injection of fixative. Completion of fixation of these tissue samples is then handled as outlined previously.

Following injection, incisions and bisection/trisection, or excision of key organs, immerse the specimen in the fixative (use 10:1 fixative:tissue ratio).

Allow fixation to proceed at room temperature for 24–72 hours depending on the size of crustacean being preserved. Longer fixation times in Davidson's AFA may be used to thoroughly decalcify the shell of crabs, lobsters, crayfish, etc.

Following fixation, the specimens should be transferred to 70% ethyl alcohol, where they can be stored for an indefinite period.

iii) Transport and shipment of preserved samples

As large volumes of alcohol should not be mailed or shipped, the following methods are recommended: Remove the specimens from the 70% ethyl alcohol. For larvae, postlarvae, or small juveniles, use leak-proof, screw-cap plastic vials if available; if glass vials must be used, pack to

prevent breakage. For larger specimens, wrap samples with white paper towels to completely cover (do not use raw or processed cotton). Place towel-wrapped specimens in a sealable plastic bag and saturate with 70% ethyl alcohol. Insert the label and seal the bag. Place the bag within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, crush-proof appropriately labelled container for shipment (for details see *Aquatic Code* Chapter 5.10 *Measures concerning international transport of aquatic animal pathogens and pathological material*).

5.4. Transmission or scanning electron microscopy

Electron microscopy (EM – transmission or scanning) is a valuable research tool for the study of disease in crustaceans. However, EM methods are not routinely used for diagnosis of the diseases listed by the OIE.

5.5. Use of molecular and antibody-based techniques for confirmatory testing and diagnosis

Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional PCR and real-time PCR, have been developed for the identification of many pathogens of aquatic animals. Real-time PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared from crustacean tissue. The technique can be used in direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well as in the diagnosis of clinically affected animals.

When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative controls, as well as validation of the PCR method chosen are important. Real-time PCR is a powerful technique particularly for analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening, diagnostic or confirmatory methods for crustaceans and can be undertaken as the standard method. Following real-time PCR-positive results, where possible, conventional PCR with sequencing of PCR products should be used for confirmation of pathogen identity.

As with all PCR protocols, optimisation may be necessary depending on the reagents, equipment and the plasticware used. PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction) may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. False-negative results (positive samples giving a negative result) may lead to unwanted transmission of pathogens and biosecurity failure.

Diagnostic samples should be tested in duplicate and both must produce positive results for a sample to be deemed positive. In instances where a sample produces one positive and one negative result, these are deemed indeterminate and should be retested. In addition, the following controls should be run with each assay: negative extraction control (e.g. a tissue [or equivalent sample that is under test]) sample from a known uninfected animal; positive control (preferably, one that can be distinguished from the pathogen genomic sequence [e.g. an artificial plasmid], thus allowing detection of any cross-contamination leading to a false positive result); no template control (all reagents with water replacing the template); internal positive control (internal housekeeping gene). All controls should produce their expected results in order for the diagnostic test result to be valid.

To minimise the risk of contamination, aerosol-preventing pipette tips should be used for all sample and PCR preparation steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the amplifications and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes, shoes and paper (e.g. workbooks). Also, ensure all work-tops and air-flow hoods used for the extractions and PCR set-up are regularly cleaned and decontaminated. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location away from the molecular biology laboratory and reagents.

5.5.1. Sample preparation and types

Samples selected for nucleic acid-based or antibody-based diagnostic tests should be handled and packaged (in new plastic sample bags or bottles) with care to minimise the potential for cross-

contamination among the sample set taken from different (wild or farmed) stocks, tanks, ponds, farms, etc. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

i) *Live specimens:* these may be processed in the field or shipped to the diagnostic laboratory for testing.

ii) *Haemolymph:* this tissue is the preferred sample for certain molecular and antibody-based diagnostic tests (see disease-specific chapters). Samples may be collected by needle and syringe through cardiac puncture, from the haemocoel (i.e. the ventral sinus in penaeids), or from a severed appendage, and immediately transferred to a tube that is half full with 90–95% ethanol or suitable nucleic acid preservative.

iii) *Iced or chilled specimens:* these are specimens that can be transported to the laboratory for testing within 24 hours. Pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory.

iv) *Frozen whole specimens:* select live specimens according to the criteria listed in disease-specific chapters in this *Aquatic Manual*, quick freeze in the field using crushed dry-ice or freeze in the field laboratories using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.

v) *Alcohol-preserved samples:* in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples for molecular tests. Alcohol-preserved samples are generally not suitable for antibody-based tests. Whole crustaceans (any life stage provided the specimen is no larger than 2–3 g), excised tissues (i.e. pleopods) from large crustaceans, or haemolymph may be preserved in 90–95% ethanol, and then packed for shipment according to the methods described in Section 5.3.1, paragraph iii (see chapter 5.10 of the *Aquatic Code* for additional details on the international transport of such samples).

5.5.2. Preservation of RNA and DNA in tissues

For routine diagnostic testing by PCR or RT-PCR, samples must be prepared to preserve the pathogen's nucleic acid. For most purposes, preservation of samples in alcohol (80–90%) is the preferred method for subsequent molecular tests. Samples preserved in this way can be stored at 4°C for 1 month, at 25°C for 1 week or indefinitely at –20°C or below. In addition, other products (e.g. nucleic acid preservatives, various lysis buffers, etc.) are commercially available for the same purpose.

5.5.3. Nucleic acid extraction

To isolate nucleic acids from tissues preserved in ethanol or nucleic acid preservative, simply remove the tissue from the fixative or preservative and treat it as though it was just harvested. Most fresh and preserved or fixed tissues can be homogenised (e.g. with a mortar and pestle or in bead-beating tubes) directly in the lysis or extraction buffer provided with commercially available DNA and RNA extraction kits. Commercial kits should be validated or undergo equivalence testing with current validated extraction procedures prior to routine use.

5.5.4. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation, fixed tissues that have been transferred to 70% ethanol are embedded in paraffin according to standard histological methods. Sections are cut at a thickness of 5 µm and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step

of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For in-situ hybridisation tests (see individual chapters for details), it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol (Qadiri *et al*., 2019; Valverde *et al*., 2017).

For further details see disease-specific chapters in this *Aquatic Manual*.

6. Additional information to be collected

Sample information should include the collector's name, organisation, date, time, and description of the geographical location. The geographical origin of samples may be described as the name or location of the sampling site or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the sample site to the storage facility or laboratory and within those facilities.

A history of the specimens should also be collected and should include species, age, weight, details of clinical signs including behavioural changes, as well as observations concerning any gross pathology which has been observed.

Information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable) should be collected. This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

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NB: FIRST ADOPTED IN 2000; MOST RECENT UPDATES ADOPTED IN 2012.

Annex 18. Item 7.1.2. – Chapter 2.2.1. AHPND

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: 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. 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 including 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).~~ None known.

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: moribund prawns sink to bottom, pale to white hepatopancreas (HP) due to pigment loss in the connective tissue capsule (NACA, 2014). ~~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

Gross pathological observations include pale-to-white 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). ~~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.woah.org/#/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, but repeated freezing and thawing of samples should be avoided.

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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology		+	+	NA		+	+	NA				
Cell culture												
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	++	++	++	2	++	++	++	2	~~++~~	~~++~~	~~++~~	~~2~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	~~1~~2
In-situ hybridisation												
Bioassay					+	+	+	NA	+	+	+	NA
LAMP		++	++	1								
Ab-ELISA												
Ag-ELISA		+	++	1		+	++	1		+	++	1
Other antigen detection methods[3]												
Other methods[3]												

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.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify 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 assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

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 $PirA^{vp}$ 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. *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 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.* (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

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
	Method 1: Han *et al.*, 2015b; GenBank KM067908		
pirA	Fwd VpPirA-F: TTG-GAC-TGT-CGA-ACC-AAA-CG Rev VpPirA-R: GCA-CCC-CAT-TGG-TAT-TGA-ATG VpPirA Probe: FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA	Fwd: 0.3 µM Rev: 0.3 µM probe: 0.1 µM	95°C/20 sec; 45 cycles 95°C/3 sec and 60°C/30 sec

~~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

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (AP1): Flegel & Lo, 2014; GenBank : KP324996; 700 bp			
pVA1	Fwd AP1F: 5CCT-TGG-GTG-TGC-TTA-GAG-GAT-G Rev AP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-C	0.2 µM each	94°C/5 min; 25–30 cycles of 94°C/30 sec, 60°C/30 sec and 72°C/60 sec; final extension step at 72°C/10 min. Reaction mixture can be held at 4°C
Method 2 (AP2): Flegel & Lo, 2014; GenBank : KP324996; 700 bp			
pVA1	Fwd AP2F: TCA-CCC-GAA-TGC-TCG-CTT-GTG-G Rev AP2R: CGT-CGC-TAC-TGT-CTA-GCT-GAA-G	0.2 µM each	94°C/5 min; 25–30 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/60 sec; final extension step at 72°C/10 min. Reaction mixture can be held at 4°C
Method 3 (AP3): Sirikharin *et al.*, 2015; GenBank: JALL01000066.1; 333 bp			
pirA^{vp}	Fwd AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA	0.2 µM each	94°C/5 min; 30 cycles of 94°C/30 sec, 53°C/30 sec, 72°C/40 sec; final elongation step at 72°C/7 min; Reaction mixture can be held at 4°C
Method 4 (TUMSAT-Vp3): Tinwongger *et al.*, 2014; GenBank : AB972427; 360 bp			
pVA1	Fwd TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CA Rev TUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA	0.6 µM each	95°C/2 min; 30 cycles of 95°C/30 sec, 56°C/30 sec, 72°C/30 sec
Method 5 (VpPirA-284): Han *et al.*, 2015a; GenBank : KM067908; 284 bp			
pirA^{vp}	Fwd VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG Rev VpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 6 (VpPirB-392): Han *et al.*, 2015a; GenBank KM067908; 392 bp			
pirB^{vp}	Fwd VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC Rev VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 7 (AP4): Dangtip *et al.*, 2015; GenBank : JPKS01000000; 1269 bp			
PirA and PirB toxin genes	Fwd AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA	0.2 µM each	94°C/2 min; 30 cycles of 94°C/30 sec, 55°C/30 sec, 72°C/90 sec; final extension step at 72°C/2 min; hold at 4°C
Method 8 (AP4): Dangtip *et al.*, 2015; GenBank : JPKS01000000; 230 bp			
PirA and PirB toxin genes	Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 µM each	94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C

~~**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 G~~ ~~AP1R: GCA AAC TAT CGC GCA GAA CAC C~~	~~*pVA1*~~	~~700bp~~	~~Flegel & Lo (2014)~~
~~AP2~~	~~AP2F: TCA CCC GAA TGC TCG CTT GTG G~~ ~~AP2R: 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 $MgCl_2$, 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 4.4.2.32.~~

~~*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 AC~~ ~~AP3-R: GTG GTA ATA GAT TGT ACA GAA~~	~~$pirA^{vp}$~~	~~333bp~~	~~Sirikharin *et al.*, 2015~~
~~TUMSAT-Vp3~~	~~TUMSAT-Vp3 F: GTG TTG CAT AAT TTT GTG CA~~ ~~TUMSAT-Vp3 R: TTG TAC AGA AAC CAC GAC TA~~	~~$pirA^{vp}$~~	~~360bp~~	~~Tinwongger *et al.*, 2014~~
~~VpPirA-284~~	~~VpPirA-284F: TGA CTA TTC TCA CGA TTG GAC TG~~ ~~VpPirA-284R: CAC GAC TAG CGC CAT TGT TA~~	~~$pirA^{vp}$~~	~~284bp~~	~~Han *et al.*, 2015a~~
~~VpPirB-392~~	~~VpPirB-392F: TGA TGA AGT GAT GGG TGC TC~~ ~~VpPirB-392R: TGT AAG CGC CGT TTA ACT CA~~	~~$pirB^{vp}$~~	~~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 $MgCl_2$, 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.~~

~~**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 $MgCl_2$, 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 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 4.4.2.73. 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
AP4 Step 1	AP4-F1: ATG AGT AAC AAT ATA AAA CAT GAA AC AP4-R1: ACG ATT TCG ACG TTC CCC AA	1269	Dangtip *et al.*, 2015
AP4 Step 2	AP4-F2: TTG AGA ATA CGG GAC GTG GG AP4-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 size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published sequences.

~~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 2×10^8 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_{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) and conventional PCR (Dangtip *et al.,* 2015) are ~~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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status [4]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. ~~Geographical~~ Hydrographical 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 PCR

ii) A positive result by ~~or~~ conventional PCR ~~methods recommended in Table 4.1~~

iii) A positive result by LAMP

iv) Histopathology~~- or cytopathological changes~~ consistent with the ~~presence of the pathogen or the~~ disease

v) A positive result by Ag-ELISA

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 at least one of the following ~~criterion~~ criteria is met:

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

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA 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

iv) A positive result by LAMP

[4] For example transboundary commodities.

v) A positive result by Ag-ELISA

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 at least one of the following ~~criterion~~ criteria is met:

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

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA 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	100[1]	100	Bioassay	Tinwongger *et al.*, 2014
Real-time PCR	Diagnosis	Clinically 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.
[1]100% sensitivity for TUMSAT-Vp3 primer set.

6.3.2. For surveillance of apparently healthy animals

Test type	Test rpose	Source pulations	Tissue or mple types	Species	DSe	DSp	Reference test	Citation

DSe = diagnostic sensitivity, DSp = diagnostic specificity, NA= Not available, PCR: = polymerase chain reaction.

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NB: There are OIE Reference Laboratories for acute hepatopancreatic necrosis disease
(please consult the OIE web site for the most up-to-date list:
https://www.woah.org/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.

CHAPTER 2.2.2.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci*, Phylum Oomycota. The disease is commonly known as crayfish plague.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Aphanomyces astaci is a water mould. The Oomycetida or Oomycota, are considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

Five groups (A–E) of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) (Dieguez-Uribeondo *et al.,* 1995; Huang *et al.,* 1994; Kozubikova *et al.,* 2011). Additional geno- or haplotypes are still being detected using molecular methods (Di Domenico *et al*., 2021). Group A (the so called *Astacus* strains) comprises strains isolated from several European crayfish species. These strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus* strains I) includes isolates from several European crayfish species and from the invasive *Pacifastacus leniusculus* in Europe as well as Lake Tahoe, USA. Imported to Europe, *P. leniusculus* probably introduced this genotype of *A. astaci* and infected the native European crayfish. Group C (*Pacifastacus* strains II) consists of a strain isolated from *P. leniusculus* from Pitt Lake, Canada. Another strain (Pc), isolated from *Procambarus clarkii* in Spain, sits in group D (*Procambarus* strains). This strain shows temperature/growth curves with higher optimum temperatures compared with groups A and B (Dieguez-Uribeondo *et al*., 1995). *Aphanomyces astaci* strains that have been present in Europe for many years (group A strains) appear to be less pathogenic than strains introduced with crayfish imports from North America since the 1960s.

2.1.2. Survival and stability in processed or stored samples

Aphanomyces astaci is poorly resistant against desiccation and does not survive long in decomposing hosts. Any treatment of the crayfish (freezing, cooking, drying) will affect the survival of the pathogen (Oidtmann *et al.,* 2002). Isolation from processed samples is not possible, however they may be suitable for molecular methods used for pathogen detection.

2.1.3. Survival and stability outside the host

Outside the host *Aphanomyces astaci* is found as zoospores that remain motile for up to 3 days and form cysts that can survive for 2 weeks in distilled water. As *A. astaci* can go through three cycles of encystment and zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension in clean water kept at 2°C for 2 months (Unestam, 1966). Survival time is probably shorter in natural waters.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

All stages of crayfish species native to Europe, including the noble crayfish (*Astacus astacus*) of north-west Europe, the white clawed crayfish (*Austropotamobius pallipes*) of south-west and west Europe, the related *Austropotamobius torrentium* (mountain streams of south-west Europe) and the slender clawed or Turkish crayfish (*Pontastacus leptodactylus*) of eastern Europe and Asia Minor are highly susceptible (e.g. Holdich *et al*., 2009). Australian species of crayfish are also highly susceptible. North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Faxonius* spp. are infected by *A. astaci*, but under normal conditions the infection does not cause clinical disease or death. All North American crayfish species investigated to date have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (reviewed by Svoboda *et al*. 2017) and it is therefore currently assumed that this is the case for any other North American species.

The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*).

2.2.2. Species with incomplete evidence for susceptibility

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

The host species susceptible to infection with *A. astaci* fall largely into two categories: those highly susceptible to infection with development of clinical disease and mortalities, and those that are infected without associated clinical disease or mortalities.

Highly susceptible species: clinical disease outbreaks caused by infection with *A. astaci* are generally known as 'crayfish plague' outbreaks. In such outbreaks, moribund and dead crayfish of a range of sizes (and therefore ages) can be found.

2.2.4. Distribution of the pathogen in the host

The tissue that becomes initially infected is the exoskeleton cuticle. Soft cuticle, as is found on the ventral abdomen and around joints, is preferentially affected. In the highly susceptible European crayfish species, the pathogen often manages to penetrate the basal lamina located underneath the epidermis cell layer. From there, *A. astaci* spreads throughout the body primarily by invading connective tissue and haemal sinuses; however, all tissues may be affected.

In North American crayfish species, infection is usually restricted to the cuticle. Based on PCR results, the tailfan (consisting of uropods and telson) and soft abdominal cuticle appear to be frequently infected (Oidtmann *et al.,* 2006; Vralstad *et al.,* 2011).

2.2.5. Aquatic animal reservoirs of infection

North American crayfish species act mostly as carriers of the infection without showing clinical signs. However, some strains, especially from group A, show lowered virulence, thus enabling normally highly susceptible European crayfish to act as carriers as well (see review by Svoboda *et al*., 2017).

Colonisation of habitats, initially occupied by highly susceptible species, by North American crayfish species carrying *A. astaci* is likely to result in an epizootic among the highly susceptible animals.

2.2.6. Vectors

Transportation of finfish may facilitate the spread of *A. astaci* through the presence of spores in the transport water or co-transport of infected crayfish specimens (Alderman *et al*., 1987; Oidtmann *et al*., 2002). There is also circumstantial evidence of spread by contaminated equipment (e.g. nets, boots, clothing, traps) (Alderman *et al*., 1987).

2.3. Disease pattern

2.3.1. Mortality, morbidity, and prevalence

When the infection first reaches a naïve population of highly susceptible crayfish species, high levels of mortality are usually observed within a short space of time, so that in areas with high crayfish densities the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread quickly from the initial outbreak site downstream, whereas upstream spread is slower. Lower water temperatures are associated with slower mortalities and a greater range of clinical signs in affected animals (Alderman *et al*., 1987). Observations from Finland suggest that at low water temperatures, noble crayfish (*Astacus astacus*) can be infected for several months without the development of noticeable mortalities (Viljamaa-Dirks *et al*., 2013).

On rare occasions, single specimens of the highly susceptible species have been found after a wave of infection with *A. astaci* has gone through a river or lake. This is most likely to be due to lack of exposure of these animals during an outbreak (animals may have been present in a tributary of a river or lake or in a part of the affected river/lake that was not reached by spores, or crayfish may have stayed in burrows during the epizootic). However, low virulent strains of *A. astaci* have been described to persist in a water way, kept alive by a weak infection in the remnant population (Viljamaa-Dirks *et al*., 2011). Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now heavily diminished (Souty-Grosset *et al*., 2006; Holdich *et al*., 2009). Populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with spores, new outbreaks of infection with *A. astaci* and large-scale mortalities will occur.

In the highly susceptible European crayfish species, exposure to *A. astaci* spores usually leads to infection and eventually to death. Prevalence of infection within a population in the early stage of an outbreak may be low (few animals in a river population may be affected). However, the pathogen is amplified in affected animals and subsequently released into the water; usually leading to 100% mortality in a contiguous population. The rate of spread from initially affected animals depends on several factors, one being water temperature. Therefore, the time from first introduction of the pathogen into a population to noticeable crayfish mortalities can vary greatly and may range from a few weeks to months. Prevalence of infection will gradually increase over this time and usually reach 100%. Data from a noble crayfish population in Finland that experienced an acute mortality event due to infection with *A. astaci* in 2001 suggest that in sparse noble crayfish populations, spread of disease throughout the host population may take several years (Viljamaa-Dirks *et al.,* 2011).

2.3.2. Clinical signs, including behavioural changes

Susceptible species

Gross clinical signs are variable and depend on challenge severity and water temperatures. The first sign of an epizootic may be the appearance of crayfish during daylight (crayfish are normally nocturnal), some of which may show loss of co-ordination, falling onto their backs and remaining unable to right themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

Often, however, the first sign of an outbreak may be the presence of large numbers of dead crayfish in a river or lake (Alderman *et al.,* 1987).

Infection with *A. astaci* may cause mass mortality of crayfish. However, investigation of mortality event should consider other causes such as environmental pollution (e.g. insecticides such as cypermethrin have been associated with initial misdiagnoses).

North American crayfish species

Infected North American crayfish may be subclinical carriers. Controlled exposure to a highly virulent strain has resulted in mortality in juvenile stages of *Pacifastacus leniusculus* as well as behavioural alterations in adults (Thomas *et al.,* 2020).

2.3.3 Gross pathology

Susceptible species

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernible despite careful examination. Infection foci are best viewed under a low power stereo microscope and are recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, or hyphae may be visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle. Sites for examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and abdomen, the joints of the pereiopods (walking legs), particularly the proximal joint, eyestalks and finally the gills.

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example, the soft abdominal cuticle and joints. These melanisations can be caused by mechanical injuries or infections with other water moulds and are non-specific. However, populations with high levels of infection can show abnormally high levels of cuticular damage in individual animals, such as missing legs and claws due to deteriorated joints.

2.3.4. Modes of transmission and life cycle

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, as may occur during movements of finfish, or 3) through colonisation of habitats by invasive North American crayfish species.

The main route of spread of *A. astaci* in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms. Subsequent spread occurs through expanding populations of invasive North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (Holdich *et al.,* 2009).

Transmission from crayfish to crayfish occurs through the release of zoospores from an infected animal and attachment of the zoospores to naïve crayfish. The life cycle of *A. astaci* is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores. These initially encyst, but then release a biflagellate zoospore (secondary zoospore). Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (Cerenius & Söderhäll, 1984). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infectivity (Soderhall & Cerenius 1999). Growth and sporulation capacity is strain-and temperature-dependent (Dieguez-Uribeondo *et al*., 1995).

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows varies slightly depending on the strain. In a study, which compared several *A. astaci* strains that had been isolated from a variety of crayfish species, mycelial growth was observed between 4 and 29.5°C, with the strain isolated from *Procambarus clarkii* growing better at higher temperatures compared to the other strains. Sporulation efficiency was similarly high for all strains tested between 4 and 20°C, but it was clearly reduced for the non-*P. clarkii* strains at 25°C and absent at 27°C. In contrast, sporulation still occurred in the *P. clarkii* strain at 27°C. The proportion of motile zoospores (out of all zoospores observed in a zoospore suspension) was almost 100% at temperatures ranging from 4–18°C, reduced to about 60% at 20°C and about 20% at 25°C in all but the *P. clarkii* strain. In the *P. clarkii* strain, 80% of the zoospores were still motile at 25°C, but no motile spores were found at 27°C (Dieguez-Uribeondo *et al*., 1995).

Field observations show that outbreaks of infection with *A. astaci* occur over a wide temperature range, and at least in the temperature range 4–20°C. The rate of spread within a population depends on several factors, including water temperature. In a temperature range between 4 and 16°C, the speed of an epizootic is enhanced by higher water temperatures.

In buffered, redistilled water, sporulation occurs between pH 5 and 8, with the optimal range being pH 5–7. The optimal pH range for swimming of zoospores appears to be pH 6.0–7.5, with a maximum range between pH 4.5 and 9.0 (Unestam, 1966).

Zoospore emergence is influenced by the presence of certain salts in the water. $CaCl_2$ stimulates zoospore emergence from primary cysts, whereas $MgCl_2$ has an inhibitory effect. In general, zoospore emergence is triggered by transferring the vegetative mycelium into a medium where nutrients are absent or low in concentration (Cerenius *et al.*, 1988).

2.3.6. Geographical distribution

In Europe the reports of large mortalities of crayfish go back to 1860. The reservoir of the original infections in the 19th century was never established. *Faxonius* (*Orconectes*) spp. were not known to have been introduced into Europe until the 1890s, but the post-1960s extensions are largely linked to more recent introductions of North American crayfish for farming (Alderman, 1996; Holdich *et al.* 2009). *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

In recent years, crayfish plague has been reported in Asia and also in North- and South America (see e.g. references in Di Domenico *et al.* 2021). The distribution of *A. astaci* in North America is likely to be much wider than reported.

Any geographical area where North American crayfish species were introduced must be considered as potentially infected if not proven otherwise. Lack of clinical disease in these carrier species may hamper the reliability in reporting the infection. For the highly susceptible species, see OIE WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level. However, even high mortalities can go unnoticed in wild populations.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No vaccines are available.

2.4.2. Chemotherapy including blocking agents

No treatments are currently known that can successfully treat the highly susceptible crayfish species, once infected.

2.4.3. Immunostimulation

No immunostimulants are currently known that can successfully protect the highly susceptible crayfish species against infection and consequent disease due to *A. astaci* infection.

2.4.4. Breeding resistant strains

A few studies suggest that there might be differences in resistance between populations of highly susceptible species (reviewed by Martin-Torrijos *et al.*, 2017; Svoboda *et al.*, 2017). The fact that North American crayfish generally do not develop clinical disease suggests that selection for resistance may be possible and laboratory studies using attenuated strains of *A. astaci* might be successful. However, there are currently no published data from such studies.

2.4.5. Inactivation methods

Aphanomyces astaci, both in culture and in infected crayfish, is inactivated by a short exposure to temperatures of 60°C or to temperatures of –20°C (or below) for 48 hours (or more) (Oidtmann *et al.*, 2002). Sodium hypochlorite at 100 ppm, free chlorine and iodophors at 100 ppm available iodine, are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection since organic matter decreases the effectiveness of disinfectants (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation (Rennerfelt, 1936).

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

If a crayfish farm for highly susceptible species is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or present upstream . If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

In an endemic area where the highly susceptible species are being farmed, the following biosecurity recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

1. General biosecurity should be in place (e.g. controlled access to premises; disinfection of boots when entering the site; investigation of mortalities if they occur; introduction of live animals (crayfish, finfish) only from sources known to be free from infection with *A. astaci*).

2. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species should be prevented.

3. If transfers of finfish or crayfish are being planned, these should not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of infection with *A. astaci* or North American carrier crayfish).

4. North American crayfish should not be brought onto the site.

5. Finfish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of infection with *A. astaci* may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment to kill *A. astaci* (see Section 2.4.5. *Inactivation methods*).

6. Any equipment that is brought onto site should be disinfected.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

For a suspected outbreak of infection with *A. astaci* in a population of highly susceptible crayfish species, sample crayfish should ideally consist of: a) live crayfish showing signs of disease, and b) live, apparently healthy crayfish. Freshly dead crayfish may also be suitable, although this will depend on their condition.

Live crayfish should be transported using insulated containers equipped with small holes to allow aeration. The temperature in the container should not exceed 16°C.

Crayfish should be transported in a moist atmosphere, for example using moistened wood shavings/wood wool, newspaper, or grass/hay. Unless transport water is sufficiently oxygenated, live crayfish should not be transported in water, as they may suffocate.

The time between sampling of live animals and delivery to the investigating laboratory should not exceed 24 hours.

Should only dead animals be found at the site of a suspected outbreak, freshly dead animals should be selected for diagnosis. Dead animals can either be: a) transported chilled (if they appear to have died only very recently), or, b) placed in non-methylated ethanol (minimum concentration 70%; see 3.5. *Preservation of samples for submission*), or c) placed in freezer at –20°C to avoid further decay and transported frozen.

When testing any population outside an acute mortality event for the presence of crayfish plague, as many individuals as possible should be inspected visually for signs of cuticular damage. Crayfish that have melanized spots or missing limbs should be selected in the first place for further analysis.

3.2. Selection of organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen. Any other soft part of the exoskeleton can be included as well. If any melanized spots or whitened areas are detected, these should be included in the sampling. From diseased animals, samples should be aseptically collected from the soft abdominal cuticle. For identification of carriers, samples should be aseptically collected from soft abdominal cuticle, and telson and uropods, separately.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended. Any other soft part of the exoskeleton can be included as well. If any melanized spots are detected, these should be included in the sampling.

3.3. Samples or tissues not suitable for pathogen detection

Autolysed material is not suitable for analysis.

3.4. Non-lethal sampling

A non-destructive sampling method that detects *A. astaci* DNA in the microbial biofilm associated with the cuticle of individual crayfish through vigorous scrubbing has been described (Pavic *et al.,* 2020), and could be considered in case of testing vulnerable populations.

3.5. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If transport of recently dead or moribund crayfish cannot be arranged, crayfish may be frozen or fixed in ethanol (minimum 70%). However, fixation may reduce test sensitivity. The crayfish:ethanol ratio should ideally be 1:10 (1 part crayfish, 10 parts ethanol).

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time in storage). Isolation is best attempted from crayfish with clinical signs delivered alive (see Section 3.1.). Fresh specimens should be kept chilled and preferably sent to the laboratory within 24 hours of collection.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

3.5.3. Samples for histopathology

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.4. Samples for other tests

Sensitive molecular methods can be used to detect *A. astaci* DNA directly from water samples (Strand *et al*. 2011, 2012) These methods require validation for diagnostic use.

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 animals should be processed and tested individually. Small life stages such as PL, can be pooled to obtain the minimum amount of material for 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts						+	+	NA				
Histopathology						+	+	NA				
~~Cell~~ Culture						+	+	NA				
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	+	+	+	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation												
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods[3]												
Other methods[3]												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available. PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 2.3.3 *Gross pathology*) and examined under a compound microscope using low-to-medium power will confirm the presence of aseptate fungal-like hyphae 7–9 µm wide. The hyphae can usually be found pervading the whole thickness of the cuticle, forming a three-dimensional network of hyphae in heavily affected areas of the cuticle. The presence of host haemocytes and possibly some melanisation closely associated with and encapsulating the hyphae give good presumptive evidence that the hyphae represent a pathogen rather than a secondary opportunist invader. In some cases, examination of the surface of such mounted cuticles will demonstrate the presence of characteristic *A. astaci* sporangia with clusters of encysted primary spores (see Section 4.3 *Culture for isolation*).

4.2. Histopathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used. However, such material does not prove that any hyphae observed are those of *A. astaci*, especially when the material comes from animals already dead by sampling.

See also Section 4.1 *Wet mounts*.

4.3. Culture for isolation

Isolation is not recommended as a routine diagnostic method (Alderman & Polglase, 1986; Cerenius *et al*., 1987; Viljamaa-Dirks, 2006). Test sensitivity and specificity of the cultivation method can be very variable depending on the experience of the examiner, but in general will be lower than the PCR. Isolation of *A. astaci* by culture from apparently healthy crayfish is challenging and molecular methods are recommended. A detailed description of this test is available from the Reference Laboratory[5].

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate. Shrimp tissues may be used as negative controls.

Live crayfish can be killed using chloroform, electric current or by mechanical destroying the nerve cords. If live or moribund animals are not available, only recently dead animals should be used for DNA extraction. The soft abdominal cuticle is the preferred sample tissue for DNA extraction. Any superficial contamination should first be removed by thoroughly wiping the soft abdominal cuticle with wet (using autoclaved H_2O) clean disposable swabs. The soft abdominal cuticle is then excised and 30–50 mg ground using a pestle and mortar.

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

[5] https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling conditions
Method 1*: Vralstad *et al.,* 2009, Strand, 2013; GenBank Accession No.AM947024			
Aphanomyces astacus & *A. fennicus* ITS	Fwd: AAG-GCT-TGT-GCT-GGG-ATG-TT Rev: CTT-CTT-GCG-AAA-CCT-TCT-GCT-A Probe: 6-FAM-TTC-GGG-ACG-ACC-C-MGBNFQ	500 nM 200 nM	50 cycles of: 95°C/15 sec and 60°C/30 sec
Alternative method 2: Strand *et al.* to be published; GenBank Accession No. AM947024			
Aphanomyces astacus ITS	Fwd: TAT-CCA-CGT-GAA-TGT-ATT-CTT-TAT Rev: GCT-AAG-TTT-ATC-AGT-ATG-TTA-TTT-A Probe: FAM-AAG-AAC-ATC-CCA-GCA-C-MGBNFQ	500 nM 200 nM	50 cycles of: 95°C/15 sec and 60°C/30 sec

*These ITS-based methods have been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).

The absolute limit of detection of method 1 was reported as approximately 5 PCR forming units (= target template copies), which is equivalent to less than one *A. astaci* genome (Vralstad *et al*., 2009). Another study reported consistent detection down to 50 fg DNA using this assay (Tuffs & Oidtmann, 2011).

Analytical test specificity has been investigated (Tuffs & Oidtmann, 2011; Vralstad *et al*., 2009) and no cross-reaction was observed in these studies. However, a novel species, *Aphanomyces fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this test at the same level as *A. astaci*. Due to this problem in specificity, the assay has been modified according to the alternative method 2 (Strand *et al*., manuscript in preparation):

Owing to the repeated discovery of new *Aphanomyces* strains, sequencing is required to determine the species of *Aphanomyces*. In the case of the real-time PCR assay, this requires separate amplification of a PCR product using primers ITS-1 and ITS-4 (see Section 4.5 *Amplicon sequencing*).

4.4.2. Conventional PCR

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling conditions
Method 1*: Oidtmann *et al.,* 2006; GenBank Accession No. AY310499 Product size: 569 bp			
Aphanomyces astacus & *A. fennicus* ITS	Fwd: GCT-TGT-GCT-GAG-GAT-GTT-CT Rev: CTA-TCC-GAC-TCC-GCA-TTC-TG-	500 nM	40 cycles of: 1 min/96°C, 1 min/59°C and 1 min/72°C

*This ITS-based method has been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).

Confirmation of the identity of the PCR product by sequencing is required as a novel species, *A. fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this assay.

The assay consistently detects down to 500 fg of genomic target DNA or the equivalent amount of ten zoospores submitted to the PCR reaction (Tuffs & Oidtmann, 2011).

4.4.3. Other nucleic acid amplification methods

Several genotype-specific molecular methods have been developed that, instead of requiring a pure growth as sample material like the RAPD-PCR assay, can be used to analyse crayfish tissue directly (Di

Domenico *et al*., 2021; Grandjean *et al*., 2014; Makkonen *et al.,* 2018; Minardi *et al*., 2018; 2019). Detection of a known genotype group combined with a positive result by a recommended conventional or real-time PCR can be used as a confirmative test in geographical areas where crayfish plague is known to be present. However, the current knowledge of the genotype variation is mostly limited to a few original host species and new genotypes or subtypes are expected to be found. Thus, the suitability of these methods is limited for initial excluding diagnosis or as confirmative tests in geographical areas not known to be infected.

PCR targeting mitochondrial DNA with *A. astaci* genotype specific primers have been shown to detect the known genotypes of *A. astaci*, but these assays may also provide positive results for some other oomycete genera (Cassabella-Herrero *et al.,* 2021).

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published sequences.

4.6. *In-situ* hybridisation

Not available.

4.7. Immunohistochemistry

Not available

4.8. Bioassay

No longer used for diagnostic purposes (see Cerenius *et al*., 1988).

4.9. Antibody- or antigen-based detection methods

Not available.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The recommended method for surveillance is real-time PCR, the modified assay by Strand *et al*. (manuscript in preparation).

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 a suspect and confirmed case 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 OIE Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status[6]

[6] For example transboundary commodities.

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 *Aphanomyces astaci* shall be suspected if at least the following criterion is met:

i) Positive result by real-time PCR
ii) Positive result by conventional PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Aphanomyces astaci* is considered to be confirmed if the following criterion is met:

i) Positive result by real-time PCR and positive result by conventional PCR followed by amplicon sequencing

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 *Aphanomyces astaci* 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) Visual observation of hyphae indicative of A. astaci

iii) Observation of hyphae indicative of *A. astaci* in stained histological sections

iv) Culture and isolation of the pathogen

v) Positive result by real-time PCR

vi) Positive result by conventional PCR

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *Aphanomyces astaci* is confirmed if the following criterion is met:

i) Positive result by real-time PCR and positive result by conventional PCR and amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *Aphanomyces astaci* are provided in Tables 6.3.1. and 6.3.2 (none available). This information can be used for the design of surveys for infection with *Aphanomyces astaci*, 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

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study.

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 specificity, *n* = number of samples used in the study.

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NB: There is an OIE Reference Laboratory for infection with *Aphanomyces astaci* (crayfish plague)
(please consult the OIE web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on
infection with *Aphanomyces astaci* (crayfish plague)

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.3.

INFECTION WITH HEPATOBACTER PENAEI (NECROTISING HEPATOPANCREATITIS)

1. Scope

Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR)-deleted ISAV, or the non-pathogenic HPR0 (non-deleted HPR) ISAV of the Genus *Isavirus* and Family *Orthomyxoviridae*.

Infection with *Candidatus Hepatobacter penaei* means infection with the pathogenic agent *Candidatus* H. penaei, an obligate intracellular bacterium of the Family Holosporaceae, Order Rickettsiales α-*Proteobacteria*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Hepatobacter penaei is a pleomorphic, Gram-negative, intracytoplasmic bacterium (Nunan *et al.,* 2013). It is a member of the α-*Proteobacteria* (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). More recently it has been suggested that it belongs to the Family Holosporaceae family within the Order Rickettsiales (Leyva *et al.,* 2018). The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). Genetic analysis of *H. penaei* associated with North and South American outbreaks suggests that the isolates are either identical or very closely related subspecies (Loy *et al.,* 1996). Recently Analysis based on the 16S rRNA confirms the high similarity among different *H. penaei* isolates in the Americas (99–100%) (Aranguren & Dhar, 2018).

2.1.2. Survival and stability in processed or stored samples

Hepatobacter penaei-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. *Hepatobacter penaei* frozen at –20°C to –70°C and –80°C have been shown to retain infectivity in experimental transmission trials with *Penaeus vannamei* (Crabtree *et al.,* 2006; Frelier *et al.,* 1992). Flash freezing *H. penaei* at –70°C to –80°C does not significantly affect the infectivity (Aranguren *et al*., 2010; Crabtree *et al.,* 2006).

2.1.3. Survival and stability outside the host

No information available.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *H. penaei* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include are: whiteleg shrimp (*P. 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 infection with *H. penaei* according to Chapter 1.5. of the *Aquatic Code* ~~include~~ are: aloha prawn (*P. marginatus*), banana prawn (*P. merguiensis*), blue shrimp (*P. stylirostris*), giant tiger prawn (*P. monodon*), northern brown shrimp (*P. aztecus*), northern pink shrimp (*P. duorarum*) and northern white shrimp (*P. setiferus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: American lobster (*Homarus americanus*) (Avila-Villa *et al.,* 2012; Bekavac *et al.,* 2022).

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Infection with *H. penaei* has been demonstrated in postlarvae (PL), juveniles, adults and broodstock of *P. vannamei* (Aranguren *et al.*, 2006).

2.2.4. Distribution of the pathogen in the host

The target tissue is the hepatopancreas: infection with *H. penaei* has been reported in all hepatopancreatic cell types (Lightner 2012). *Hepatobacter penaei* is also present in the faeces (Brinez *et al.,* 2003).

2.2.5. Aquatic animal reservoirs of infection

Some members of *P. vannamei* populations that survive infection with *H. penaei* may carry the intracellular bacteria for life and transmit it to other populations by horizontal transmission (Aranguren *et al.,* 2006; Lightner, 2005; Morales-Covarrubias, 2010; Vincent & Lotz, 2005).

2.2.6. Vectors

No vectors are known in natural infections.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Infection with *H. penaei* often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adults and broodstock, the incubation period and severity of the disease are somewhat size or age dependent. Infection with *H. penaei* results in the mortalities approaching 100% in *P. vannamei*, 5.6–15% in *P. duorarum*, and 5–17% in *P. aztecus* (Aguirre-Guzman *et al.,* 2010).

The prevalence was reported as 0.77% in cultured *P. vannamei* and 0.43% in cultured *P. stylirostris* in Peru (Lightner & Redman, 1994), 5–86.2% in Mexico (Ibarra-Gamez *et al.,* 2007), and 0.6–1.3% in *P. vannamei* in Belize, Brazil, Guatemala, Honduras, Mexico, Nicaragua and Venezuela (Morales-Covarrubias *et al.*, 2011).

NHP-affected broodstock ponds in Colombia reported mortalities of up to 85%, while non NHP-affected broodstock ponds in the same farm experienced mortalities of 40–50% (Aranguren *et al.,* 2006).

2.3.2. Clinical signs, including behavioural changes

A wide range of gross signs can be used to indicate the possible presence of infection with *H. penaei*. These include lethargy, reduced food intake, atrophied hepatopancreas, anorexia and empty guts, noticeably reduced growth and poor length weight ratios ('thin tails')~~; soft shells and flaccid bodies; black or darkened gills; heavy surface fouling by epicomensals organisms; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic. (Lightner, 1996; Loy *et al.,* 1996)~~.

2.3.3 Gross pathology

~~Infection with *H. penaei* often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adult and broodstock, the incubation period and severity of the disease are somewhat size or age dependent.~~ Gross signs are not specific, but shrimp with acute infection with *H. penaei* show atrophied hepatopancreas, empty guts, soft shells and flaccid bodies; black or darkened gills; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic. (Lightner, 1996; Loy *et al.,* 1996) ~~a marked reduction in food consumption, followed by changes in behaviour and appearance including pale discoloration of the hepatopancreas with further size reduction~~.

2.3.4. Modes of transmission and life cycle

Horizontal transmission of *H. penaei* can be through cannibalism or by contaminated water (Aranguren *et al.,* 2006; 2010; Frelier *et al.,* 1993; Gracia-Valenzuela *et al.*, 2011; Vincent *et al.*, 2004). *Hepatobacter penaei* in faeces shed into pond water has also been suggested as a source of contamination (Aranguren *et al.,* 2006; Briñez *et al.,* 2003; Morales-Covarrubias *et al.*, 2006). *Hepatobacter penaei*-positive broodstock females produce PL that were also *H. penaei*-positive, which suggests that a transmission from broodstock to progeny can occur~~s~~ (Aranguren *et al.,* 2006).

2.3.5. Environmental factors

The occurrence of infection with *H. penaei* in farms may increase during long periods of high temperatures (>29°C) and high salinity (20–38 ppt) (Morales-Covarrubias, 2010). In the months when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

2.3.6. Geographical distribution

Hepatobacter penaei appears to have a Western Hemisphere distribution in both wild and cultured penaeid shrimp (Aguirre-Guzman *et al.,* 2010; Del Rio-Rodriguez *et al.,* 2006). In the Western Hemisphere, *H. penaei* is commonly found in cultured penaeid shrimp in the Americas (Aranguren *et al.,* 2010; Frelier *et al.,* 1992; Ibarra-Gamez *et al.,* 2007; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2011). *Hepatobacter penaei*, was introduced into Africa from North America via movement of infected *P.vannamei* broodstock, however NHP was later eradicated by fallowing (Lightner *et al.,* 2012).

See OIE WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

Early detection (initial phase) of clinical infection with *H. penaei* is important for successful treatment because of the potential for cannibalism to amplify and transmit the disease. Shrimp starvation and cannibalism of infected shrimp, and positive conditions for *H. penaei* ~~enaei~~ multiplication, are important factors for the spread of *H. penaei* in *P. vannamei*. Preventive measures include raking, tilling, and removing sediments from the bottom of the ponds, prolonged drying (through exposure to sunlight) of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, extensive liming of ponds and the use of ponds liners. The use of specific pathogen-free (SPF) broodstock is an effective preventive measure. NHP, particularly in the initial phase, can be treated by using antibiotics in medicated feeds. ~~*Hepatobacter penaei* is sensitive to oxytetracycline (Lightner & Redman, 1994).~~

2.4.1. Vaccination

No scientifically confirmed reports.

2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Breeding resistant strains

One population from Latin America that has been selected for several generations for resistance to Taura syndrome virus in the presence of infection with *H. penaei*, seems to be more resistant to NHP disease than the Kona line under experimental conditions (Aranguren *et al.,* 2010).

2.4.5. Inactivation methods

The use of hydrated lime ($Ca(OH)_2$) to treat the bottom of ponds during pond preparation before stocking can help reduce infection with *H. penaei*.

2.4.6. Disinfection of eggs and larvae

Disinfection of eggs and larvae is a good management practice and is recommended for its potential to reduce *H. penaei* contamination of spawned eggs and larvae (and contamination by other disease agents).

2.4.7. General husbandry

The prevalence and severity of infection with *H. penaei* may be increased by rearing shrimp in relatively crowded or stressful conditions. Some husbandry practices have been successfully applied to the prevention of infection with *H. penaei*. Among these has been the application of PCR to pre-screening of wild or pond-reared broodstock.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Suitable specimens for testing for infection with *H. penaei* are the following life stages: PL, juveniles and adults.

3.2. Selection of organs or tissues

Hepatobacter penaei infects most enteric tissue. The principal target tissue for *H. penaei* is the hepatopancreas and this organ should be selected preferentially (Lightner, 2012).

3.3. Samples or tissues not suitable for pathogen detection

Hepatobacter penaei does not replicate in the midgut, caeca, connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells. Samples of pleopods or haemolymph are not recommended for *H. penaei* detection by PCR.

3.4. Non-lethal sampling

Hepatobacter penaei can be detected in faeces samples collected from clinically affected populations of *Penaeus vannamei* ~~may be collected and used for testing (usually by PCR),~~ when non-lethal testing of ~~valuable~~ broodstock is necessary (Brinez *et al.,* 2003~~; Frelier et al., 1993; Lightner, 1996~~). However, the use of faeces samples to detect NHP in apparently healthy shrimp has not been evaluated. ~~Faeces samples have not been validated to the same level as hepatopancreas samples.~~

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 *General information* (*diseases of crustaceans*)

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time 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 alternate storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples of hepatopancreas or faeces for PCR testing should be preserved in 70–95% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen, but repeated freezing and thawing should be avoided.

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 5.3 of Chapter 2.2.0.

3.5.4. Samples for other tests

No scientifically confirmed reports.

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 shrimp should be processed and tested individually. Small life stages such as PL or specimens up to 0.5 g can be pooled to obtain the minimum amount of material for *H. penaei* 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts						+	+	NA				
Histopathology						++	++	NA				
Cell culture												
Real-time PCR	++	+++	+++	1	++	+++	+++	1	++	++	++	1
Conventional PCR	++	++~~+~~	++~~+~~	1	++	+++	+++	1	~~++~~	~~+++~~	~~+++~~	~~1~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation					+	++	++	NA	+	++	++	NA
Bioassay					+	+	+	NA	+	+	+	NA
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods[3]												
Other methods[3]												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]~~Specify the test used.~~ Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Wet mount squash examination of hepatopancreas tissue is generally conducted to detect presumptive infection with *H. penaei*. The hepatopancreas may be atrophied and have any of the following characteristics: soft and watery; fluid filled centre; pale colour with or without black stripes (melanised tubules). Hepatopancreatic tubules show deformity at the distal portion; multifocal melanisation initially at the distal portion of the tubule and, later on, in the medial and proximal portion; reduced or absence of lipid droplets (Lightner, 2012).

4.2. Histopathology and cytopathology

Histological methods can be useful for indicating acute and chronic infection with *H. penaei*.

Initial infection with *H. penaei* is difficult to diagnose using routine H&E histological methods. Therefore, molecular methods are recommended for initial *H. penaei* detection (e.g. by PCR or application of *H. penaei*-specific DNA probes or *in-situ* hybridisation ~~[ISH]~~ of histological sections).

Acute infection with *H. penaei* is characterised by atrophied hepatopancreas with moderate atrophy of the tubule epithelia, presence of bacterial cells and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations). Hypertrophic cells, individual epithelial cells, appeared to be separated from adjacent cells, undergo necrosis and desquamation into the tubular lumen. The tubular epithelial cell lipid content is variable.

The transitional phase of infection with *H. penaei* is characterised by haemocytic inflammation of the intertubular spaces in response to necrosis, cytolysis, and sloughing of hepatopancreas tubule epithelial cells. The hepatopancreas tubule epithelium is markedly atrophied, resulting in the formation of large oedematous (fluid filled or 'watery') areas in the hepatopancreas. Tubule epithelial cells within multifocal encapsulation are typically atrophied and reduced from simple columnar to cuboidal morphology. They contain little or no stored lipid vacuoles, markedly reduced or no secretory vacuoles and masses of bacteria. At this phase haemocyte nodules are observed in the presence of masses of bacteria in the centre of the nodule.

In the chronic phase of infection with *H. penaei*, tubular lesions, multifocal encapsulation and oedematous areas decline in abundance and severity and are replaced by infiltration and accumulation of haemocytes at the sites of necrosis. There are areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells with masses of bacteria in the cytoplasm and low numbers of haemocyte nodules.

4.3. Cell culture for isolation

Hepatobacter penaei has not been grown *in vitro*. No crustacean cell lines exist (Vincent & Lotz, 2007).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

PCR methods including PCR and real-time PCR have been developed that target several *H. penaei* genes including 16S rRNA and Flg E genes (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Loy *et al.*, 1996).

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

~~*DNA extraction*~~

A general DNA extraction method may be used to extract DNA from the hepatopancreatic tissue of putatively infected shrimp. The amount of template DNA in a 10–25 µl PCR reaction volume should be in the range of 10–100 ng of total DNA

4.4.1. Real-time PCR

Real-time PCR methods for detection of *H. penaei* have the advantages of speed, specificity and sensitivity. The sensitivity of real-time PCR is ~100 copies of the target sequence from the *H. penaei* genome (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Vincent & Lotz, 2005).

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Aranguren *et al.,* 2010; GenBank U65509			
16S rRNA gene	Fwd NHP1300F: CGT-TCA-CGG-GCC-TTG-TAC-AC Rev NHP1366R: GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A Probe: CCG-CCC-GTC-AAG-CCA-TGG-AA	300 nM 100 nM	40 cycles: 95°C/15 sec and 60°C/1 min
Method 2: Aranguren & Dhar 2018; GenBank JQAJ01000001.1			
Flagella hook protein	Fwd NHP FlgE3qF: AAC-ACC-CTG-TCT-CCC-CAA-TTC Rev FlgE3qR: CCA-GCC-TTG-GAC-AAA-CAC-CTT Probe: CGC-CCC-AAA-GCA-TGC-CGC	500 nM 100 nM	40 cycles: 95°C/1 sec and 60°C/20 sec

Protocol 1

The real-time PCR method using TaqMan chemistry described below for *H. penaei* based on the 16S rRNA gene generally follows the method used in Aranguren *et al.* (2010).

i) The PCR primers and TaqMan probe are selected from the 16S, rRNA gene of *H. penaei* (GenBank U65509) (Loy & Frelier, 1996). The primers and TaqMan probe were designed by the Primer Express software version 2.0 (Applied Biosystems). The upstream (NHP1300F) and downstream (NHP1366R) primer sequences are: 5'-CGT-TCA-CGG-GCC-TTG-TACAC-3' and 5'-GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A-3', respectively. The TaqMan probe NHP: 5'-CCG-CCC-GTC-AAG-CCA-TGG-AA-3', which corresponds to the region from nucleotides 1321–1340, is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

ii) *The real-time PCR reaction mixture contains:* TaqMan One-step real-time PCR SuperMix (Quanta, Biosciences), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iii) Amplification is performed with the master cycler Realplex 2.0 (Eppendorf). The cycling consists of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. After each cycle, the levels of fluorescence are measured.

iv) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from *H. penaei*-infected hepatopancreas.

Protocol 2

Another real-time PCR method using TaqMan chemistry described below for *H. penaei* is based on the flagella gene (flagella hook protein, flgE) (Aranguren & Dhar, 2018).

i) The PCR primers and TaqMan probe were selected from the Flg E gene of *H. penaei* (GenBank JQAJ01000001.1) (Aranguren & Dhar, 2018). The primers and TaqMan probe were designed by the Primer Express software version 3.0 (Applied Biosystems). The upstream (NHP FlgE3qF) and downstream (FlgE3qR) primer sequences are: 5'-AAC-ACC-CTG-TCT-CCC-CAA-TTC-3'; and 5'-CCA-GCC-TTG-GAC-AAA-CAC-CTT-3', respectively. The TaqMan probe NHP: 5'-CGC-CCC-AAA-GCA-TGC-CGC-3', is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

ii) *The real-time PCR reaction mixture contains:* The amplification reactions were conducted as follows: 0.5 µM of each primer, 0.1 µM TaqMan probe, 1× TaqMan Fast Virus 1-Step Master Mix (Life Technologies), 5–50 ng DNA template and HPLC water in a reaction volume of 10 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iii) The real-time PCR profile consists of 20 seconds at 95°C followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Amplification detection and data analysis for real-time PCR assays are carried out with the StepOnePlus real-time PCR system (Life Technologies).

iv) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, or DNA extracted from *H. penaei*-infected hepatopancreas.

4.4.2. Conventional PCR

Hepatopancreas may be assayed for *H. penaei* using PCR. Two different PCR methods have been developed for *H. penaei* detection using 16S rRNA gene and Flg E gene separately.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Aranguren *et al.*, 2010; GenBank: MH230908.1; 379 bp			
16S rRNA gene	Fwd NHPF2: CGT-TGG-AGG-TTC-GTC-CTT-CAG-T Rev NHPR2: GCC-ATG-AGG-ACC-TGA-CAT-CAT-C	200 nM	35 cycles: 95°C/30 sec, 60°C/30 sec and 72°C/30 sec
Method 2: Aranguren & Dhar, 2018; JQAJ01000001.1; 333 bp			
Flagella hook protein	Fwd FlgE 1143F: AGG-CAA-ACA-AAC-CCT-TG Rev FlgE 1475R: GCG-TTG-GGA-AAG-TT	0.2 µM	35 cycles,: 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec

Protocol 1

The PCR based on 16S rRNA is based on Aranguren *et al.* (2010). Primers designated as NHPF2: 5'-CGT-TGG-AGG-TTC-GTC-CTT-CAGT-3' and NHPR2: 5'-GCC-ATG-AGG-ACC-TGA-CAT-CAT-C-3', amplify a 379-base pair (bp) fragment corresponding to the 16S rRNA of *H. penaei*. The PCR method outlined below generally follows the method described in Aranguren *et al.* (2010).

i) The following controls should be included when performing the PCR assay a) known *H. penaei* negative tissue sample; b) a known *H. penaei* positive sample (hepatopancreas); and c) a 'no template' control.

ii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

~~iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of *H. penaei* in shrimp hepatopancreas samples are: primers (0.2 µM each), dNTPs (200 µM each), Taq polymerase (0.1 U µl^{-1}), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.~~

~~iv) The cycling parameters are: Step 1: 95°C for 5 minutes, 1 cycle; Step 2: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, 35 cycles; Step 3: 60°C for 1 minute, 72°C for 2 minutes, 1 cycle; 4°C infinite hold.~~

~~**Protocol 2**~~

~~The PCR based on flagella gene (flagella hook protein, flgE) is based on Aranguren & Dhar (2018). Primers designated as NHP FlgE 1143F (5'-AGG CAA ACA AAC CCT TG-3') and and the NHP FlgE 1475R (5'- GCG TTG GGA AAG TT-3') amplify a 333-base pair (bp) fragment corresponding to the Flg E of *H. penaei*.~~

~~i) The following controls should be included when performing the PCR assay a) known *H. penaei* negative tissue sample; b) a known *H. penaei* positive sample (hepatopancreas); and c) a 'no template' control.~~

~~ii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.~~

~~iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of *H. penaei* in shrimp hepatopancreas samples are: primers (0.2 µM each), dNTPs (200 µM each), Taq polymerase (0.1 U µl^{-1}), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.~~

~~iv) The cycling parameters are: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes followed by 4°C infinite hold.~~

~~Note: The conditions should be optimised for each thermal cycler using known positive controls.~~

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published sequences.

~~PCR products may be cloned and sequenced or sequenced directly when necessary to confirm infection with *H. penaei* or to identify false positives or nonspecific amplification (Aranguren *et al.*, 2010; Aranguren & Dhar, 2018; Vincent & Lotz, 2005).~~

4.6. *In-situ* hybridisation

The ISH method of Loy & Frelier (1996) and Lightner (1996) provides greater diagnostic sensitivity than do more traditional methods for *H. penaei* detection and diagnosis of infection that employ classical histological methods (Lightner, 1996; Morales-Covarrubias, 2010). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled DNA probe to *H. penaei* 16S rRNA provides a definitive diagnosis of infection with *H. penaei* (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.*, 2006). Pathognomonic *H. penaei* positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the DNA probes. (See Chapter 2.2.4 *Infection with infectious hypodermal and*

haematopoietic necrosis virus for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

4.7. Immunohistochemistry

Immunohistochemistry (IHC) tests using monoclonal antibodies (MAbs) to *H. penaei*, according to the methods described in Bradley-Dunlop *et al.* (2004), ~~are available~~ exist for *H. penaei* detection.

4.8. Bioassay

Confirmation of infection with *H. penaei* may be accomplished by bioassay of suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the intracellular bacteria (Aranguren *et al.,* 2010; Lightner, 2005). Oral protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped hepatopancreas of suspect shrimp to SPF juvenile *P. vannamei* in small tanks. The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the hepatopancreas feeding (*per os*) protocol is used to bioassay for *H. penaei*, *positive* indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of infection with *H. penaei* and unusual mortalities.

4.9. Antibody- or antigen-based detection methods

Serological tests are not applicable because shrimp are invertebrate animals that do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to *H. penaei*.

4.10. Other methods

No scientifically confirmed reports.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR ~~are~~ is the recommended test for surveillance to demonstrate freedom from infection with *H. penaei* in apparently healthy populations as described in Section 4.4.1~~ and 4.4.2~~.

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 a suspect and confirmed case 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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status [7]

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

i) A positive result by real-time PCR

ii) A positive result by conventional PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *H. penaei* is considered to be confirmed if at least one of the following criteria is met:

i) A positive result by two different probe-based real-time PCR tests targeting different region of the *H. penaei* genome

ii) A positive result by real-time PCR and conventional PCR targeting different region of the *H. penaei* genome followed by amplicon sequencing

~~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 *H. penaei* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs consistent with *H. penaei* infection

ii) Histopathology consistent with *H. penaei* infection

iii) A positive result by real-time PCR

iv) A positive result by conventional PCR

v) A positive result by *in-situ* hybridisation

vi) A positive result by bioassay

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *H. penaei* is considered to be confirmed if at least at least one of the following criteria is met:

i) A positive result by two different probe-based real-time PCR tests targeting different regions of the *H. penaei* genome

[7] For example transboundary commodities.

ii) A positive result by real-time PCR and conventional PCR targeting different regions of the *H. penaei* genome followed by amplicon sequencing

iii) ~~Histopathology consistent with *H. penaei* and positive *in-situ* hybridisation test~~ A positive result by *in-situ* hybridisation and real-time PCR

iv) A positive result by *in-situ* hybridisation and conventional PCR followed by amplicon sequencing

v) A positive result by bioassay followed by real-time PCR

vi) A positive result by bioassay followed by conventional PCR followed by amplicon sequencing

~~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 *H. penaei* are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with *H. penaei*, 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

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction, ND = Not determined.

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 specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.

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*
* *

NB: There is an OIE Reference Laboratory for infection with *Hepatobacter penaei*
(necrotising hepatopancreatitis)
(please consult the OIE web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on
infection with *Hepatobacter penaei* (necrotising hepatopancreatitis).

NB: FIRST ADOPTED IN 2012; MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.4.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infection with infectious hypodermal and haematopoietic necrosis virus means *infection* with the *pathogenic agent* *Decapod penstylhamaparvovirus* 1, of the Genus *Penstylhamaparvovirus* and Family *Parvoviridae* ~~infection with the pathogenic agent infectious hypodermal and haematopoietic necrosis virus (IHHNV), Family *Parvoviridae*, subfamily *Hamaparvovirinae*, Genus *Penstylhamaparvovirus* with IHHNV (*Decapod penstylhamaparvovirus 1*) as the Type species (Penez *et al.,* 2020)~~.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

IHHNV is the smallest of the known penaeid shrimp viruses. The virion is a 20–22 nm, non-enveloped icosahedron, with a density of 1.40 g ml^{-1} in CsCl that contains linear single-stranded DNA with an estimated size of 3.9 kb (GenBank NC_002190), and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (Bonami *et al.,* 1990; Nunan *et al.,* 2000~~; GenBank NC_002190~~) .

At least two distinct genotypes of IHHNV have been identified (Tang *et al.,* 2003): Type 1 is from the Americas and South-East Asia (principally the Philippines) and Type 2 is from South-East Asia. These genotypes were shown to be ~~are~~ infectious to *Penaeus vannamei* and *P. monodon* (Tang et al., 2003). Two sequences homologous to part of the IHHNV genome are found embedded in the genome of penaeids. These were initially described as Type 3A from East Africa, India and Australia, and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al*., 2007). Tissues containing the IHHNV-homologous sequences (also known as endogenous viral elements; Taengchaiyaphum *et al*., 2021) in the *P. monodon* genome are not infectious to susceptible host species (Lightner *et al*., 2009; Tang & Lightner, 2006; Tang *et al*., 2007).

2.1.2. Survival and stability in processed or stored samples

IHHNV is believed to be the most stable virus of the known penaeid shrimp viruses. Infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner *et al.,* 1987; Lightner *et al.,* 2009).

2.1.3. Survival and stability outside the host

No data.

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 infection with IHHNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: yellowleg shrimp (*Penaeus californiensis*), giant tiger

prawn (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*Penaeus stylirostris*), and white leg 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 infection with IHHNV according to Chapter 1.5 of the *Aquatic Code* are: northern brown shrimp (*Penaeus aztecus*). ~~Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.~~

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: ~~giant river prawn (*Macrobrachium rosenbergii*), northern pink shrimp (*Penaeus duorarum*), western white shrimp (*P. occidentalis*), kuruma prawn (*P. japonicus*), green tiger prawn (*P. semisulcatus*), *Hemigrapsus penicillatus*, Argentine stiletto shrimp (*Artemesia longinaris*), Cuata swimcrab (*Callinectes arcuatus*), Mazatlan sole (*Achirus mazatlanus*), yellowfin mojarra (*Gerres cinereus*), tilapias (*Oreochromis* sp.), Pacific piquitinga (*Lile stolifera*) and blackfin snook (*Centropomus medius*).~~

Family	Scientific name	Common name
Achiridae	*Achirus mazatlanus*	Mazatlan sole
Centropomidae	*Centropomus medius*	blackfin snook
Cichlidae	*Oreochromis* sp.	tilapias
Clupeidae	*Lile stolifera*	Pacific piquitinga
Gerreidae	*Gerres cinereus*	yellowfin mojarra
Palaemonidae	*Macrobrachium rosenbergii*	giant river prawn
Penaeidae	*Penaeus duorarum*	northern pink shrimp
	Penaeus occidentalis	western white shrimp
	Penaeus japonicus	*kuruma prawn*
	Penaeus semisulcatus	green tiger prawn
	Artemesia longinaris	Argentine stiletto shrimp
Portunoidea	*Callinectes arcuatus*	Cuata swimcrab
Varunidae	*Hemigrapsus penicillatus*	

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

IHHNV has been detected in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei*. Nauplii produced from infected broodstock have a high prevalence of infection with IHHNV (Motte *et al.*, 2003).

2.2.4. Distribution of the pathogen in the host

IHHNV targets gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, lymphoid organ, parenchymal cells, connective tissue cells and ovaries (Chayaburakul, 2005; Lightner, 1996; Lightner & Redman, 1998).

2.2.5. Aquatic animal reservoirs of infection

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infection may carry the virus subclinically and infect their progeny or other populations by vertical and horizontal transmission (Bell & Lightner, 1984; Lightner, 1996; Motte *et al.*, 2003).

2.2.6. Vectors

IHHNV ~~was found in wild crabs~~ has been detected in many crustacean and non-crustacean species however their ~~(*Hemigrapsus penicillatus*, *Neohelice granulata*), but there were no clinical signs. Adults of *Macrobrachium rosenbergii* are carriers of IHHNV without apparent signs. Although the mussel *Mytilus edulis* is an important reservoir of IHHNV (Wei *et al.*, 2017), its~~ capacity to transmit virus is unknown.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The effects of infection with IHHNV varies among shrimp species and populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with IHHNV results in acute, usually catastrophic, disease with mortalities approaching 100%. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size- or age-dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Lightner, 1996; Lightner *et al.*, 1983).

In contrast, in populations of *P. vannamei*, some selected lines of *P. stylirostris*, and some populations of *P. monodon*, infection with IHHNV results in a more subtle, chronic disease, runt-deformity syndrome (RDS), in which high mortalities are unusual, but where growth suppression and cuticular deformities are common (Kalagayan *et al.*, 1991; Sellars *et al.,* 2019). The severity and prevalence of RDS in infected populations of juvenile or older *P. vannamei* may be related to infection during the larval or early postlarval stages.

~~Infection with IHHNV interferes with normal egg, larval, and postlarval development. When broodstock are used from wild or farmed stocks where the disease is enzootic, hatching success of eggs may be reduced, and survival and culture performance of the larval and postlarval stages lowered (Motte *et al.,* 2003).~~

There was no mortality or clinical signs of disease in *P. vannamei*, *P. monodon* or *P. stylirostris* when experimentally challenged with IHHNV genotypes from Ecuador and Peru (Aranguen Caro *et al.*, 2022). The IHHNV genotypes were found to be within a separate lineage of IHHNV type 2 genotypes circulating within these countries (Aranguen Caro *et al.*, 2022).

In the past, stocks of *P. stylirostris*, juveniles, ~~subadults,~~ and adults showed persistently high mortality rates due to infection with IHHNV. However, selected lines of *P. stylirostris* do not show mortality and appear to be tolerant to this virus. ~~*Penaeus vannamei* and *P. monodon* stocks infected with IHHNV show poor and highly disparate growth and cuticular deformities, particularly bent rostrums and deformed sixth abdominal segments (Jagadeesan *et al.,* 2019; Sellars *et al.,* 2019).~~

In regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *P. stylirostris* in the lower and upper Gulf of California, respectively (Pantoja *et al.,* 1999); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (Morales-Covarrubias *et al.,* 1999); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (Nunan *et al.,* 2001); from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte *et al.,* 2003), and from 6 to 63% in *P. vannamei* broodstock and 49.5% in post-larvae from Mexico (Fernando *et al*., 2016). In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence is typical (Aly *et al*., 2021; Chayaburakul *et al.,* 2004; Lightner, 1996; Lightner *et al.,* 1983).

2.3.2. Clinical signs, including behavioural changes

Animals with this disease may show one or more of these signs, but the pathogen may still be present in the absence of any signs. Clinical signs are non-specific, but juvenile *P. stylirostris* with acute infection with IHHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHHNV have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings.

Certain cuticular deformities, specifically a deformed rostrum bent to the left or right, which may be presented by *P. vannamei* and *P. stylirostris* with RDS, are indicative of infection with IHHNV ~~(see Section 2.3.3 *Gross pathology: Infection with IHHNV in* Penaeus vannamei)~~. However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV.

In acute disease, *P. stylirostris* may present behavioural changes ~~(see Section 2.3.3 *Gross pathology: Infection with IHHNV in* Penaeus stylirostris)~~ but with RDS, no consistent behavioural changes have been reported for affected shrimp.

<u>Infection with IHHNV interferes with normal egg, larval, and postlarval development. When broodstock are used from wild or farmed stocks where the disease is enzootic, hatching success of eggs may be reduced, and survival and culture performance of the larval and postlarval stages lowered (Motte *et al.*, 2003).</u>

2.3.3. Gross pathology

~~*Infection with IHHNV in* Penaeus stylirostris~~

~~Infection with IHHNV may result in acute disease with very high mortalities in juveniles. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size- or age-dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Lightner, 1996; Lightner *et al.,* 1983). Gross signs are non-specific, but juvenile P. stylirostris with acute infection with IHHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHHNV have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. *Penaeus stylirostris* at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *P. stylirostris* and individuals become more bluish. In *P. stylirostris* and P. monodon with terminal-phase infection with IHHNV, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Lightner *et al.,* 1983).~~

~~*Infection with IHHNV in* Penaeus vannamei~~

~~RDS, a chronic form of infection with IHHNV, occurs in *P. vannamei*. The severity and prevalence of RDS in infected populations of juvenile or older *P. vannamei* may be related to infection during the larval or early postlarval stages. RDS has also been reported in cultured stocks of *P. stylirostris* and *P. monodon*.~~ Juvenile shrimp with RDS may display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp with RDS display disparate growth with a wide distribution of sizes and many smaller than expected ('runted') shrimp. The coefficient of variation (CV = the standard deviation divided by the mean of different size groups within a population) for populations with RDS is typically greater than 30% and may approach 90%, while populations of juvenile *P. vannamei* and *P. stylirostris* free from infection with IHHNV (and thus RDS-free) usually show CVs of 10–30% (Lightner, 1996; Primavera & Quinitio, 2000).

2.3.4. Modes of transmission and life cycle

Transmission of IHHNV can be by horizontal or vertical. Horizontal transmission has been demonstrated by cannibalism or by contaminated water (Lightner, 1996; Lightner *et al.,* 1983), and vertical transmission via infected eggs (Motte *et al.,* 2003).

2.3.5. Environmental factors

The replication rate of IHHNV at high water temperatures was significantly reduced in a study in which viral replication was compared in *P. vannamei* experimentally infected and held at 24°C and 32°C. After

a suitable incubation period, shrimp held at 32°C had approximately 10^2 times lower viral load than shrimp held at 24°C (Montgomery-Brock *et al*., 2007).

2.3.6. Geographical distribution

Infection with IHHNV has been reported from cultured shrimp in most of the major shrimp-culturing regions of the world including Asia, Oceania, North and South America and the Middle East.

IHHNV homologous sequences have been found within the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al*., 2007). These sequences do not represent viral DNA (refer Section 2.1.1 *Aetiological agent*).

See OIE WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

None available.

2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Breeding resistant strains

Selected stocks of *P. stylirostris* that are resistant to infection with IHHNV have been developed and these have had some successful application in shrimp farms (Lightner, 1996). However, lines of *P. stylirostris* bred for resistance to infection with IHHNV (Tang *et al.,* 2000) do not have increased resistance to other diseases, such as white spot syndrome virus (WSSV), so their use has been limited. In some stocks a genetic basis for IHHNV susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.,* 1997).

2.4.5. Inactivation methods

IHHNV is a stable shrimp virus; infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner et al., 2009).

2.4.6. Disinfection of eggs and larvae

IHHNV is transmitted vertically by the transovarian route (Motte *et al.,* 2003). Disinfection of eggs and larvae is good management practice (Chen *et al.,* 1992) that may reduce IHHNV contamination of spawned eggs and larvae but is not effective for preventing transovarian transmission of IHHNV (Motte *et al.,* 2003).

2.4.7. General husbandry

Some husbandry practices have been successful in preventing the spread of IHHNV. Among these has been the application of PCR pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Motte *et al.,* 2003), as well as the development of specific pathogen-free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 2005). The latter has proven to be the most successful husbandry practice for the prevention and control of infection with IHHNV (Lightner, 2005).

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

Infection with IHHNV may be below detection limits in spawned eggs and larval stages, so these life stages are not suitable for surveillance to demonstrate freedom from infection with IHHNV.

3.2. Selection of organs or tissues

IHHNV infects tissues of ectodermal and mesodermal origin. The principal target tissues for IHHNV include connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells (Lightner, 1996; Lightner & Redman, 1998). Hence, whole shrimp (e.g. larvae or postlarvae) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

3.3. Samples or tissues not suitable for pathogen detection

Enteric tissues (e.g. the hepatopancreas, the midgut or its caeca) are inappropriate samples for detection of IHHNV (Lightner, 1996; Lightner & Redman, 1998). Shrimp eyes contain PCR inhibitors and their use should be avoided.

3.4. Non-lethal sampling

Haemolymph or excised pleopods may be collected and used for testing when non-lethal testing of valuable broodstock is necessary (Lightner, 1996; Lightner & Redman, 1998).

3.5. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time 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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.5 of Chapter 2.2.0 *General information* (diseases of crustaceans).

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.2 of Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.4. Samples for other tests

Not relevant.

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 shrimp should be processed and tested individually. Small life stages such as PL can be pooled to obtain the minimum amount of material for ~~virus 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology						++	++	NA		~~++~~	~~++~~	~~NA~~
Cell culture												
Real-time PCR	~~++~~	+++	+++	1	++	+++	+++	1	++	++	++	1
Conventional PCR	~~+~~	++	++	1	++	++	++	1	~~++~~	~~++~~	~~++~~	~~1~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation						+	+	1		++	++	1
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods[3]												
Other methods[3]												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction;
LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

4.2. Histopathology and cytopathology

Presumptive acute infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained sections whereas chronic infection are much more difficult to diagnose using these staining methods. For diagnosis of chronic infections and confirmation of acute infections however, the use of molecular methods is required for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or *in-situ* hybridisation [ISH] of histological sections).

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies, provides a provisional diagnosis of infection with IHHNV. These characteristic IHHNV inclusion bodies are eosinophilic and often haloed (with H&E stains of tissues preserved with fixatives that contain acetic acid, such as Davidson's AFA and Bouin's solution) (Bell & Lightner, 1988; Lightner, 1996), intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies caused by infection with IHHNV may be easily confused with developing intranuclear inclusion bodies caused by WSSV infection. ISH assay (see Section 4.6 In-situ *hybridisation*) of such sections with a DNA probe specific to IHHNV provides a definitive diagnosis of infection with IHHNV (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

The use of Davidson's fixative (containing 33% ethyl alcohol [95%], 22% formalin [approximately 37% formaldehyde], 11.5% glacial acetic acid and 33.5% distilled or tap water) is highly recommended for all routine histological studies of shrimp (Bell & Lightner, 1988; Lightner, 1996a). To obtain the best results, dead shrimp should not be used. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are killed by injection of fixative directly into the hepatopancreas; the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline is opened with fine-pointed surgical scissors to enhance fixative penetration (the abdomen may be removed and discarded), the whole shrimp (or cephalothorax) is immersed in fixative for 24 to 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags.

4.3. Cell culture for isolation

IHHNV has not been grown *in vitro*. No crustacean cell lines exist.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

There are multiple ~~geographical~~ variants of IHHNV, some of which are not detected by ~~all of the~~ some available methods. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (Tang *et al*., 2000; 2007). However, these tests also detect non-infectious endogenous viral elements (EVE) within the *P. monodon* genome (previously known as types 3A and 3B), which are inserted into the genome of certain stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (Saksmerprome *et al.,* 2011; Taengchaiyaphum *et al.,* 2022; Tang & Lightner, 2006; Tang *et al.,* 2007). As these PCR methods may result in positive test results in uninfected *P. monodon,* positive results should be confirmed by a method that detects IHHNV but not the IHHNV-related EVEs.

PCR primers have been developed that can detect the IHHNV sequence but do not amplify IHHNV-related EVEs present in the *P. monodon* stocks from Africa, Australia (Tang *et al.,* 2007), or Thailand (Saksmerprome *et al.,* 2011). Primer set 309F/R amplifies only a genomic segment of IHHNV types 1 and 2 (the infectious forms of IHHNV), but not the non-infectious EVEs within the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Primer set MG831F/R reacts only with non-infectious EVEs within the *P. monodon* genome (Tang *et al.,* 2007). Hence, confirmation of unexpected positive or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. histology, bioassay, ISH) is highly recommended.

4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Real-time PCR methods have been developed for the detection of IHHNV (Dhar *et al.,* 2001; Tang & Lightner, 2001). A highly sensitive SYBR Green real-time PCR targeting a segment of the IHHNV genome that is considered less susceptible to endogenisation was developed (Encinas-Garcia *et al.,* 2015). More recently, A TaqMan real-time assay capable of developed to differentiate endogenous virus element EVEs from infectious form of IHHNV in *P. monodon* has been reported (Cowley *et al.,* 2018); however, analysis of a *P. monodon* whole genome sequence has identified 100% primer and probe sequence matches to EVEs (Taengchaiyaphum *et al.,* 2022). The real-time PCR method using TaqMan chemistry described in Table 4.4.1 below for IHHNV generally follows the method used in Tang & Lightner (2001).

Table 4.4.1. Primers and probes for real-time PCR detection of IHHNV

Pathogen/ target gene	**Primer/probe (5'–3')**	**Concentration**	**Cycling parameters**
Method 1* Tang & Lightner, 2001; GenBank Acc. No AF218266			
IHHNV and IHHNV-related EVEs non-structural protein	Fwd IHHNV1608F: TAC-TCC-GGA-CAC-CCA-ACC-A Rev IHHNV1688R: GGC-TCT-GGC-AGC-AAA-GGT-AA Probe: FAM-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-TAMRA	300 nM primers 150 nM probe	40 cycles of: 95°C/1 sec and 60°C/20 sec

****NOTE*** *– this method will amplify EVEs within the genome of* P. monodon. *Positive results in this species must be confirmed by a method that does not react with IHHNV EVEs.*

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5'-TAC-TCC-GGA-CAC-CCA-ACC-A-3' and 5'-GGC-TCT-GGC-AGC-AAA-GGT-AA-3', respectively. The TaqMan probe 5'-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3'), is synthesised and labelled with FAM on the 5' end and TAMRA on the 3' end.

ii) Preparation of DNA template: DNA extracted from tissues or haemolymph that was preserved in 95% ethanol and then dried. A control consisting of tissues or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods. Commercial DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega), or DNazol (Life Technologies). Spectrophotometric readings of the final DNA will indicate the purity of the DNA and the amount of total DNA extracted from the sample.

iii) The real-time PCR reaction mixture contains: TaqMan Fast virus 1-step Master Mix (Life Technologies, or commercially available equivalent reagents), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 20 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

~~iv) The cycling profile is: initial denaturation of 20 seconds at 95°C, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.~~

4.4.2. Conventional PCR

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

Several one-step PCR methods (Krabsetsve *et al.,* 2004; Nunan *at al.,* 2000; Shike *et al.,* 2000; Tang *et al.,* 2000; 2007), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available. In addition to IHHNV, some of these methods will amplify EVEs in *Penaeus monodon*. Positive results in *P. monodon* should be followed up with other methods that will not react with EVEs. ~~In the event that results are ambiguous using the 389F/R 'universal' primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012F/77353R or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.~~

Table 4.4.2.1. Recommended primer sets for ~~one-step~~ conventional PCR detection of IHHNV

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1* Tang *et al.,* 2007; GenBank Acc. No. AF218266; 389bp product			
IHHNV and IHHNV-related EVEs Non-structural protein	Fwd 389F: CGG-AAC-ACA-ACC-CGA-CTT-TA Rev 389R: GGC-CAA-GAC-CAA-AAT-ACG-AA	200 nM	35 cycles of: 94°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 2* Nunan *et al.,* 2000; GenBank Acc. No AF218266; 356bp product			
IHHNV and IHHNV-related EVEs Between the non-structural and capsid protein-coding regions	Fwd 77012F: TAC-TCC-GGA-CAC-CCA-ACC-A Rev 77353R: GGC-TCT-GGC-AGC-AAA-GGT-AA	1000 nM	35 cycles of: 95°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 3* Tang *et al.,* 2000; GenBank Acc. No AF218266; 392bp product			
IHHNV and IHHNV-related EVEs Non-structural protein	Fwd 392F: GGG-CGA-ACC-AGA-ATC-ACT-TA Rev 392R: ATC-CGG-AGG-AAT-CTG-ATG-TG	300 nM	35 cycles of: 95°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 4 Tang *et al.,* 2007; GenBank Acc. No AF218266; 309bp product			
IHHNV ORF1	Fwd 309F: TCC-AAC-ACT-TAG-TCA-AAA-CCA-A Rev 309R: TGT-CTG-CTA-CGA-TGA-TTA-TCC-A	200 nM	35 cycles of: 94°C/30 sec, 55°C/30 sec, and 72°C/30 sec

****NOTE*** – *these methods will amplify EVEs within the genome of* P. monodon. *Positive results in this species must be confirmed by a method that does not react with IHHNV EVEs.*

~~Primer~~	~~Product~~	~~Sequence (5'-3')~~	~~G+C%/Temp.~~	~~GenBank & References~~	~~Specificity~~
~~389F~~	~~389 bp~~	~~CGG-AAC-ACA-ACC-CGA-CTT-TA~~	~~50%/72°C~~	~~AF218266~~	~~All genetic variants of IHHNV~~
~~389R~~		~~GGC-CAA-GAC-CAA-AAT-ACG-AA~~	~~45%/71°C~~	~~(Tang *et al.,* 2007)~~	and ~~IHHNV-related EVEs~~
~~77012F~~	~~356 bp~~	~~ATC-GGT-GCA-CTA-CTC-GGA~~	~~50%/68°C~~	~~AF218266~~	~~Not given in the reference~~

Primer	Product	Sequence (5′-3′)	G+C%/Temp.	GenBank & References	Specificity
77353R		TCG-TAC-TGG-CTG-TTC-ATC	55%/63°C	(Nunan *et al.*, 2000)	
392F	392 bp	GGG-CGA-ACC-AGA-ATC-ACT-TA	50%/68°C	AF218266	All genetic variants of IHHNV and IHHNV-related EVEs
392R		ATC-CGG-AGG-AAT-CTG-ATG-TG	50%/71°C	(Tang *et al.*, 2000)	
309F	309 bp	TCC-AAC-ACT-TAG-TCA-AAA-CCA-A	36%/68°C	AF218266	IHHNV but not IHHNV-related EVEs
309R		TGT-CTG-CTA-CGA-TGA-TTA-TCC-A	40%/69°C	(Tang *et al.*, 2007)	
MG831F	831 bp	TTG-GGG-ATG-CAG-CAA-TAT-CT	45%/58°C	DQ228358	IHHNV-related EVEs but not IHHNV
MG831R		GTC-CAT-CCA-CTG-ATC-GGA-CT	55%/62°C	(Tang *et al.*, 2007)	

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region of the IHHNV genome. Primers 77012F/77353R are from a region in between the nonstructural and capsid protein-coding region of the genome. In the event that results are ambiguous using the 389F/R 'universal' primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012F/77353R or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.

General PCR method for IHHNV: the PCR method described below for IHHNV generally follows the methods outlined in Tang *et al.* (2007) and Nunan *et al.* (2000). However, recent minor modifications including the sources of the reagents and the use of an automated DNA extraction instrument are acceptable. The modifications include DNA extraction method, choice of primers (Table 4.4.2.1), and the volume of reaction. These slightly modified methods have been validated in accordance with Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases* and do not affect the diagnostic performance of the assay.

i) Use as a template, the extraction of DNA templates is the same as that described above. Use 1–5 µl of extracted DNA as a template per 25 µl reaction volume.

ii) The following controls should be included in every PCR assay for IHHNV: (a) DNA from a known negative tissue sample; (b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and (c) a 'no template' control.

iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV-infected material. Prepare primers at 10 µM in distilled water.

iv) If PuReTaq™ Ready-To-Go PCR Beads (GE Healthcare) are used, the PCR profile involves a 3–5 minutes at 95°C to denature DNA followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

v) Prepare a 'Master Mix' consisting of water and primers.

vi) For a 25 µl reaction mix, add 24 µl Master Mix to each tube and then add 1 µl of the DNA template to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. Insert tubes into the thermal cycler and start the PCR program.

viii) After PCR, run 6–10 µl of the sample in a 1.5% agarose gel (containing SYBR™ Safe (Thermo Fisher Scientific) or equivalent to stain the DNA). Look for the 389 bp band (if using primers 389F and 389R) or for the 356 bp band (if using primers 77012F and 77353R). Bands are not always seen, as it is necessary to have at least 10 ng DNA µl^{-1} to see DNA in a gel. A direct sequencing of amplified products can be performed through gel extraction of a PCR band with correct size and the sequencing primer(s) used for amplification to confirm the presence of IHHNV.

4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification (LAMP) assays and a real-time isothermal recombinase polymerase amplification (RPA) assay ~~are available~~ to detect ~~and confirm~~ IHHNV ~~infection~~ have been published (Arunrut *et al*., 2011; Sun *et al*., 2006; Xia *et al*., 2015), however, they are currently not recommended as they are not sufficiently validated.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published sequences.

~~PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified products from the infectious form of the virus and demonstrate the presence of the non-infectious IHHNV-related EVEs in the host genome (Tang & Lighter, 2006).~~

4.6. *In-situ* hybridisation

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. The ISH method uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al*. (1993) and Lightner (1996).

Gene probe and PCR methods provide greater diagnostic specificity and sensitivity than traditional techniques that employ classic histological approaches. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp. A haemolymph sample may be taken with a tuberculin syringe, or an appendage (a pleopod for example) may be biopsied (Bell *et al*., 1990), and used as the sample for a dot-blot hybridisation test.

4.7. Immunohistochemistry

Not relevant.

4.8. Bioassay

If SPF shrimp are available, the following bioassay method is based on Tang *et al.* (2000), is suitable for IHHNV diagnosis.

i) For bioassay, feed the minced shrimp tissue suspected of being infected with IHHNV to the indicator shrimp species (e.g. SPF *P. vannamei* and *P. stylirostris* at the PLs or juvenile stage) at 10% of their body weight twice daily for 1 day~~s~~.

ii) For the following, the indicator shrimp were maintained on a pelletised ration.

iii) Examine moribund shrimp grossly or by using the methods described above. There may be no apparent mortality during the experimental period.

iv) If at 30 days after feeding there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

Known IHHNV positive and negative control groups should be included in the bioassay.

4.9. Antibody- or antigen-based detection methods

None has been successfully developed.

4.10. Other methods

Not available.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Conventional PCR and/or real-time PCR are the recommended test for surveillance to demonstrate freedom from infection with IHHNV in apparently healthy populations as described in Sections 4.4.1 and 4.4.2.

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 a suspect and confirmed case 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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status [8]

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

i) Positive result by conventional PCR

ii) Positive result by real-time PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with IHHNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time PCR and a positive result by conventional PCR ~~targeting non-overlapping regions of the viral genome and~~ followed by amplicon sequencing

ii) ~~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 IHHNV shall be suspected if at least one of the following criteria is met:

[8] For example transboundary commodities.

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality

ii) Histopathology consistent with IHHNV infection

iii) Positive result by conventional PCR

~~iii~~ iv) Positive result by real-time PCR

~~iv) Histopathology consistent with IHHNV infection~~

v) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with IHHNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time PCR and a positive result by conventional PCR ~~targeting non-overlapping regions of the viral genome and~~ followed by amplicon sequencing

ii) ~~Histopathology consistent with IHHNV infection coupled with~~ A positive result by *in-situ* hybridisation and ~~detection of IHHNV~~ a positive result by real-time PCR

iii) ~~Histopathology consistent with IHHNV infection coupled with~~ A positive result by *in-situ* hybridisation and ~~detection of IHHNV by~~ a positive result by conventional PCR ~~and~~ followed by amplicon sequencing

~~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 [under study]

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHHNV is provided in Table 6.3.1 (none available). This information can be used for the design of surveys for infection with IHHNV, 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

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.

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 specificity, *n* = number of samples used in the study,

PCR: = polymerase chain reaction.

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* *

NB: There are OIE Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus (please consult the OIE web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on
infection with infectious hypodermal and haematopoietic necrosis virus

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS;
MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 2.2.5.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

1. Scope

Infection with infectious myonecrosis virus means infection with the pathogenic agent infectious myonecrosis virus (IMNV) that is tentatively assigned to the Family *Totiviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Phylogenetic analysis of its RNA-dependent RNA polymerase (RdRp) gene coding sequence indicates that IMNV is most closely related to *Giardia lamblia virus*, a member of the family *Totiviridae* (Fauquet *et al.,* 2005; Lightner, 2011; Nibert, 2007; Poulos *et al.,* 2006).

IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml^{-1} in caesium chloride. The genome consists of a single, double-stranded (ds) RNA molecule of 8226–8230 bp (Loy *et al.*, 2015; Naim *et al.,* 2015). Sequencing of the viral genome reveals two non-overlapping open reading frames (ORFs). The first ORF (ORF1, 470–5596 nt) encodes a putative RNA-binding protein and a capsid protein. The coding region of the RNA-binding protein is located in the first half of ORF1 and contains a dsRNA-binding motif in the first 60 amino acids. The second half of ORF1 encodes a capsid protein, as determined by amino acid sequencing, with a molecular mass of 106 kDa. The second ORF (ORF2, 5884–8133 nt) encodes a putative RdRp (Poulos *et al.,* 2006). The most variable region of IMNV genome is located in the first half of ORF1, coinciding with a region which probably encodes the capsid protrusions (Dantas *et al*., 2015).

The complete genomes of IMNV types originating from Brazil and Indonesia have been sequenced and found to be 99.6% identical at the nucleotide level (Poulos *et al.,* 2006; Senapin *et al.,* 2007). The 99.6% full genome sequence identity (and anecdotal information on the introduction of *Penaeus vannamei* stocks from Brazil) indicate that the disease was introduced from Brazil to Indonesia in 2006. A new genotype was analysed in infected samples in 2018 in Indonesia, including an isolate that contains a deletion of 622 amino acids (Mai *et al.,* 2019).

2.1.2. Survival and stability in processed or stored samples

No data.

2.1.3. Survival and stability outside the host

No information available.

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 infection with IMNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: brown tiger prawn (*Penaeus esculentus*), banana prawn (*P. merguiensis*), and whiteleg shrimp (*P. 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 infection with IMNV according to Chapter 1.5 of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*) and blue shrimp (*P. stylirostris*).

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: southern brown shrimp (*P. subtilis*).

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Juveniles and subadults of *P. vannamei*, farmed in marine, brackish, and low salinity brackish water, appear to be most severely affected by infection with IMNV (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006).

2.2.4. Distribution of the pathogen in the host

The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells (Lightner, 2011; Lightner *et al.,* 2004; Poulos *et al.,* 2006; Tang *et al.,* 2005).

2.2.5. Aquatic animal reservoirs of infection

Some members of populations of *P. vannamei* that survive IMNV infections or epizootics may carry the virus.

2.2.6. Vectors

Experimental studies have demonstrated that brine shrimp *Artemia franciscana* can act as a vector for IMNV (da Silva *et al*., 2015).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In early juvenile, juvenile, or adult *P. vannamei* in regions where infection with IMNV is enzootic, outbreaks of IMNV infections associated with sudden high morbidity and mortality may follow 'stress' events such as capture by cast-netting, feeding and sudden changes in water salinity or temperature (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006). Feed conversion ratios of affected populations can increase from a normal value of ~ 1.5 up to 4.0 or higher (Andrade *et al.,* 2007). Mortalities from infection with IMNV can range from 40% to 70% in cultivated *P. vannamei*.

In regions where infection with IMNV is enzootic in farmed stocks of *P. vannamei*, its prevalence may reach 100% (Andrade *et al.,* 2007; Nunes *et al.,* 2004).

2.3.2. Clinical signs, including behavioural changes

Affected shrimp present with visibly white tails. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. High mortality can occur suddenly and continue for several days. Clinical signs may have a sudden onset following stress events (e.g. capture by cast-netting, feeding, and sudden changes in temperature or salinity).

Only shrimp in the acute phase of disease present behavioural changes. Typically, severely affected shrimp become lethargic during or soon after stress events such as capture by cast-netting, feeding, sudden changes in water temperature, sudden reductions in water salinity, etc.

2.3.3 Gross pathology

Shrimp in the acute phase of disease present focal-to-extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp.

Exposing the paired lymphoid organs (LO) by simple dissection will show that they are hypertrophied (3–4 times their normal size) (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

2.3.4. Modes of transmission and life cycle

IMNV has been demonstrated to be transmitted horizontally by cannibalism (Lightner, 2011; Poulos *et al.,* 2006). Transmission via water probably occurs. Although vertical transmission is suspected from anecdotal evidence, it is not known whether this occurs via transovarial mechanism or by surface contamination of newly spawned eggs.

2.3.5. Environmental factors

Temperature and salinity effects are likely predisposing factors to disease outbreaks, but no experimental data are available (Nunes *et al.,* 2004).

2.3.6. Geographical distribution

Infection with IMNV has been reported to occur in some countries in the Americas, Asia and Africa (Aly *et al.,* 2021; Andrade *et al.,* 2007; Lightner *et al.,* 2004; Naim *et al.,* 2014; Nunes *et al.,* 2004; Poulos *et al.,* 2006; Sahul *et al*., 2017).

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No effective vaccines for infection with IMNV are available.

2.4.2. Chemotherapy including blocking agents

Ctn[15-34], a cathelicidin-derived eicosapeptide was found to demonstrate antiviral activity against IMNV in primary haemocyte cultures (Vieira-Girao *et al.,* 2017).

2.4.3. Immunostimulation

No data.

2.4.4. Breeding resistant strains

There are anecdotal reports of some selected lines of *P. vannamei* having better survival and culture performance in farms where infection with IMNV is enzootic. During a 20-day controlled laboratory study in which the shrimp were challenged with IMNV, some domesticated lines of *P. vannamei* were found to survive better than other lines (White-Noble *et al.,* 2010).

Penaeus monodon and *P. stylirostris*, for which there is incomplete evidence of susceptibility (see section 2.2.2), are considered to be more resistant to infection with IMNV than *P. vannamei* (Tang *et al.,* 2005).

2.4.5. Inactivation methods

No data.

2.4.6. Disinfection of eggs and larvae

While IMNV is believed to be transmitted vertically, there are no scientific data confirming this route of transmission. Disinfection of eggs and larvae (Chen *et al.,* 1992) is a good management practice

recommended to reduce the potential for transmission of a number of penaeid shrimp diseases from female spawners to their eggs or larvae, and the practice may reduce IMNV contamination of spawned eggs and larvae produced from them.

2.4.7. General husbandry

Management practices in endemic areas principally involves exclusion of IMNV from shrimp farms. Broodstock or their spawned eggs or nauplii are PCR-tested and those that test positive are discarded (Andrade *et al.,* 2007). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P. vannamei* most suited to local culture conditions has proven to be the most successful for preventing and controlling other virus diseases of shrimp, and should be applicable to control and prevent infection with IMNV (Lee & O'Bryen, 2003; Lightner, 2005; Lightner *et al.,* 2009; Moss & Moss, 2009).

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

Specimens suitable for testing for infection with IMNV using molecular methods (e.g. RT-PCR, nested RT-PCR, real-time RT-PCR, etc.) include postlarvae (PL), juveniles, subadults and adults. While IMNV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in larval stages, so these life stages may not be suitable for demonstrating freedom from infection with IMNV unless validated for those life stages.

3.2. Selection of organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of infection with IMNV are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue.

3.3. Samples or tissues not suitable for pathogen detection

IMNV replicates systemically but does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of IMNV infection.

3.4. Non-lethal sampling

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.5. Preservation of samples for submission

Several factors can affect specimen quality during collection, handling and storage, such as exposure to light, heat, desiccation, and incomplete preservation. Hence, standard operating protocols or recommended practices should be followed at all steps of the diagnostic process.

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0.

3.5.1. Samples for pathogen isolation

Not applicable.

3.5.2. Preservation of samples for molecular detection

Tissue samples (pleopods, cephalothorax, muscle, haemolymph) for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.2 of Chapter 2.3.0 *General information* (diseases of fish).

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 shrimp should be processed and tested individually. Small life stages such as PL or fry can be pooled to obtain the minimum amount of material for 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts					+	+	+	1				
Histopathology					++	++	++	2				
Cell culture												
Real-time RT-PCR	+	++	++	1	++	++	++	2	++	++	++	2
Conventional RT-PCR	+	++	++	1	++	++	++	1				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation					+	+	+	1	+	++	++	1
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods												
Other methods												

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; IFAT = indirect fluorescent antibody test. [give definitions of abbreviations as appropriate; nPCR = nested PCR, etc. NB "RT-PCR" is reserved for reverse-transcription polymerase chain reaction methods. "real-time PCR" should always be stated in full and refers to probe-based and SYBR green assays]
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Stained or unstained tissue squashes of affected skeletal muscle or of the LO may show abnormalities. Tissue squashes of skeletal muscle when examined with phase or reduced light microscopy may show loss of the normal striations. Fragmentation of muscle fibres may also be apparent. Squashes of the LO may show the presence of significant accumulations of spherical masses of cells called lymphoid organ spheroids (LOS) amongst normal LO tubules.

4.2. Histopathology and cytopathology

Infection with IMNV in the acute and chronic phases can be presumptively diagnosed using histology (Bell & Lightner, 1988; Lightner, 2011; Lightner *et al.,* 2004; Poulos *et al.,* 2006). However, the lesions in striated muscles and LO are not pathognomonic for infection with IMNV. White tail disease of penaeid shrimp caused by the *P. vannamei* nodavirus (PvNV) can mimic infection with IMNV (Tang *et al.,* 2007).

Haematoxylin and eosin stained tissue sections from shrimp with acute-phase infection with IMNV present myonecrosis with characteristic coagulative necrosis of striated (skeletal) muscle fibres, often with marked oedema among affected muscle fibres. Some shrimp may present a mix of acute and older lesions. The affected muscle fibres appear to progress from presenting coagulative necrosis to liquefactive necrosis, which is accompanied by moderate infiltration and accumulation of haemocytes. In the most advanced lesions, haemocytes and inflamed muscle fibres are replaced by a loose matrix of fibrocytes and connective tissue fibres that are interspersed with haemocytes and foci of (presumed) regenerating muscle fibres (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

Significant hypertrophy of the LO caused by accumulations of LOS is a highly consistent lesion in shrimp with acute or chronic-phase infection with IMNV lesions. Often, many ectopic LOS are found in other tissues not near the main body of the LO. Common locations for ectopic LOS include the haemocoelom in the gills, heart, near the antennal gland tubules, and ventral nerve cord (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

4.3. Cell culture for isolation

No crustacean cell lines exist, but IMNV was observed to propagate in C6/36 subclone of *Aedes albopictus* cell line (Kumar *et al.,* 2020). Performance of the test should be confirmed before being recommended.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and quantitative real-time RT-PCR (Andrade *et al.,* 2007; Poulos *et al.,* 2006; Tang *et al.,* 2005). A nested RT-PCR kit for detection of the virus is available commercially.

4.4.1. Real-time RT-PCR

A real-time RT-PCR method was developed to detect and quantify IMNV in shrimp tissue. The method which can detect as few as 10 IMNV RNA copies μl^{-1} total RNA (Andrade *et al.,* 2007) is summarised below.

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters

Method 1: Andrade *et al.,* 2007; GenBank Accession No. AY570982			
IMNV Capsid protein gene	Fwd IMNV412F: GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA Rev IMNV545R: AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT Probe: CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG	300 Nm 200 nM	40 cycles of: 95°C/3 sec and 60°C/30 sec

4.4.2. Conventional PCR

The nested RT-PCR method to detect IMNV uses two PCR primer sets that produce a 328 bp one-step amplicon and139 bp two-step amplicon. The 1-step PCR can detect as little as 100 IMNV RNA copies and the 2-step PCR can detect in the order of 10 IMNV RNA copies (Poulos & Lightner, 2006).

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Poulos & Lightner, 2006; GenBank : KJ636783.2; 328/139 bp			
IMNV Capsid protein gene (nested-PCR)	Outer Fwd 4587F: CGA-CGC-TGC-TAA-CCA-TAC-AA Rev 4914R: ACT-CGG-CTG-TTC-GAT-CAA-GT Inner Fwd 4725 NF: GGC-ACA-TGC-TCA-GAG-ACA Rev 4863 NR: AGC-GCT-GAG-TCC-AGT-CTT-G	200 nM 620 nM	45 cycles of: 95°C/45 sec; 60°C/45 sec; 60°C/7 min 39 cycles of: 95°C/30 sec, 65°C/30 sec, 72°C/30 sec; 72°C/2 min

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published sequences.

4.6. *In-situ* hybridisation

DNA probe for ISH detection of IMNV

A cDNA library was generated from RNA extracted from purified IMNV. A IMNV-specific ISH DNA probe is prepared from clone IMNV-317 by PCR labelling with digoxigenin-11-dUTP (DIG). The PCR primers used for amplification of the 993 bp probe are IMNV993F (5'-AAC-ACA-AAA-TCT-GCC-AGC-AA-3') and IMNV993R (5'-CCC-AAC-CAC-CCA-AAT-TCA-TA-3'). Following PCR, the DIG-labelled DNA probe is precipitated with ethanol, re-suspended in water and stored at –20°C until used. The ISH procedure for detecting IMNV follows that outlined by Tang *et al.* (2005). Negative and positive controls should be sourced from PCR-confirmed uninfected and infected shrimp, respectively.

4.7. Immunohistochemistry

Monoclonal antibodies have been generated using recombinant IMNV capsid protein fragments to immunise mice (Kunanopparat et al., 2011). Immunohistochemical analysis demonstrated strong reactivity in muscle, gill, heart, LO and connective tissue derived from IMNV-infected *P. vannamei* similar to that demonstrated by *in-situ* hybidisation (Tang *et al*., 2005). There was no cross-reactivity to tissues derived from uninfected shrimp or shrimp infected with other viral pathogens such as WSSV, YHV, TSV among others.

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

None are recommended, however an immunochromatographic strip test has been developed (Chaivisuthangkura *et al.*, 2013) using the monoclonal antibodies developed by Kunanopparat *et al.* (2011). While the test is simple, fast and low-cost it is approximately 300-fold less sensitive than one-step RT-PCR (Chaivisuthangkura *et al.*, 2013).

4.10. Other methods

A chromatographic method for detection of PCR amplicons has been developed (Koiwai *et al.,* 2018).

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time RT-PCR is the recommended test for surveillance to demonstrate freedom of infection with IMNV in apparently healthy populations as described in Section 4.1.1.

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 a suspect and confirmed case 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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status[9]

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

i) Histopathology consistent with the presence of the pathogen or the disease

ii) Positive result by real-time RT-PCR

iii) Positive result by conventional RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with IMNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time RT-PCR and positive result by conventional RT-PCR followed by amplicon sequencing

[9] For example transboundary commodities.

ii) Histopathology consistent with IMNV infection coupled with *in-situ* hybridisation and detection of IMNV in a tissue sample by real-time RT-PCR

iii) Histopathology consistent with IMNV infection coupled with *in-situ* hybridisation and detection of IMNV in a tissue sample by conventional RT-PCR followed by amplicon sequencing

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 IMNV 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) Positive result by conventional RT-PCR

iii) Positive result by real-time RT-PCR

iii) Histopathology consistent with the presence of the pathogen or the disease

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with IMNV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by amplicon sequencing

ii) Positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR

iii) Positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IMNV are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with IMNV, 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
Real-time PCR	Diagnosis	Experimentally infected SPF *P. vannamei*	abdominal muscle	*P. vannamei*	100 (30)	100 (30)	Histopathology	Andrade *et al.* (2007)

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

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
Real-time PCR								

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

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NB: At the time of publication (2022) there was no OIE Reference Laboratory for infection with infectious myonecrosis virus (please consult the OIE web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2009. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.7.

INFECTION WITH TAURA SYNDROME VIRUS

1. Scope

Infection with Taura syndrome virus means infection with the pathogenic agent Taura syndrome virus (TSV), Genus Aparavirus, Family *Dicistroviridae*, Order Picornavirales.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

TSV was described as the cause of the disease commonly known as Taura syndrome by Hasson *et al.* (1995), Bonami *et al.* (1997) and Mari *et al.* (1998; 2002). At least four genotypes (strains) of TSV have been documented based on the gene sequence encoding VP1 the largest and presumably dominant of the three major structural proteins of the virus. Based on VP1 sequence variations, these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; 3) the Belize group; and 4) the Venezuelan group (Nielsen *et al.,* 2005; Tang & Lightner, 2005; Wertheim *et al.,* 2009).

At least two distinct antigenic variants of TSV have been identified by their differential reactivity to monoclonal antibody MAb 1A1, produced using a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (Poulos *et al.,* 1999) as the immunogen: Type A represents those that react with MAb 1A1 (in the enzyme-linked immunosorbent assay [ELISA], Western blots and immunohistochemistry (IHC) with infected tissues) and those that do not were subdivided into Type B (TSV 98 Sinaloa, Mexico) and Type C (TSV 02 Belize), based on host species and virulence. All TSV isolates of the Americas and most, if not all, South-East Asian genotypes react with MAb 1A1. In marked contrast, none of the Belize genotype group reacts with MAb 1A1 (Robles-Sikisaka *et al.,* 2002), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.

TSV particles are 32 nm in diameter, non-enveloped icosahedrons and have a buoyant density of 1.338 g ml^{-1} in CsCl. The genome of TSV consists of a linear, positive-sense single-stranded RNA 10,205 nucleotides in length, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for non-structural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (Bonami *et al.,* 1997; Mari *et al.,* 1998; 2002; Robles-Sikisaka *et al.,* 2001).

Other reported causes of Taura syndrome: TS in Ecuador was initially linked to fungicide contamination of shrimp farms, a contention that was supported by litigation for ~16 years after the disease was scientifically shown to have a viral aetiology (Brock *et al.,* 1995; Hasson *et al.,* 1995). Hence, several papers in the literature propose a toxic aetiology for TS (Intriago *et al.,* 1997; Jimenez, 1992; Jimenez *et al.,* 2000).

2.1.2. Survival and stability in processed or stored samples

No information available.

2.1.3. Survival and stability outside the host

No information available.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with TSV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: blue shrimp (*Penaeus stylirostris*), giant tiger prawn (*Penaeus monodon*), greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), northern white shrimp (*Penaeus setiferus*), 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 infection with TSV according to Chapter 1.5 of the *Aquatic Code* are: fleshy prawn (*Penaeus chinensis*), giant river prawn (*Macrobrachium rosenbergii*), the copepod *Ergasilus manicatus*, and the barnacles *Chelonibia patula* and *Octolasmis muelleri.* Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is TSV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: blue crab (*Callinectes sapidus*), the crabs *Uca vocans* and *Sesarma mederi*, gulf killifish (*Fundulus grandis*), Indo-Pacific swamp crab (*Scylla serrata*), kuruma prawn (*Penaeus japonicus*), northern pink shrimp (*Penaeus duorarum*) and southern white shrimp (*P. schmitti*).

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Infection with TSV has been documented in all life stages (i.e. post-larvae [PL], juveniles and adults) of *P. vannamei* except eggs, zygotes and larvae (Lightner, 1996a).

2.2.4. Distribution of the pathogen in the host

Using injection and *per os* challenge experiments, Nunan *et al.* (2004) demonstrated TSV could be detected in different body parts including gills, head, whole tail, tail muscle, pleopod and tail fan (Nunan *et al.,* 2004). While there was no significant difference in the viral copy number contained in different body parts when TSV was administered via injection, there was a statistically significant difference between tail/gills, tail/head, tail/tail fan, whole tail/tail fan and pleopods/tail fan when the viral inoculum was administered *per os*. The tail samples had the lower viral copy numbers, as did the whole tail and pleopods when compared to the tail fan (Nunan *et al.,* 2004).

2.2.5. Aquatic animal reservoirs of infection

Not demonstrated unequivocally

2.2.6. Vectors

Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV-infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus gallus,* used as a laboratory surrogate for all shrimp-eating birds) thus suggesting that the virus can retain infectivity when passed through the gastro-intestinal system of any bird species. These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (Garza *et al.,* 1997; Vanpatten *et al.,* 2004).

Aquatic insects: the water boatman (*Trichocorixa reticulata* [*Corixidae*], an aquatic insect that feeds on shrimp carcasses in shrimp farm ponds) have been demonstrated to transport TSV within their intestinal contents, but are not directly infected by the virus (Brock, 1997; Lightner, 1996a; 1996b; reviewed in Dhar *et al.,* 2004).

2.3. Disease pattern

Infection with TSV is best known as a disease of nursery- or grow-out-phase *P. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with TSV infection are typically small (~0.05 g to <5 g) juveniles. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults (Brock, 1997; Brock *et al.,* 1995; Lightner, 1996a, 1996b; Lotz, 1997).

2.3.1. Mortality, morbidity and prevalence

At the farm level, outbreaks of infection with TSV involving stocks of *P. vannamei* (the principal host species for infection with TSV) not selected for resistance, typical cumulative mortalities range from 40 to >90% in cultured populations of PL, juvenile, and subadult life stages. TSV-resistant lines of *P. vannamei* are available which show survival rates of up to 100% in laboratory challenge with all four TSV genotypes (Lightner *et al.,* 2009).

In regions where the virus is enzootic in farmed stocks, the prevalence of infection with TSV has been found in various surveys to range from 0 to 100% (Brock, 1997; Jimenez *et al.,* 2000).

2.3.2. Clinical signs, including behavioural changes

Only acute-phase clinical infection with TSV can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase clinical infection with TSV.

Only shrimp with acute-phase clinical infection with TSV present behavioural changes. Typically, severely affected shrimp apparently become hypoxic and move to the pond edges or pond surface where dissolved oxygen levels are higher. Such shrimp may attract seabirds in large numbers. In many disease outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicates the presence of a serious disease outbreak (which is often either infection with TSV or white spot syndrome virus) to the farm manager.

2.3.3. Gross pathology

Infection with TSV has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b; Lightner *et al.,* 1995). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of infection with TSV are unique and provide a suspicion of infection.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase infection with TSV include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish colouration and making the tail fan and pleopods distinctly red; hence 'red tail' disease was one of the names given by farmers when the disease first appeared in Ecuador (Lightner *et al.,* 1995). In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with a ×10 hand lens reveals signs of focal epithelial necrosis. Shrimp showing these gross signs of acute infection with TSV typically have soft shells, an empty gut and are often in the late D stages of the moult cycle. Acutely affected shrimp usually die during ecdysis.

Transition (*recovery*) *phase:* although only present for a few days during outbreaks of infection with TSV, the gross signs presented by shrimp in the transition phase can provide a suspicion of infection with TSV. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites of resolving TSV lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (Brock, 1997; Hasson *et al.,* 1999b; Lightner, 1996a).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of infection with TSV in which persistently infected shrimp show no obvious signs of disease (Brock, 1997; Hasson *et al.,* 1999b; Lightner, 1996a; 1996b; Lightner *et al.,* 1995). However, *P. vannamei* that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp.

2.3.4. Modes of transmission and life cycle

Not applicable.

2.3.5. Environmental factors

Outbreaks of infection with TSV are more frequent when salinities are below 30 ppt (Jimenez *et al.,* 2000).

2.3.6. Geographical distribution

TSV is now widely distributed in the shrimp-farming regions of the Americas, South-East Asia and the Middle East (Brock, 1997; Hasson *et al.,* 1999a; Lightner, 1996a, 1996b; Lightner *et al.,* 2012; Lotz *et al.,* 2005; Nielsen *et al.,* 2005; Tang & Lightner, 2005; Tu *et al.,* 1999; Wertheim *et al.,* 2009; Vergel *et al.,* 2019; Yu & Song, 2000).

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No effective vaccines for TSV are available.

2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Breeding resistant strains

After TSV emerged in Ecuador in 1992–1994, *P. stylirostris* were found that possessed resistance to infection with TSV (genotype 1, MAb 1A1 Type A). Following on from this discovery and due to the disease occurrence in Mexico in 1994 where it caused crop failures of *P. vannamei,* selected lines of TSV-resistant *P. stylirostris* became the dominant shrimp farmed in western Mexico from 1995. However, in 1998–1999, a new 'strain' of TSV (Type B; Fegan & Clifford, 2001; Lightner, 1999; 2005; Zarain-Herzberg & Ascencio, 2001) emerged and caused massive epizootics in *P. stylirostris*. The emergence of this new 'strain' of TSV was soon followed in late 1999 by the introduction of white spot syndrome virus (WSSV) into shrimp farms in western Mexico, to which *P. stylirostris* had no resistance, effectively ending any interest in the culture of *P. stylirostris* in Mexico.

TSV-resistant domesticated stocks of *P. vannamei* and *P. stylirostris* have been developed. Some domesticated lines of TSV-resistant *P. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (Clifford, 1998; White *et al.,* 2002). After the appearance of infection with TSV in Central America, improved TSV resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region. Currently all genetic lines of *P. vannamei* shrimp that are being cultured in Asia and the Americas contain varying levels of tolerance/resistance to TSV.

2.4.5. Inactivation methods

No information available.

2.4.6. Disinfection of eggs and larvae

It is possible that TSV might be transmitted vertically (transovarian transmission), despite the lack of published reports documenting this route of transmission. Disinfection of eggs and larvae (Chen *et al.,* 1992) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.7. General husbandry

Some husbandry and disease control and management practices have been used successfully to reduce the risks of infection with TSV occurring during farm grow-out. These include the application of PCR assays for pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen-free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Wyban 1992). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of infection with TSV.

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

Suitable specimens for testing for infection with TSV include PL, juveniles and adults. While TSV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for TSV detection or certification of freedom from infection with TSV.

3.2. Selection of organs or tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of infection with TSV is the cuticular epithelium. In chronic infections the lymphoid organ (LO) is the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.3. Samples or tissues not suitable for pathogen detection

TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection with TSV.

3.4. Non-lethal sampling

Haemolymph or pleopods can be collected without sacrificing the animals and used as non-lethal sampling of genetically valuable broodstock.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0. *General information* (*diseases of crustaceans*).

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (which is influenced by time since collection and time 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

animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be preserved in ethanol it may be frozen.

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Chapter 2.2.0. *General information* (*diseases of crustaceans*).

3.5.4. Samples for other tests

Haemolymph could be used for PCR-based detection of TSV.

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. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger animals should be processed and tested individually.

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, 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 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts						+	+	NA				
Histopathology		+	+	NA	+	+	+	NA				
Cell culture												
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	1	+++	+++	+++	1
Conventional RT-PCR	++	++	++	1	++	++	++	1				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP												
IFAT												
ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay, respectively.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).
[2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Direct microscopy of simple unstained wet mounts from excised pieces of the gills, appendage tips, etc., examined by phase- or reduced-light microscopy may be used to demonstrate (and make a tentative diagnosis of acute-phase infection with TSV) focal lesions of acute-phase infection with TSV in cuticular epithelial cells. Preparations presenting acute-phase infection with TSV will contain numerous spherical structures (see the histopathological methods in Section 4.2.3 above), which are pyknotic and karyorrhectic nuclei and cytoplasmic remnants of necrotic cells.

4.2. Histopathology and cytopathology

Histopathology is a useful method to detect infection with TSV in the acute and chronic phases of infection (Hasson *et al.,* 1999b; Lightner, 1996a). In chronic infections with TSV, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.,* 1999b), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When histological lesions are observed and infection with TSV is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR) must be used for confirmation of infection with TSV (see Section 6).

4.2.1. Acute phase of Taura syndrome

The acute phase of the disease is characterised by multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres basal to affected cuticular epithelium are occasionally affected. In some severe cases of acute-phase infection with TSV, the antennal gland tubule epithelium is also destroyed. Prominent in the multifocal cuticular lesions are conspicuous foci of affected cells that display an increased eosinophilia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often extremely abundant in these infections with TSV acute-phase lesions and these are generally presented as spherical bodies (1–20 µm in diameter) that range in staining from eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give acute-phase TS lesions a characteristic ‘peppered’ or ‘buckshot-riddled’ appearance, which is considered to be pathognomonic for the infection when there is no concurrent necrosis of the parenchymal cells of the LO tubules. The absence of necrosis of the LO in acute-phase infection with TSV distinguishes it from acute-phase infection with yellowhead virus genotype 1 in which similar patterns of necrosis to those induced by infection with TSV may occur in the cuticular epithelium and gills (Lightner, 1996a).

In TSV-infected tissues, pyknotic or karyorrhectic nuclei give a positive (for DNA) Feulgen reaction, which distinguishes them from the less basophilic to eosinophilic cytoplasmic inclusions that do not contain DNA. The absence of haemocytic infiltration or other signs of a significant host-inflammatory response distinguishes the acute phase of infection with TSV from the transitional phase of the disease (Brock, 1997; Brock *et al.,* 1995; Hasson *et al.,* 1995; 1999a; 1999b; Lightner, 1996a; Lightner *et al.,* 1995).

4.2.2. Transition (recovery) phase of infection with Taura syndrome virus

In the transitional phase of infection with TSV, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by *Vibrio* spp. (Hasson *et al.,* 1999b; Lightner, 1996a). Sections of the LO during the transition phase of infection with TSV may appear normal with H&E staining. However, when sections of the LO are assayed for TSV by ISH with a specific cDNA probe (or by ISH with MAb 1A1 for TSV type A, genotype 1), large quantities of TSV are shown accumulating in the more peripheral parenchymal cells of the LO tubules (Hasson *et al.,* 1999b; Srisuvan *et al.,* 2005).

4.2.3. Chronic phase of infection with Taura syndrome virus

Shrimp in the chronic phase of infection with TSV display no gross signs of infection, and histologically the only sign of infection is the presence of numerous prominent LOS, which may remain associated with the main body of the paired LO, or which may detach and become ectopic LOS bodies that lodge in

constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connective tissues, etc.). Such LOS are spherical accumulations of LO cells and haemocytes and may be distinguished from normal LO tissues by their spherical nature and the lack of the central vessel that is typical of normal LO tubules. When assayed by ISH with a cDNA probe for TSV (or with MAb 1A1 using ISH) some cells in the LOS give positive reactions to the virus, while no other target tissues react (Hasson *et al.,* 1999b; Lightner, 1996a; 1996b).

4.3. Cell culture for virus isolation

TSV has not been grown *in vitro*, as no crustacean cell lines exist (Lightner, 1996a; Pantoja *et al.,* 2004). Although one publication incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle *et al.,* 2003), two other laboratories that repeated the study both found that TSV does not infect or replicate in primate or human cell lines that are known to have susceptibility to human picornaviruses (Luo *et al.,* 2004; Pantoja *et al.,* 2004).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

4.4.1. Real-time reverse-transcription (RT)-PCR

Real-time RT-PCR methods have been developed for the detection of TSV. These methods have the advantage of speed, specificity and sensitivity. The sensitivity of real time RT-PCR is approximately equal to 100 copies of the target sequence from the TSV genome (Dhar *et al.,* 2002; Tang *et al.,* 2004).

The real-time RT-PCR method described below for TSV follows the method used in Tang *et al.,* 2004.

Primer and probe sequences, real time RT-PCR

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Tang *et al.,*2004) GenBank Accession No. AFAF277675			
ORF-1 Nt 1024 to 1051	Fwd: TSV1004: TTG-GGC-ACC-AAA-CGA-CAT-T- Rev: TSV1075 GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT Probe: TSV-P1 FAM-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-TAMRA,	300 nM of each primer 100 nM of probe	Reverse transcription at 50°C/30 min 40 cycles of 95°C/3 sec and 60°C/30 sec

4.4.2. Conventional RT-PCR

Tissue samples (haemolymph, pleopods, whole small shrimp etc) may be assayed for TSV using RT-PCR. The RT-PCR method outlined below for TSV follows the method used in Nunan *et al.* (1998).

Primer and probe sequences, conventional RT-PCR

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters

Method 1 (Nunan *et al.,* 1998); product size 231 bp			
ORF 2	Fwd: 9992: AAG-TAG-ACA-GCC-GCG-CTT Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC	Primers/620 nM each	Reverse transcription 60°C/30 min 40 cycles: 94°C/45 sec, 60°C/45 sec

4.4.3. Other nucleic acid amplification methods

None currently available.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published sequences.

4.6. *In-situ* hybridisation (ISH)

4.6.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled cDNA probes for detection of TSV may be produced in the laboratory. The ISH method provides greater diagnostic sensitivity than do more traditional methods for TSV detection and diagnosis that employ classic histological methods (Hasson *et al.,* 1999a; Lightner, 1996a; 1999; Lightner & Redman 1998; Mari *et al.,* 1998). The ISH assay of routine histological sections of acute- and transition-phase lesions in the cuticular epithelium, other tissues, and of LOS in transition and chronic phase with a specific DIG-labelled cDNA probe to TSV, provides a definitive diagnosis of infection with TSV (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b). Pathognomonic TSV-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. Not reacting to the probe are the prominent karyorrhectic nuclear fragments and pyknotic nuclei that contribute to the pathognomonic 'buckshot riddled' appearance of TS lesions (Lightner, 1996a; Mari *et al.,* 1998). (See Chapter 2.2.4 *Infection with infectious hypodermal and haematopoietic necrosis virus* for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

False-negative ISH results may occur with Davidson's fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson's fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This hydrolysis can be prevented by avoiding fixation times over 24 hours (Hasson *et al.,* 1997; Lightner, 1996a; Lightner & Redman 1998).

4.7. Immunohistochemistry

Not suitable.

4.8. Bioassay

Confirmation of infection with TSV may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the virus (Garza *et al.,* 1997; Hasson *et al.,* 1999b; 1995; Lightner, 1996a; Lotz, 1997; Overstreet *et al.,* 1997). Oral or injection protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped carcasses of suspect shrimp to SPF juvenile *P. vannamei* in small tanks (White *et al.,* 2002). The use of a negative control tank of indicator shrimp, which receive only SPF (TSV-free) tissue and normal shrimp feed is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, TSV-positive indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of disease and unusual mortalities (Hasson *et al.,* 1999b; Lightner, 1996a; White *et al.,* 2002).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during an outbreak of infection with TSV. Heads only should be used if shrimp display gross transition-phase lesions (multifocal melanised spots on the cuticle) or no clinical signs of infection (chronic phase) as the virus, if present, will be concentrated in the LO (Hasson *et al.,* 1999b; Lightner, 1996a). For non-lethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (Lightner, 1996a).

4.9. Antibody- or antigen-based detection methods

Not recommended.

4.10. Other methods

4.10.1. Dot-blot immunoassay method

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore)[10].

ii) After air drying, the wells are blocked for 1 hour at room temperature with 200 µl of a buffer containing phosphate-buffered saline and 0.05% Tween 20 (PBST) mixed with 10% normal goat serum (Life Technologies) and 2% Hammersten casein (Amersham Life Sciences).

iii) The wells are washed three times with PBST and then reacted with 100 µl primary antibody (MAb or mouse polyclonal antibodies) for 30 minutes at room temperature.

iv) Alkaline-phosphatase-labelled goat anti-mouse IgG, γ chain specific, secondary antibody (Zymed) diluted 1/1000 in PBST plus 10% normal goat serum is used for detection (30 minutes at room temperature).

v) After washing three times with PBST, once with PBS and once with distilled water, the reactions are visualised by development for 15 minutes at room temperature with nitroblue tetrazolium and bromo-chloro-indoyl phosphate (Roche Diagnostics in 100 mM Tris-HCl, 100 mM NaCl buffer containing 50 mM $MgCl_2$, pH 9.5.

vi) Reactions are stopped with distilled water.

vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semi-purified TSV. A negative reaction is one in which no coloured spot is visible in the well.

5. Test(s) recommended for surveillance to demonstrate disease freedom in apparently healthy populations

Real-time RT-PCR is the recommended test for surveillance to demonstrate freedom of infection with TSV in apparently healthy populations as described in Section 4.1.1.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (6.1) or presence of clinical signs (6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case 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

[10] 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*.

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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status [11]

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

i) Histopathological changes consistent with the presence of the pathogen or the disease

ii) A positive result by real-time RT-PCR

iii) A positive result by conventional RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with TSV is considered to be confirmed if at least one of the following criteria is met:

i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by sequencing of the amplicon

ii) A positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR

iii) A positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.2. Clinically affected animals

No clinical signs are 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 TSV 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) Histopathological changes consistent with TSV infection

iii) Positive result by real-time RT-PCR

iv) Positive result by conventional RT-PCR

v) Positive result of a bioassay

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with TSV is considered to be confirmed if at least at least one of the following criteria is met:

i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by sequencing of the amplicon

ii) A positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR

11 For example transboundary commodities.

iii) A positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with TSV are provided in Table 6.3.1 (none currently available). This information can be used for the design of surveys for infection with TSV, 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 surveillance of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

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 specificity.

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NB: There is an OIE Reference Laboratory for infection with Taura syndrome virus
(please consult the OIE Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact OIE Reference Laboratories for any further information on
infection with Taura syndrome virus

NB: FIRST ADOPTED IN 2006. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

1. Scope

Infection with white spot syndrome virus means infection with the pathogenic agent white spot syndrome virus (WSSV), Genus *Whispovirus,* Family *Nimaviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

WSSV was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus *Whispovirus* within the *Nimaviridae* family. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 80–120 nm in diameter and 250–380 nm in length. A flagella-like extension (appendage) may be observed at one end of the virion. WSSV has been divided into three groups: isolates originating from Thailand (WSSV-TH-96-II), isolates originating from India (WSSV-IN-07-I), and another Indian isolate (WSSV-IN-06-I). Most strains of WSSV were speculated to have originated from the Indian Ocean and then spread across the world (Zeng, 2021). Today, although various geographical isolates with genotypic variability have been identified, they are all classified as a single species (WSSV) within the genus *Whispovirus* (Lo *et al.,* 2012; Wang *et al*., 2019).

2.1.2. Survival and stability in processed or stored samples

Viable WSSV was found in frozen commodity shrimp imported to Australia from Southeast Asia (McColl *et al.,* 2004). The virulence of WSSV was retained for 14 months at –80°C in a filtered tissue homogenate prepared from moribund shrimp with hepatopancreas and abdomen removed (Momoyama *et al.,* 1998). The virus originally collected from the haemolymph of moribund shrimp could maintain its virulence for at least 16 months at –80°C (Wu *et al.,* 2002). However, WSSV might be inactivated by multiple freeze-thaw cycles due to damage the viral envelopes or nucleocapsids (Durand *et al.,* 2000; Hasson *et al.,* 2006).

2.1.3. Survival and stability outside the host

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al.,* 1998); and is viable in ponds for at least 3–4 days (Nakano *et al.*, 1998). Laboratory emulations of drainable and non-drainable ponds suggest that the virus is no longer infective after 21 days of sun-drying or after 40 days in waterlogged pond sediment (Satheesh Kumar *et al*., 2013).

WSSV with an initial viral load of 1000 virions ml^{-1} was found to be viable for a period of 12 days in seawater of 27 ppt salinity, pH of 7.5 at 29–33°C. In shrimp pond sediment (with initial viral load of 211,500 copies g^{-1}), the virus was viable and infective up to 19 days despite sun-drying. In the case of non-drainable conditions, WSSV (753,600 copies g^{-1}) remained infective for a period of 35 days (Satheesh Kumar *et al*., 2013).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Of all the species that have been tested to date, no decapod (order Decapoda) crustacean from marine, brackish or freshwater sources has been reported to be refractory to infection with WSSV (Flegel, 1997; Lightner, 1996; Lo & Kou, 1998; Maeda *et al.,* 2000; Stentiford *et al.,* 2009).

2.2.2. Species with incomplete evidence for susceptibility

All life stages are potentially susceptible, from eggs to broodstock (Lightner, 1996; Venegas *et al.,* 1999). WSSV genetic material has been detected in reproductive organs (Lo *et al.,* 1997), but susceptibility of the gametes to WSSV infection has not been determined definitively.

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

The best life stages of crustaceans for detection of WSSV are late postlarvae (PL) stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms).

2.2.4. Distribution of the pathogen in the host

The major target tissues of WSSV are of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama *et al.*, 1994; Wongteerasupaya *et al.*, 1995). Although WSSV infects the underlying connective tissue in the crustacean hepatopancreas and midgut, the tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

2.2.5. Aquatic animal reservoirs of infection

Wild decapods known to be reservoirs of infection with WSSV include *Mysis* sp. (Huang *et al.,* 1995), *Acetes* sp., *Alpheus* sp., *Callianassa* sp., *Exopalaemon* sp., *Helice* sp., *Hemigrapsus* sp. *Macrophthalmus* sp., *Macrophthel* sp., *Metaplax* sp., *Orithyia* sp., *Palaemonoidea* sp., *Scylla* sp., *Sesarma* sp., and *Stomatopoda* sp. (Desrina *et al*., 2022; He & Zhou, 1996; Lei *et al.,* 2002). These species can express the disease under suitable environmental conditions. However, non-decapodal crustaceans, such as copepods (Huang *et al.,* 1995), rotifers (Yan *et al.,* 2004), *Balanus* sp. (Lei *et al.,* 2002), *Artemia* (Li *et al*., 2004; Zhang *et al*., 2010) and *Tachypleidue* sp. (He & Zhou, 1996) may be apparently healthy carrier animals. Other marine molluscs, polychaete worms (Vijayan *et al.,* 2005), as well as non-crustacean aquatic arthropods such as sea slaters (*Isopoda*), and Euphydradae insect larvae can mechanically carry the virus without evidence of infection (Lo & Kou, 1998).

2.2.6. Vectors

The harpacticoid copepod *Nitocra* sp. (Zhang *et al*., 2008), microalgae (Liu *et al*., 2007), and the polychaete, *Dendronereis* spp. (Peters) (Desrina *et al*., 2013; Haryadi *et al.,* 2015) are vectors for WSSV.

2.3. Disease pattern

Infection with WSSV sometimes causes clinical disease (Tsai *et al*., 1999), depending on factors that are poorly understood but related to species tolerance and environmental triggers. With an appropriate infection dose to allow sufficient time before mortality, animals susceptible to disease show large numbers of virions circulating in the haemolymph (Lo *et al*., 1997), but this may also occur for tolerant species that show no mortality. Thus, high viral loads *per se* do not cause disease or mortality for all susceptible species.

2.3.1. Mortality, morbidity and prevalence

All penaeid shrimp species are highly susceptible to infection with WSSV, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection with WSSV, but morbidity and mortality as a consequence of infection are highly variable (Lo & Kou, 1998). High level infections with WSSV are known in some decapods in the absence of clinical disease.

Prevalence of infection with WSSV is highly variable, from <1% in infected wild populations to up to 100% in captive populations (Lo & Kou, 1998).

2.3.2. Clinical signs, including behavioural changes

White spots embedded within the exoskeleton are the most commonly observed clinical sign. In most shrimp, these spots range from barely visible to 3 mm in diameter, and they sometimes coalesce into larger plates. However, it should be noted that environmental stress factors, such as high alkalinity, or bacterial disease can also cause white spots on the carapace of shrimp, and that moribund shrimp with infection with WSSV may have few, if any, white spots. Therefore, the appearance of white spots is not a reliable diagnostic sign of infection with WSSV infection. High degrees of colour variation with a predominance of reddish or pinkish discoloured shrimp are seen in diseased populations.

WSSV infections can be subclinical or manifest as clinical disease. The penaeid shrimp in aquaculture will generally show clinical signs associated with high morbidity and mortality. Some animals may die without showing any clinical signs. Non-penaeid species (e.g. crab, lobster) generally have subclinical infections under natural conditions.

The affected animals can show lethargy, decreased or absent feed consumption and abnormal swimming behaviour – slow swimming, swimming on side, swimming near water surface and gathering around edges of rearing units (Corbel *et al.,* 2001; Sahul Hameed *et al.,* 1998; 2001). A very high mortality rate in the shrimp population can be expected within a few days of the onset of behavioural signs.

2.3.3 Gross pathology

In addition to the clinical and behavioural signs in Section 2.3.2. above, the following gross pathology has been reported in clinically affected penaeid shrimp: loosened attachment of the carapace with underlying cuticular epithelium (Sanchez-Paz, 2010), so that the carapace can be easily removed (Zhan *et al.,* 1998); empty gastro-intestinal tract due to anorexia (Escobedo-Bonilla, 2008); delayed clotting of haemolymph (Heidarieh *et al.,* 2013); excessive fouling of gills (Wu *et al.,* 2013) and exoskeleton.

2.3.4. Modes of transmission and life cycle

Infection with WSSV can be transmitted horizontally by consumption of infected tissue (e.g. cannibalism, predation, fomites, etc.), by water-borne routes, and by other routes of transmission (e.g. via sea birds, anthropogenic movements, feeding, rotifer, copepods, etc) (Haryadi *et al.,* 2015; Vanpatten *et al.,* 2004; Zhang *et al.,* 2006; 2008). Transmission of WSSV can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (Lo & Kou, 1998). Microalgae could serve as a potential horizontal transmission pathway for WSSV (Liu *et al.,* 2007).

True vertical transmission (intra-ovum) of WSSV to the progeny has not been demonstrated.

In-vitro studies with primary cell cultures and *in-vivo* studies with postlarvae show that the replication cycle is approximately 20 hours at 25°C (Chang *et al.*, 1996; Chen *et al.*, 2011; Wang *et al.*, 2000).

2.3.5. Environmental factors

Disease outbreaks may be induced by stressors, such as rapid changes in salinity. Water temperature has a profound effect on disease expression, with average water temperatures of between 18 and 30°C being conducive to WSSV outbreaks (Song *et al.*, 1996; Vidal *et al.*, 2001). Under experimental challenge condition, WSSV-induced mortality in shrimp is reduced when the temperature increases above 32°C (Vidal *et al.,* 2001).

2.3.6. Geographical distribution

Infection with WSSV has been identified from crustaceans in Asia, the Mediterranean (Stentiford & Lightner, 2011), the Middle East, Oceania (Moody *et al.*, 2022) and the Americas. Zones and compartments free from infection with WSSV are known within these regions (Lo *et al.,* 2012).

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No consistently effective vaccination methods have been developed for infection with WSSV.

2.4.2. Chemotherapy including blocking agents

No published or validated methods.

2.4.3. Immunostimulation

Several reports have shown that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to infection with WSSV (Chang *et al.*, 2003; Chotigeat *et al.*, 2004).

2.4.4. Breeding resistant strains

Progress in breeding *P. vannamei* for resistance to infections with WSSV has been reported (Cuellar-Anjel *et al.,* 2012; Huang *et al.,* 2012).

2.4.5. Inactivation methods

Method	Treatment	Reference
Heat	55°C/90 min 70°C/5 min	Chang *et al.*, 1998
	50°C/60 min 60°C/1 min 70°C/0.2 min	Nakano *et al.*, 1998
pH	pH 3/60 min pH 12/10 min	Chang *et al.*, 1998; Balasubramanian *et al.,* 2006
UV	9.30×10^5 µWs/cm^2	Chang *et al.*, 1998
Ozone	0.5 µg ml^{-1}/10 min	Chang *et al.*, 1998
Chlorine	100 ppm/10 min	Chang *et al.*, 1998; Balasubramanian *et al.,* 2006
Iodophore	100 ppm/10 min	Chang *et al.*, 1998

2.4.6. Disinfection of eggs and larvae

For transovum transmission, disinfection of egg is likely to be effective (Lo & Kou, 1998), but this has not yet been confirmed in formal scientific trials.

2.4.7. General husbandry

Management practices in endemic areas principally involve the exclusion of WSSV from production populations or avoiding risk factors for development of clinical disease. Examples include avoiding stocking in the cold season, use of specific pathogen-free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, use of biosecure water and culture systems (Withyachumnarnkul, 1999). Polyculture of shrimp and fish has been proposed to reduce WSSV transmission in infected populations (Wang *et al.*, 2021).

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 or exhibit behavioural changes (Sections 2.3) should be selected for detection of WSSV.

3.2. Selection of organs or tissues

Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular connective tissues, as well as other target tissues (e.g. antennal gland, haematopoietic organ, etc.), are the main target tissues for infection with WSSV. Samples from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission (Lo *et al.*, 1997).

3.3. Samples or tissues not suitable for pathogen detection

Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the columnar epithelial cells of these two organs are of endodermal embryonic origin (Lo *et al.*, 1997) and are not appropriate tissues for detection. The compound eye may contain a PCR inhibitor (Lo *et al.*, 1997) and is therefore not suitable for PCR-based diagnosis.

3.4. Non-lethal sampling

Gill, haemolymph or pleopod are suitable tissues for non-lethal sampling and screening by PCR.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time 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 animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for histological techniques can be found in Chapter 2.2.0 *General information* (diseases of crustaceans).

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Chapter 2.2.0 *General information* (diseases of crustaceans).

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 virus 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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology					+	+	+	1				
Cell culture												
Real-time PCR	+++	+++	+++	4	+++	+++	+++	4	+++	+++	+++	4
Conventional PCR	++	++	++	2	++	++	++	2				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	2
In-situ hybridisation					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP	++	++	++	1	++	++	++	1	+	+	+	1
Ab-ELISA					+	+	+	1				
Ag-ELISA					+	+	+	1				
Other antigen detection methods					+	+	+	1				
Other methods												

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; IFAT = indirect fluorescent antibody test. [give definitions of abbreviations as appropriate; nPCR = nested PCR, etc. NB "RT-PCR" is reserved for reverse-transcription polymerase chain reaction methods. "real-time PCR" should always be stated in full and refers to probe-based and SYBR green assays]
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained.

T-E staining

A T-E staining solution may be prepared from Trypan blue 0.6%, Eosin Y 0.2%, NaCl 0.5%, phenol 0.5%, and glycerol 20% (Huang & Yu, 1995) and used as follows:

i) Place a piece of diseased tissue (e.g. a piece of gill or stomach epithelium without the cuticle) on a slide and mince with a scalpel.

ii) Add 1–2 drops of the T-E staining solution to the minced tissue, mix and allow to stain for 3–5 minutes.

iii) Lay a cover glass over the stained tissue and cover with several pieces of absorbent paper. Use a thumb to squash the mince into a single layer of cells.

If the sample was taken from a heavily infected shrimp, hypertrophied nuclei and intranuclear eosinophilic or vacuolation-like inclusion bodies can be observed using light microscopy (400–1000× magnification).

4.2. Histopathology and cytopathology

Smears

Demonstration of aggregates of WSSV virions in unstained smear preparations of haemolymph by dark-field microscopy.

NOTE: This is the simplest of the microscopic techniques and is recommended for people with limited expertise in diagnosing infection with WSSV. The aggregates appear as small reflective spots of 0.5 μm in diameter (Momoyama *et al.*, 1995).

Fixed sections

Histological changes commonly reported in susceptible species include: Hypertrophied nuclei with marginated chromatin material in virus-infected cells; eosinophilic to pale basophilic (with haematoxylin & eosin stain) stained intranuclear viral inclusions within hypertrophied nuclei and multifocal necrosis associated with pyknotic and karyorrhectic nuclei in affected tissues of ectodermal and mesodermal origin. The infection with infectious hypodermal and hematopoietic necrosis virus, another DNA virus, produces similar inclusions that need to be differentiated from those of WSSV.

4.3. Cell culture for isolation

WSSV can be isolated from primary cultures of lymphoid or ovary cells. However, it is NOT recommended to use cell culture as a routine isolation method because of: 1) the high risk of contamination, and, 2) the composition of the medium varies depending on the tissue type, host species and experimental purpose; that is, to date there is no standard or recognised medium that can be recommended. As primary cell culture is so difficult to initiate and maintain for virus isolation purposes, bioassay should be the primary means for virus propagation.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

4.4.1. Real-time PCR

The real-time PCR methods described by Durand & Lightner (2002) and Sritunyalucksana *et al.* (2006) are described here as modified and validated by Moody *et al.,* (2022).

Pathogen/ Target	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Durand & Lightner, 2002[1]; GenBank Accession No. [to be included])			
WSSV ORF X	Fwd WSS1011F: TGG-TCC-CGT-CCT-CAT-CTC-AG Rev WSS1079R: GCT-GCC-TTG-CCG-GAA-ATT-A Probe: AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A	900 nM 900 nM 250 nM	45 cycles of: 95°C/15 sec and 60°C/1 min
Method 2 (Sritunyalucksana, 2006[1]; GenBank Accession No. [to be included])			
WSSV ORF X	Fwd CSIRO WSSV-F: CCG ACG CCA AGG GAA CT Rev CSIRO WSSV-R: TTC AGA TTC GTT ACC GTT TCC A Probe: 6FAM-CGC TTC AGC CAT GCC AGCCG-TAMRA	900 nM 900 nM 250 nM	45 cycles of: 95°C/15 sec and 60°C/1 min

[1]Method described here as modified and validated by Moody *et al.,* 2022

4.4.2. Conventional PCR

Pathogen/ Target	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Lo *et al.*, 1996a; GenBank Accession No. , 1447/941 bp)			
WSSV (nested PCR)	Outer Fwd: ACT-ACT-AAC-TTC-AGC-CTA-TCTAG Rev; 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A Inner Fwd 146F2: GTA-ACT-GCC-CCT-TCC-ATC-TCC-A Rev 146R2 TAC-GGC-AGC-TGC-TGC-ACC-TTG	100 pmol 100 pmol 100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min 39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min

Commercial PCR kits are available. Please consult the OIE Register for kits that have been certified by the OIE (https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5).

4.4.3. Loop-mediated isothermal amplification (LAMP) method

The protocol described here is from Kono *et al.* (2004). The LAMP method is sensitive and rapid, and it amplifies the target nucleic acids under isothermal conditions, therefore needing no sophisticated machine for thermal cycling.

DNA extraction

DNA extraction could be performed according to the protocol described in Section 4.4.2 *Conventional PCR* or by other suitable methods or by commercial kits.

LAMP reaction

i) Add DNA to a tube to set up a 25 µl reaction mixture (20 mM Tris/HCl, pH 8.8, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8M Betaine, 1.4 mM of each dNTP, 40 pmol of WSSV-FIP and -BIP primers, 5 pmol of WSSV-F3 and -B3 primers).

ii) The primer sequences are WSSV-FIP: 5’-GGG-TCG-TCG-AAT-GTT-GCC-CAT-TTT-GCC-TAC-GCA-CCA-ATC-TGT-G-3’, WSSV-BIP: 5’-AAA-GGA-CAA-TCC-CTC-TCC-TGC-GTT-TTA-GAA-CGG-AAG-AAA-CTG-CC-TT-3’, WSSV-F3: ACG-GAC-GGA-GGA-CCC-AAA-TCG-A-3’, WSSV-B3: 5’-GCC-TCT-GCA-ACA-TCC-TTT-CC-3’.

iii) Heat the mixture at 50°C for 5 minutes and at 95°C for 5 minutes, then chill on ice, and add 1 µl (8 U) of *Bst* DNA polymerase.

iv) Incubate the mixture at 65°C for 60 minutes, and then terminate the reaction at 80°C for 10 minutes.

v) To visualise, electrophorese 2 µl LAMP reaction products on 2% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}. This reaction produces WSSV-specific LAMP products with multiple bands of various sizes from approximately 200 bp to the loading well.

Reliable LAMP commercial kits may be an alternative for WSSV diagnosis.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published sequences.

4.6. *In-situ* hybridisation

Use of WSSV-specific DNA probes with histological sections is useful to demonstrate the presence of WSSV nucleic acid in infected cells (Nunan & Lightner, 1997). See Chapter 2.2.0 Section 5.5.4 for general comments on *in-situ* hybridisation.

4.7. Immunohistochemistry

See Section 4.9.

4.8. Bioassay

If SPF shrimp are available, the bioassay method based on Nunan *et al.* (1998) and Durand *et al*. (2000), is suitable for WSSV diagnosis.

4.9. Antigen detection methods

Both polyclonal and monoclonal antibodies raised against either the virus or a recombinant viral structural protein have been used in various immunological assays including western blot analysis, immunodot assay, indirect fluorescent antibody test (IFAT), immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA) to detect WSSV (Huang *et al*., 1995; Poulos *et al.*, 2001; Sithigorngul *et al.*, 2006; Yoganandhan *et al.*, 2004).

4.10. Other methods

Lateral flow tests are commercially available but their performance needs to be evaluated before they can be recommended.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance to demonstrate freedom of infection with WSSV in apparently healthy populations as described in Section 4.4.1.

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 a suspect and confirmed case 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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status[12]

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

i) Positive result by conventional PCR

ii) Positive result by real-time PCR

iii) Positive result by LAMP method

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with WSSV is considered to be confirmed if at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR

iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing

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

i) Gross pathology or clinical signs consistent with the disease as described in this chapter, with or without elevated mortality

ii) Histopathology consistent with WSSV infection

iii) Positive result by conventional PCR

iv) Positive result by real-time PCR

v) Positive result by LAMP method

[12] For example transboundary commodities.

vi) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with WSSV is considered to be confirmed if at least at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR

iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with WSSV are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with WSSV, 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
Real-time PCR (Durand & Lightner, 2002)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100%	100%	Real-time PCR	Moody *et al.*, 2022
Real-time PCR (Sritunyalucksana *et al.*, 2006)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100%	100%	Real-time PCR	Moody *et al.*, 2022

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.
*The nested PCR (Lo *et al.*, 1996a) is linked to false positives for WSSV when they are used to test species of *Cherax quadricarinatus* (Claydon *et al.*, 2004).

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
Real-time PCR (Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	76.8%	99.7%	Bayesian latent class analysis	Moody *et al.*, 2022
Real- time PCR (Sritunyalucksana et al., 2006)	Surveillance in apparently	Wild populations	Gill, pleopod	*Penaeus merguiensis, P. esculentus,*	82.9%	99.7%	Bayesian latent class analysis	Moody *et al.*, 2022

	healthy animals	of crustaceans		*P. plebejus, Metapenaeus endeavouri, M. bennettae*				
Two real-time PCR methods in parallel (Sritunyalucksana *et al.,* 2006 and Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	98.3%	99.4%	Bayesian latent class analysis	Moody *et al.,* 2022

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

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NB: There are OIE Reference Laboratories for infection with white spot syndrome virus
(please consult the OIE web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/).
Please contact the OIE Reference Laboratories for any further information on
infection with white spot syndrome virus

NB: FIRST ADOPTED IN 1997 AS WHITE SPOT DISEASE. MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 2.3.1.

INFECTION WITH APHANOMYCES INVADANS (EPIZOOTIC ULCERATIVE SYNDROME)

1. Scope

Infection with *Aphanomyces invadans* means all infections caused by the oomycete ~~fungus~~ *A. invadans* of the Genus *Aphanomyces* and Family *Leptolegniaceae.*

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Infection with *A. invadans* is most commonly known as epizootic ulcerative syndrome (EUS) ~~(Food and Agriculture Organization of the United Nations [FAO], 1986).~~ It has also been known as red spot disease (RSD) (Mckenzie & Hall, 1976), mycotic granulomatosis (MG) (Egusa & Masuda, 1971; Hanjavanit, 1997) and ulcerative mycosis (UM) (Noga & Dykstra, 1986). The disease is caused by the oomycete *Aphanomyces invadans*.

Infection with *A. invadans* is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish. It is clinically characterised by the presence of invasive, non-septate hyphae in skeletal muscle, usually leading to a granulomatous response. Infections with *A. invadans* have spread widely since the first outbreak in 1971 in Japan and to date a high degree of genetic homogeneity is observed for this species based on publicly available genome sequences (Dieguez-Uribeondo *et al.,* 2009; European Food Safety Authority [EFSA] 2011a; Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018; Lilley *et al.,* 2003). Other pathogenic viruses (mostly rhabdoviruses), bacteria (mainly *Aeromonas hydrophila*), fungi, oomycetes and parasites are routinely co-isolated from *A. invadans*-infected fish (Iberahim *et al.,* 2018).

Aphanomyces invadans is within a group of organisms commonly known as the water moulds. Although long-regarded as fungi because of their characteristic filamentous growth, this group, the Oomycota, is not considered a member of the Eumycota (true fungi) but is classified with diatoms and brown algae in a group called the Heterokonta or Stramenopiles within the Kingdom Chromista (Cavalier-Smith & Chao 2006; Tsui et al., 2009). Junior synonyms of *A. invadans* include *Aphanomyces piscicida* and *Aphanomyces invaderis*.

2.1.2. Survival and stability in processed or stored samples

There is limited published data on the stability of the pathogen in host tissues. It is not clear whether the pathogen continues to grow for some time following the death of the host (Oidtmann, 2012).

Aphanomyces invadans cultures can be maintained for extended periods in glucose phosphate broth (6 weeks at 10°C), agar slopes and sodium phosphate buffer (over 6 months at 20°C) (Lilley *et al.,* 1998).

2.1.3. Survival and stability outside the host

How *A. invadans* survives outside the host is unclear (Oidtmann, 2012). It is assumed that the motile zoospores, which are released from an infected fish, will encyst when unsuccessful in finding a suitable substrate to grow on (Oidtmann, 2012). Encysted zoospores of *A. invadans* are capable of releasing a

new zoospore generation instead of germinating in a process called repeated zoospore emergence (Dieguez-Uribeondo *et al.,* 2009). There is no suitable method to recover or isolate the encysted zoospore from affected fish ponds (Afzali *et al.,* 2013). How long the encysted spore can survive in water or on a non-fish substrate is unclear. In an *in-vitro* experiment, the encysted zoospore survived for at least 19 days (Lilley *et al.,* 2001).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Table 2.1. Fish species susceptible to infection with Aphanomyces invadans

Family	Scientific name	Common name
Alestidae	*Brycinus lateralis*	striped robber
	Hydrocynus vittatus	tigerfish
	Micralestes acutidens	silver robber
Ambassidae	*Ambassis agassizii*	chanda perch
Apogonidae	*Glossamia aprion*	mouth almighty
Ariidae	*Arius sp.*	fork-tailed catfish
Belonidae	*Strongylura kreffti*	long tom
Centrarchidae	*Lepomis macrochirus*	bluegill
	Micropterus salmoides	largemouth black bass
Channidae	*Channa marulius*	great snakehead fish
	Channa striatus	striped snakehead
Cichlidae	*Coptodon rendalli*	redbreast tilapia
	Oreochromis andersoni	three-spoted tilapia
	Oreochromis machrochir	greenhead tilapia
	Sargochromis carlottae	rainbow bream
	Sargochromis codringtonii	green bream
	Sargochromis giardi	pink bream
	Serranochromis angusticeps	thinface largemouth
	Serranochromis robustus	Nembwe
	Tilapia sparrmanii	banded tilapia
Clariidae	*Clarias gariepinus*	sharptooth African catfish
	Clarias ngamensis	blunt-toothed African catfish
	Clarius batrachus	walking catfish
Clupeidae	*Alosa sapidissima*	American shad
	Brevoortia tyrannus	Atlantic menhaden
	Nematalosa erebi	bony bream
Cyprinidae	*Barbus paludinosus*	straightfin barb
	Barbus poechii	dashtail barb
	Barbus thamalakanensis	Thamalakane barb
	Barbus unitaeniatus	longbeard barb
	Carassius auratus	goldfish
	Catla catla	catla
	Cirrhinus mrigala	mrigal
	Esomus sp.	flying barb
	Labeo cylindricus	red-eye labeo
	Labeo lunatus	upper Zambezi labeo
	Labeo rohita	rohu
	Puntius gonionotus	silver barb
	Puntius sophore	pool barb

Family	Scientific name	Common name
	Rohtee sp.	keti-Bangladeshi
Eleotridae	*Oxyeleotris lineolatus*	sleepy cod
	Oxyeleotris marmoratus	marble goby
Gobiidae	*Glossogobius giuris*	bar-eyed goby
	Glossogobius sp.	goby
	Tridentiger obscures obscures	dusky tripletooth goby
Helostomatidae	*Helostoma temmincki*	kissing gourami
Hepsetidae	*Hepsetus odoe*	African pike
Ictaluridae	*Ameiurus melas*	black bullhead
	Ameiurus nebulosus	black bullhead
	Amniataba percoides	striped grunter
	Ictalurus punctatus	channel catfish
Kurtidae	*Kurtus gulliveri*	nursery fish
Latidae	*Lates calcarifer*	barramundi or sea bass
Lutjanidae	*Lutjanus argentimaculatus*	mangrove jack
Melanotaeniidae	*Melanotaenia splendida*	rainbow fish
Mormyridae	*Marcusenius macrolepidotus*	bulldog
	Petrocephalus catostoma	churchill
Mugilidae	*Mugilidae (Mugil spp.; Liza spp.)*	mullets
	Mugil cephalus	grey mullet or striped mullet
	Mugil curema	white mullet
	Myxus petardi	mullet
Osmeroidei	*Plecoglossus altivelis*	ayu
Osphronemidae	*Colisa lalia*	dwarf gourami
	Osphronemus goramy	giant gourami
	Trichogaster pectoralis	snakeskin gourami
	Trichogaster trichopterus	three-spot gourami
Osteoglossidae	*Scleropages jardini*	saratoga
Percichthyidae	*Maccullochella ikei*	freshwater cod
	Maccullochella peelii	Murray cod
	Macquaria ambigua	golden perch
	Macquaria novemaculeata	Australian bass
Platycephalidae	*Platycephalus fuscus*	dusky flathead
Psettodidae	*Psettodes sp.*	spiny turbot
Salmonidae	*Oncorhynchus mykiss*	rainbow trout
Scatophagidae	*Scatophagus argus*	spotted scat
	Selenotoca multifasciata	striped scat
Schilbeidae	*Schilbe intermedius*	silver catfish
	Schilbe mystus	African butter catfish
Sciaenidae	*Bairdiella chrysoura*	drums or croakers
	Pogonias cromis	black drum
Sillaginae	*Sillago ciliata*	sand whiting
Siluridae	*Silurus glanis*	wels catfish
Soleidae	*Aseraggodes macleayanus*	narrow banded sole
Sparidae	*Acanthopagrus australis*	yellowfin sea bream
	Acanthopagrus berda	black bream
	Archosargus probatocephalus	sheepshead
Synbranchidae	*Fluta alba*	swamp eel
Terapontidae	*Anabas testudineus*	climbing perch
	Bidyanus bidyanus	silver perch
	Leiopotherapon unicolor	spangled perch

Family	Scientific name	Common name
	Scortum barcoo	Barcoo Grunter
	Therapon sp.	therapon
Toxotidae	*Toxotes chatareus*	common archerfish
	Toxotes lorentzi	primitive acherfish

2.2.2. Species with incomplete evidence for susceptibility [under study]

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *A. invadans* according to Chapter 1.5 of the *Aquatic Code* are: [under study]

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Subadult and adult fish are usually described as the susceptible life stages to natural outbreaks of EUS (FAO, 2009). However, there are reports of infection with *A. invadans* being found in early life stages (fish fry or fish larvae) (Baldock et al., 2005; EFSA 2011a). While the size of the fish does not determine an EUS outbreak (Cruz-Lacierda & Shariff, 1995), younger fish seem to be more prone to EUS compared with adult fish (Gomo *et al.,* 2016; Pagrut *et al.,* 2017).

An experimental injection of *A. invadans* into the yearling life stage of ~~Indian major carp,~~ catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), revealed resistance to *A. invadans* (Pradhan *et al.,* 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish (*Carassius auratus*) are susceptible (Hatai *et al.,* 1977; 1994), but common carp (*Cyprinus carpio*) (Wada *et al.,* 1996), Nile tilapia (*Oreochromis niloticus*) (Khan *et al.,* 1998) and European eel (*Anguilla anguilla*), (Oidtmann *et al.,* 2008) are considered resistant.

2.2.4. Distribution of the pathogen in the host

During the course of an infection with *A. invadans*, the free-swimming zoospore attaches to the skin of a fish host, encysts and germinates to develop hyphae invading and ramifying through host tissues (Kiryu *et al.,* 2003; Lilley *et al.,* 1998). The hyphal invasion and associated pathology are not confined to the region of dermal ulcers. The hyphae readily invade the body cavity and produce mycotic granulomas in all the visceral organs (Vishwanath *et al.,* 1998). In fish either suspected or confirmed to be infected with *A.~~phanomyces~~ invadans,* hyphae have also been observed in kidney, liver, spleen, pancreatic tissue, gut, parietal peritoneum, swim bladder, gonads, spinal cord, meninges, vertebrae, inter-muscular bones, the mouth region, and the orbits (Chinabut & Roberts, 1999; Vishwanath *et al.,* 1998; Wada *et al.,* 1996).

2.2.5. Aquatic animal reservoirs of infection

~~There is no information to indicate that fish can be lifelong carriers of *A. invadans*.~~ Generally, most infected fish die during an outbreak. Although some fish with mild or moderate infections could recover, they are unlikely to be lifelong carriers of *A. invadans*.

2.2.6. Vectors

No data available.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The prevalence of infection with *A. invadans* in the wild and in aquaculture farms may be high (20–90%), in endemic areas with high levels of mortality observed. Mortality patterns appear to be seasonal and can vary substantially (Herbert *et al.,* 2019).

2.3.2. Clinical signs, including behavioural changes

Fish usually develop red spots or small-to-large ulcerative lesions on the body. The occurrence of skin lesions and ultimately mortality varies according to fish species. Fish presenting with lesions are usually

weak, appear darker in colour, have a reduced appetite, are immobile and may float at the surface of the water. Generally infected fish are encountered in shallow water and present a retarded ability to escape capture occasionally followed by short lived bouts of hyperactivity characterised by jerky movements (Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018).

2.3.3 Gross pathology

Early-stage lesions or mildly infected fish are characterised by red spots observed on the lateral body surface, head, operculum or caudal peduncle of the infected fish. Scales of infected fish are often protruding or lost. In severe cases, swollen haemorrhagic areas, massive inflammation and large deep ulcers exposing the underlying necrotic muscle tissue are observed (Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018). In advanced stages of the disease, the severity of the disease results in death of the fish (Hawke *et al.,* 2003; Iberahim *et al.,* 2018).

2.3.4. Modes of transmission and life cycle

Aphanomyces invadans has an aseptate fungal-like mycelia structure. This oomycete has two typical zoospore forms. The primary zoospore consists of round cells that develop inside the sporangium. The primary zoospore is released to the tip of the sporangium where it forms a spore cluster. It quickly transforms into the secondary zoospore, which is a reniform, laterally biflagellate cell and can swim freely in the water. The secondary zoospore remains motile for a period that depends on the environmental conditions and presence of the fish host or substratum. Typically, the zoospore encysts and germinates to produce new hyphae, although further tertiary generations of zoospores may be released from cysts (repeated zoospore emergence or polyplanetism) (Lilley *et al.,* 1998). The *A. invadans* zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the secondary zoospores or free-swimming stage zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the secondary zoospores cannot find the susceptible species or encounter unfavourable conditions, they can encyst in the pond environment. The cysts may wait for conditions that favour their transformation into tertiary generations of zoospores that are also in the free-swimming stage. The encysting property of *A. invadans* may play an important role in the cycle of outbreaks in endemic areas.

2.3.5. Environmental factors

Under natural conditions, infection with *A. invadans* has been reported at water temperatures in the range 10–33°C (Bondad-Reantaso *et al.,* 1992; Hawke *et al.,* 2003) often associated with massive rainfall (Bondad-Reantaso *et al.,* 1992). These conditions favour sporulation of *A. invadans* (Lumanlan-Mayo *et al.*, 1997), and temperatures of 17–19°C have been shown to delay the inflammatory response of fish to oomycete infection (Catap & Munday, 1998, Chinabut *et al.*, 1995). In some countries, outbreaks occur in wild fish first and then spread to fish ponds. Normally, a bath infection of *A. invadans* in healthy susceptible fish species does not result in clinical signs of disease. The presence of other pathogens (viruses, bacteria or ectoparasites, skin damage, water temperature (between 18 and 22 °C), low pH (6.0–7.0) and low oxygen concentration in the water have all been hypothesised as predisposing factors for infection or factors influencing the expression of the disease (Oidtmann, 2012; Iberahim *et al.,* 2018).

Movements of live ornamental fish from countries from which infection with *A. invadans* is confirmed may spread the disease as was the case with the outbreak in Sri Lanka (Balasuriya, 1994). Flooding also caused the spread of infection with *A. invadans* in Bangladesh and Pakistan (Lilley *et al.,* 1998). Once an outbreak occurs in rivers/canals, the disease can spread downstream as well as upstream where the susceptible fish species exist.

Aphanomyces invadans grows best at 20–30°C; it does not grow *in-vitro* at 37°C. Water salinity over 2 parts per thousand (ppt) can stop spread of the agent. Under laboratory conditions the optimal growth temperature range for *A. invadans* is 19–22°C, while under natural conditions *A. invadans* seems to be more robust (Hawke *et al.,* 2003).

2.3.6. Geographical distribution

Infection with *A. invadans* was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Asia in 1971 (Egusa & Masuda, 1971). It was later reported in estuarine fish, particularly grey mullet (*Mugil*

cephalus) in eastern Australia in 1972 (Fraser *et al.*, 1992; Mckenzie & Hall, 1976). Infection with *A. invadans* has extended its range into South-East and South Asia, and into West Asia (Lilley *et al.*, 1998; Tonguthai, 1985). Outbreaks of ulcerative disease in menhaden (*Brevoortia tyrannus*) in North America had the same aetiological agent as the disease observed in Asia (Blazer *et al.*, 1999; Lilley *et al.*, 1997a; Vandersea *et al.*, 2006). The first confirmed outbreaks of infection with *A. invadans* on the African continent occurred in 2007, and were connected to the Zambezi-Chobe river system (Andrew *et al.,* 2008; FAO, 2009; Huchzermeyer & Van der Waal, 2012; McHugh *et al.,* 2014). In 2010 and 2011, infection with *A. invadans* appeared in wild freshwater fish in Southern Africa and in wild brown bullhead fish in North America. Infection with *A. invadans* has been reported from more than 20 countries in four continents: North America, Southern Africa, Asia and Australia.

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

There is no protective vaccine available.

2.4.2. Chemotherapy including blocking agents

There is no effective treatment for *A. invadans*-infected fish in the wild and in aquaculture ponds.

2.4.3. Immunostimulation

Experimentally infected snakehead fish fed a vitamin-supplemented feed exhibited clinical signs of infection with *A. invadans* but had higher survival than controls (Miles *et al.*, 2001).

2.4.4. Breeding resistant strains

No data available.

2.4.5. Inactivation methods

To minimise fish losses in infected fish ponds water exchange should be stopped and lime or hydrated lime and/or salt should be applied (Lilley *et al.,* 1998). Preparing fish ponds by sun-drying and liming are effective disinfection methods for *A. invadans* (EFSA 2011b; Kumar *et al.,* 2020; Oidtmann, 2012). Similar to other oomycetes or water moulds, general disinfection chemicals effectively destroy *A. invadans* that might contaminate farms, fish ponds or fishing gear (Iberahim *et al.,* 2018).

2.4.6. Disinfection of eggs and larvae

Routine disinfection of fish eggs and larvae against water moulds is effective against *A. invadans*. It should be noted that there is no report of the presence of *A. invadans* in fish eggs or larvae.

2.4.7. General husbandry

Control of *A. invadans* in natural waters is probably impossible. In outbreaks occurring in small, closed water bodies or fish ponds, treating water with agricultural limes and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease. Preventing entry of water from *A. invadans*-infected water bodies into fish ponds can prevents spread of the disease into farms. Sodium chloride or salt and agricultural lime are safe and effective chemicals for treating or preventing the spread of *A. invadans.*

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Scoop net, cast net or seine net represent the best choices for catching diseased fish in natural waters or in fish ponds (FAO 2009).

Fish with characteristic EUS-like lesions should be sampled from affected populations

3.2. Selection of organs or tissues

The motile zoospore plays an important role in the spread of the disease. Once the motile spore attaches to the skin of the fish, the spore will germinate under suitable conditions and its hyphae will invade the fish skin, muscular tissue and reach the internal organs. Fish skeletal muscle is the target organ and exhibits major clinical signs of infection with *A. invadans* with mycotic granulomas (Iberahim *et al.,* 2018). Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae. Instead, samples should be taken from the leading edge of the infected area or lesion and where possible, multiple samples should be taken from an infected individual to obtain viable hyphae. Fungal hyphae can be seen in tissue squash mounts and histological sections at the leading edge of the infected area. Attempting to culture *A. invadans* from severe ulcers is often constrained because of contaminating bacteria, but still should be attempted. PCR on tissue taken from the leading edge of the ulcer also should be attempted.

3.3. Samples or tissues not suitable for pathogen detection

Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae.

3.4. Non-lethal sampling

None available.

3.5. Preservation of samples for submission

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Samples must not be frozen since ~~the fungus~~ *A. invadans* is killed by freezing. Fish collected from remote areas should be anesthetised and can be fixed in normal 10% formalin or 10% phosphate-buffered formalin for at least 1–2 days. The fixed specimens are then transferred to double-layer plastic bags with formalin-moistened tissue paper.

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0 *General information* (*diseases of fish*).

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time 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. Multiple samples should be taken from each lesion to increase the chances of obtaining viable hyphae.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1.

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

Standard methods for histopathology can be found in Chapter 2.3.0.

3.5.4. Samples for other tests

None

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where 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 animals should be processed and tested individually are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
~~Squash mounts~~ Clinical signs	±	±	±	NA	+	+	+	NA				
Squash mounts					±	±	±	1	±	±	±	1
Histopathology					++	++	++	1	++	++	++	1
Cytopathology												
Cell or artificial media culture					++	++	++	1	+	+	+	1
Real-time PCR												
Conventional PCR					++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation									++	++	++	1
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
Shading indicates the test is inappropriate or should not be used for this purpose.

~~Diagnosis of infection with *A. invadans* in clinically affected fish may be achieved by histopathology, oomycete isolation or polymerase chain reaction amplification.~~

4.1. Squash mounts

Aphanomyces invadans can be detected using microscopic examination of squash preparations prepared as follows:

i) Remove ulcer surface using a sharp scalpel blade.

ii) Cut the muscular tissue at the edge of the ulcer.

iii) Place the pieces of tissue on a cutting board then make thin slices using a sharp scalpel blade.

iv) Place the thinly sliced tissue between two glass slides and squeeze gently with fingers.

v) Remove one of the glass slides and cover the tissue with a cover-slip. View under a light microscope to find the nonseptate hyphae structure of *A. invadans* (12–25 µm in diameter).

4.2. Histopathology and cytopathology

Aphanomyces invadans can be detected using microscopic examination of fixed sections, prepared as follows:

i) Sample only live or moribund specimens of fish with clinical lesions.

ii) Take samples of skin/muscle (<1 cm^3), including the leading edge of the lesion and the surrounding tissue.

iii) Fix the tissues immediately in 10% formalin. The amount of formalin should be 10 times the volume of the tissue to be fixed.

4.2.1. Histological procedure

Standard methods for processing are provided in chapter 2.3.0. H&E and general fungus stains (e.g. Grocott's stain) will demonstrate typical granulomas and invasive hyphae.

4.2.2 Histopathological changes

Early lesions are caused by erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesions progress from a mild chronic active dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae.

4.3. Cell culture for isolation

4.3.1. Isolation of *Aphanomyces invadans* from internal tissues

The following are two methods of isolation of *A. invadans* adapted from Lilley *et al.* (1998) and Willoughby & Roberts (1994).

Method 1: Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through the stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 mm^3, and place on a Petri dish containing glucose/peptone (GP) agar (see Table 4.1) with penicillin G (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}). Seal plates, incubate at room temperature or at 25°C and examine daily. Repeatedly transfer emerging hyphal tips on to fresh plates of GP agar with antibiotics until cultures are free of contamination.

Method 2: Lesions located on the flank or tail of fish <20 cm in length can be sampled by cutting the fish in two using a sterile scalpel and slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2–4 mm^3) from beneath the lesion and place it in a Petri dish of GP medium (see Table 4.1) with 100 units ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin. Instruments should not contact the contaminated external surface of the fish. Incubate inoculated medium at approximately 25°C and examine under a microscope (preferably an inverted microscope) within 12 hours. Repeatedly transfer emerging hyphal tips to plates of GP medium with 12 g $litre^{-1}$ technical agar, 100 units ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin until axenic cultures are obtained. The oomycete isolate can also be maintained at 25°C on glucose/yeast extract (GY) agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

4.3.2. Identification of *Aphanomyces invadans*

Aphanomyces invadans does not produce any sexual structures and should thus not be diagnosed by morphological criteria alone. However, the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces* spp., as described in Lilley *et al.,* 1998. *Aphanomyces invadans* is characteristically slow-growing in culture and fails to grow at 37°C on GPY agar (Table 4.1). Detailed temperature–growth profiles are given in Lilley & Roberts (1997). *A. invadans* can be identified by polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

4.3.3. Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing glucose/peptone/yeast (GPY) broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water (Table 4.1 2), and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope.

Table 4.1 2. Media for isolation, growth and sporulation of Aphanomyces invadans *cultures*

GP (glucose/peptone) medium	GPY (glucose/peptone / yeast) broth	GPY agar	GY agar (glucose/ yeast)	Autoclaved pond water
3 g $litre^{-1}$ glucose 1 g $litre^{-1}$ peptone 0.128 g $litre^{-1}$ $MgSO_4.7H_2O$ 0.014 g $litre^{-1}$ KH_2PO_4 0.029 g $litre^{-1}$ $CaCl_2.2H_2O$ 2.4 mg $litre^{-1}$ $FeCl_3.6H_2O$ 1.8 mg $litre^{-1}$ $MnCl_2.4H_2O$ 3.9 mg $litre^{-1}$ $CuSO_4.5H_2O$ 0.4 mg $litre^{-1}$ $ZnSO_4.7H_2O$	GP broth + 0.5 g $litre^{-1}$ yeast extract	GPY broth + 12 g $litre^{-1}$ technical agar	1% glucose, 0.25% yeast extract, 1.5% agar	Sample pond/lake water known to support oomycete growth. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave. pH to 6–7.

Agent purification

Maintaining *A. invadans* in the axenic culture is necessary. As it is characteristically slow-growing, it easily becomes contaminated with other micro-organisms, such as bacteria and other fast-growing oomycetes and fungi. Attempts to purify or isolate *A. invadans* from contaminated cultures usually fail.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or running a gel.

4.4.1. Real-time PCR

No real-time PCR methods for detecting *A. invadans* in fish tissues are available.

4.4.2. Conventional PCR

DNA preparation from *A. invadans* isolate

DNA is extracted from an actively growing colony of *A. invadans* culture in GY broth at about 4 days or when young mycelia reach 0.5–1.0 cm in diameter. The mycelia are transferred to sterile 100-mm Petri dishes, washed twice with PBS and then placed on tissue paper for liquid removal. Hyphal tips (~50–250 mg) are excised with a sterile scalpel blade and transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Commercial DNA extraction kits have been used successfully (Phadee *et al.*, 2004b; Vandersea *et al.*, 2006).

DNA preparation from *A. invadans*-infected tissue

Small pieces of *A. invadans*-infected tissue (25–50 mg) are suitable for DNA extractions (Phadee *et al.*, 2004a).

Diagnostic PCR technique

Three published techniques are specific to *A. invadans*.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Vandersea *et al.*, 2006) GenBank Accession No. AF396684, Product size 234bp)			
Aphanomyces invadans (ITS1)	Fwd Ainvad-2F: TCA-TTG-TGA-GTG-AAA-CGG-TG Rev Ainvad-ITSR1: GCT-AAG-GTT-TCA-GTA-TGT-AG	0.025 nM 0.025 nM	35 cycles: 95°C/30 sec, 56°C/45 sec,95°C/30 sec 72°C/2.5 min, 95°C/30 sec
Method 2 (Phadee *et al.*, 2004b) GenBank Accession No.AF396683, Product size 550bp)			
Aphanomyces invadans (ITS1-ITS2)	Fwd ITS11: GCC-GAA-GTT-TCG-CAA-GAA-AC Rev ITS23: CGT-ATA-GAC-ACA-AGC-ACA-CCA	500 nM 500 nM	35 cycles: 94°C/30 sec, 65°C/45 sec, 72°C/1 min
Method 3 (Oidtmann *et al.*, 2008) GenBank Accession No. EU422990 Product size 564bp)			
Aphanomyces invadans (ITS1-ITS2)	Fwd BO73: CTT-GTG-CTG-AGC-TCA-CAC-TC Rev BO639: ACA-CCA-GAT-TAC-ACT-ATC-TC	600 nM 600 nM	35 cycles: 96°C/1 min, 58°C/1 min, 72°C/1 min

The species-specific forward primer site is located near the 3' end of the SSU (small subunit) gene and a species-specific reverse primer site is located in the ITS1 region for Ainvad-2F (5'-TCA-TTG-TGA-GTG-AAA-CGG-TG-3') and Ainvad-ITSR1 (5'-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3'). The PCR mixture contained 25 pM of each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase and 20 ng of genomic DNA (either from an *Aphanomyces* isolate or from infected tissue) for a total volume of 50 µl. DNA is amplified in a thermocycle machine under the following cycle conditions: 2 minutes at 95°C; 35 cycles, each consisting of 30 seconds at 95°C, 45 seconds at 56°C, 2.5 minutes at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 234 bp (Vandersea *et al.*, 2006).

~~*Method 2*~~

~~The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is ITS11 (5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3') and the reverse is ITS23 (5'-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3'). The PCR mixture contains 0.5 µM of each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.6 U of *Taq* DNA polymerase and 20 ng of genomic DNA (from an *Aphanomyces* isolate) for a total volume of 25 µl. The DNA is amplified under the following cycle conditions: 5 minutes at 94°C; 25 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 550 bp. PCR amplification using the DNA template from the infected tissue is similar to the above protocol except that 5 ng of the DNA template is used for 35 cycles (Phadee *et al.*, 2004b).~~

~~*Method 3*~~

~~The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is BO73 (5'-CTT-GTG-CTG-AGC-TCA-CAC-TC-3') and the reverse is BO639 (5'-ACA-CCA-GAT-TAC-ACT-ATC-TC-3'). The PCR mixture contains 0.6 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.625 units of Taq DNA polymerase, and approximately 5 ng of genomic DNA (or 2.5 µl of DNA template extracted from 25 mg of infected tissue and suspended in 100 µl buffer) in a 50 µl reaction volume (Oidtmann *et al.*, 2008). The DNA is amplified under the following cycle conditions: 96°C for 5 minutes; 35 cycles of 1 minute at 96°C, 1 minute at 58°C and 1 minute at 72°C; followed by a final extension at 72°C for 5 minutes (Oidtmann, pers. comm.). The PCR product is analysed by agarose gel electrophoresis and the target product is 564 bp.~~

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

Nucleotide sequencing of all conventional PCR amplicons (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. *Aphanomyces invadans*-specific sequences will share a high degree of nucleotide similarity to one of the published reference sequences for *A. invadans* (Genbank accession AF396684).

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published sequences.

4.6. *In-situ* hybridisation

A fluorescent peptide nucleic acid *in-situ* hybridisation (FISH) technique has demonstrated a high specificity for *A. invadans.* The technique can directly detect the mycelia-like structure of the oomycete in thinly sliced tissues of affected organs of susceptible fish. The fluorescein (FLU) probe designed to hybridise the small subunit of the rRNA *A. invadans* (bp 621 to 635; GenBank acc. AF396684) is 5'-FLU-GTA-CTG-ACA-TTT-CGT-3' or Ainv-FLU3.

The *A. invadans*-affected tissue is fixed and hybridised as soon as possible after the fish are collected to minimise RNA degradation. Tissue (~20 mg) is dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtitre plate. One ml ethanol-saline fixative (44 ml of 95% ethanol, 10 ml of deionised H_2O, and 6 ml of 25 × SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-enesorbitan monolaurate (Tween 20) is added to enhance tissue permeabilisation. The microtitre plate is gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 hours. The fixed tissues are rinsed (twice for 15 minutes each time) with 0.5 ml of hybridisation buffer (5 × SET, 0.1% [v/v] Igepal-CA630 and 25 µg ml^{-1} poly[A]) containing 3% Tween 20. The hybridisation buffer is removed, and the tissues are resuspended in 0.5 ml of hybridisation buffer containing 3% Tween 20 and 100 nM Ainv-FLU3 probe. "No-probe" control specimens are incubated with 0.5 ml of hybridisation buffer/3% Tween 20. All tissues are incubated at 60°C for 1 hour in the dark. Following incubation,

the tissues are rinsed twice with 1 ml of pre-warmed (60°C) 5 × SET buffer containing 3% Tween 20 to remove residual probe. The tissue specimens are mounted onto poly-L-lysine-coated microscope slides. One drop of the light anti-fade solution is placed on the specimens, which are then overlaid with a cover-slip. Analyses are performed by light and epifluorescence microscopy. The camera and microscope settings for epifluorescent analyses are held constant so that comparative analyses of relative fluorescence intensity can be made between probed and non-probed specimens. The fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The above detailed protocols are published by Vandersea *et al*. (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed tissue.

4.7. Immunohistochemistry

None.

4.8. Bioassay

Fish can be experimentally infected by intramuscular injection of 0.1 ml suspension of 100+ motile zoospores into fish susceptible to infection with *A. invadans* at 20°C. Histological growth of aseptate hyphae, 12–25 µm in diameter, should be demonstrated in the muscle of fish sampled after 7 days, and typical mycotic granulomas should be demonstrated in the muscle of fish sampled after 10–14 days.

4.9. Antibody or antigen detection methods

Polyclonal antibodies against *A. invadans* or *Aphanomyces* saprophyte showed cross-reactivity to each other using protein gel electrophoresis and Western blot analysis and immunohistochemistry. (Lilley *et al*., 1997b). However, a specific monoclonal antibody against *A. invadans* ~~developed later~~ was found to have high specificity and high sensitivity to *A. invadans* using immunofluorescence. This monoclonal antibody could detect *A. invadans* hyphae at the early stage of infection (Miles *et al*., 2003).

A monoclonal antibody-based flow-through immunoassay was developed by Adil *et al*. (2013). This assay was found to have high analytical (0.007mg ml^{-1}) and diagnostic specificity comparable to PCR.

4.10. Other methods

Serological methods for detection and identification of *A. invadans* in diseased specimens are not practical. If necessary, the monoclonal antibody offers a better specificity and sensitivity than polyclonal antibody for serological detection or identification of *A. invadans* in diseased specimens or in pathogen isolates.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The test for targeted surveillance to declare freedom from infection with *A. invadans* is examination of target populations for gross signs of infection with *A. invadans*. Surveys should be conducted during seasons that favour clinical manifestation of infection with *A. invadans* or when water temperatures are in the range 18–25°C.

Using the gross sign test for targeted surveillance, a large sample of the fish population should be examined live with a sample size sufficient to meet survey design assumptions as described in Chapter 1.4 of the Aquatic Code.

If fish show gross signs consistent with infection with *A. invadans*, they should be categorised as suspect fish, and the location/farm/compartment/zone should be considered suspect. Suspect specimens should be further tested using the methods listed under presumptive diagnosis followed by confirmative diagnosis as described in the Table 4.1.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (6.1) or presence of clinical signs (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.

6.1. Apparently healthy animals or animals of unknown health status [13]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. ~~Geographical~~ Hydrographical 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 populations

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Observation of clinical signs consistent with infection with *A. invadans* [14]

ii) A positive result obtained by any of the diagnostic techniques described in Section 4.

6.1.2. Definition of confirmed case in apparently healthy populations

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Histopathology consistent with infection with *A. invadans* and positive result by PCR and amplicon sequencing

ii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iii) Artificial media culture and positive result by PCR and sequencing of the amplicon

6.2 Clinically affected animals

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with *A. invadans* as described in this chapter, with or without elevated mortality

ii) Positive result by a recommended molecular detection test

iii) Histological changes consistent with infection with *A. invadans*

iv) Visual observation of hyphae characteristic (direct or by microscopy) of *A. invadans*

v) Culture and isolation of *A. invadans*-type colonies

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Visualisation of hyphae under squash mounts and a positive result by PCR and sequencing of the amplicon

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[13] For example transboundary commodities.

[14] Note that surveillance of apparently healthy populations for EUS is based on examination of target populations for clinical signs of infection with *A. invadans* (see Section 5).

ii) Histopathological changes consistent with infection with *A. invadans* and a positive result by PCR and sequencing of the amplicon

iii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iv) Artificial media culture and a positive result by PCR and sequencing of the amplicon

v) Positive result for *in-situ* hybridisation and a positive result by PCR and sequencing of the amplicon

6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *A. invadans* is provided in Table 6.3.1. (**note**: no data are currently available). This information can be used for the design of surveys for infection with *A. invadans*, 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 is 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

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

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 specificity.

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* *

NB: There is currently (2022) no OIE Reference Laboratories for infection with *Aphanomyces invadans*
(please consult the OIE web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1995 AS EPIZOOTIC ULCERATIVE SYNDROME;
MOST RECENT UPDATES ADOPTED IN 2013.

CHAPTER 2.3.2.

INFECTION WITH EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infection with epizootic haematopoietic necrosis virus means infection with the pathogenic agent *epizootic haematopoietic necrosis virus* (EHNV) of the Genus *Ranavirus* of the Family *Iridoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

EHNV is a species of the genus *Ranavirus* in the Family *Iridoviridae* (Chinchar *et al.,* 2005). In addition to fish, ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (Chinchar*,* 2002; Drury *et al.,* 2002; Fijan *et al.,* 1991; Hyatt *et al.*, 2002; Speare & Smith, 1992; Whittington *et al.,* 2010; Wolf *et al*., 1968; Zupanovic *et al*., 1998). Ranaviruses have large (150–180 nm), icosahedral virions, a double-stranded DNA genome 150–170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (Chinchar *et al.,* 2005).

Since the recognition of disease due to EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish. These include catfish (*Ictalurus melas*) in France (European catfish virus, ECV) (Pozet *et al*., 1992), sheatfish (*Silurus glanis*) in Germany (European sheatfish virus, ESV) (Ahne *et al.,* 1989; 1990), turbot (*Scophthalmus maximus*) in Denmark (Bloch & Larsen, 1993), and cod (*Gadus morhua*) in Denmark (Cod iridovirus, CodV) (Ariel *et al.,* 2010). EHNV, ECV, ESV, and CodV share >98% nucleotide identity across concatenated sequences across the RNR-α, DNApol, RNR-β, RNAse II and MCP gene regions (Ariel *et al*., 2010).

EHNV and ECV can be differentiated using genomic analysis (Ahne *et al.,* 1998; Holopainen *et al*., 2009; Hyatt *et al*., 2000; Mao *et al*., 1996; 1997; Marsh *et al*., 2002). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV), and differentiation of these from ranavirus occurrences in amphibians.

2.1.2. Survival and stability in processed or stored samples

EHNV can persist in frozen fish tissues for more than 2 years (Langdon, 1989) and frozen fish carcases for at least a year (Whittington *et al*., 1996).

2.1.3. Survival and stability outside the host

EHNV is resistant to drying and remained infective for 97 days at 15°C and 300 days at 4°C in water (Langdon, 1989). For these reasons, it is presumed that EHNV would persist for months to years on a fish farm in water and sediment, as well as on plants and equipment.

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 infection with EHNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

Family	Scientific name	Common name
Esocidae	*Esox lucius*	Northern pike
Galaxiidae	*Galaxias olidus*	Mountain galaxias
Ictaluridae	*Ameiurus melas*	Black bullhead
Melanotaeniidae	*Melanotaenia fluviatilis*	Crimson spotted rainbow fish
Percidae	*Perca fluviatilis*	European perch
	Sander lucioperca	Pike-perch
Percichthyidae	*Macquaria australasica*	Macquarie perch
Poeciliidae	*Gambusia holbrooki*	Eastern mosquito fish
	Gambusia affinis	Mosquito fish
Salmonidae	*Oncorhynchus mykiss*	Rainbow trout
Terapontidae	*Bidyanus bidyanus*	Silver perch

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 infection with EHNV according to Chapter 1.5 of the *Aquatic Code* are: none known.

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: Atlantic salmon (*Salmo salar*), freshwater catfish (*Tandanus tandanus*), golden perch (*Macquaria ambigua*), Murray cod (*Maccullochella peelii*) and purple spotted gudgeon (*Mogurnda adspersa*).

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

Natural infections and disease have been limited to European perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) in Australia. The disease is more severe in European perch and in juveniles compared with adult fish (Whittington *et al.,* 2010). There are no descriptions of infection of eggs or early life stages of any other fish species.

For the purposes of Table 4.1, larvae and fry up to approximately 5 g in weight may be considered to be early life stages, fingerlings and grower fish up to 500 g may be considered to be juveniles, and fish above 500 g may be considered to be adults.

2.2.4. Distribution of the pathogen in the host

Target organs and tissues infected with the virus are kidney, spleen and liver. It is not known if EHNV can be detected in gonadal tissues, ovarian fluid or milt or whether these tissues are suitable for surveillance of broodstock.

2.2.5. Aquatic animal reservoirs of infection

Rainbow trout: The high case fatality rate and low prevalence of infection with EHNV in natural infections in rainbow trout means that the recruitment rate of carriers is likely to be very low (<2%) (Whittington *et al.,* 1994). EHNV has been detected in growout fish but histopathological lesions consistent with infection with EHNV indicated an active infection rather than a carrier state (Whittington *et al.,* 1999). Anti-EHNV serum antibodies were not detected in fingerlings during or after an outbreak but were detected in a low proportion of growout fish, hence, it is uncertain whether these were survivors of the outbreak (Whittington *et al.,* 1994; 1999). There are data for European stocks of rainbow trout in experimental infections where potential carriers were identified (Ariel & Bang Jensen, 2009).

European perch: EHNV was isolated from 2 of 40 apparently healthy adult European perch during epizootics in juveniles in Victoria, Australia (Langdon & Humphrey, 1987), but as the incubation period

extends for up to 28 days (Whittington & Reddacliff, 1995), these fish may have been in the preclinical phase.

2.2.6. Vectors

None demonstrated. ~~Birds are potential vectors for EHNV, it being carried in the gut, on feathers, feet and the bill (Whittington *et al.,* 1996).~~

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Rainbow trout: It appears that under natural farm conditions EHNV is poorly infective but once infected, most fish succumb to the disease ~~has a high case fatality rate~~. Infection with EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. Infection with EHNV has most often been reported in young fingerlings <125 mm fork length with daily mortality of less than 0.2% and total mortality of up to 4%. However, rainbow trout of all ages may be susceptible, although infection has not yet been seen in broodstock (Whittington *et al.*, 1994; 1999). There is a low direct economic impact because of the low mortality rate. Differences in susceptibility between European and Australian stocks of rainbow trout may exist (Ariel & Bang Jensen, 2009).

European perch: There is a very high rate of infection and mortality in natural outbreaks that, over time, leads to loss in wild fish populations (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1996). Experimental bath inoculation with as few as 0.08 $TCID_{50}$ ml^{-1} was lethal, and doses too low to be detected by virus isolation in BF-2 cells were fatal by intraperitoneal inoculation (Whittington & Reddacliff, 1995). Differences in susceptibility between European and Australian stocks of European perch may exist (Ariel & Bang Jensen, 2009).

2.3.2. Clinical signs, including behavioural changes

Moribund fish may have loss of equilibrium, flared opercula and may be dark in colour (Reddacliff & Whittington, 1996). Clinical signs are usually more obvious in fingerlings and juvenile fish than adults of both rainbow trout and European perch. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality, manifesting as skin, fin and gill lesions (Reddacliff & Whittington, 1996).

2.3.3 Gross pathology

There may be no gross lesions in affected fish. A small proportion of fish may have enlargement of kidney, liver or spleen. There may be focal white to yellow lesions in the liver corresponding to areas of necrosis (Reddacliff & Whittington, 1996).

2.3.4. Modes of transmission and life cycle

Rainbow trout: EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (Langdon *et al.,* 1988; Whittington *et al.*, 1994; 1999). The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading fish. There are no data on possible vertical transmission of EHNV on or within ova, and disinfection protocols for ova have not been evaluated. EHNV has not yet been isolated from ovarian tissues or from broodstock. Annual recurrence in farmed rainbow trout may be due to reinfection of successive batches of fish or from wild European perch present in the same catchment.

European perch: The occurrence of infection with EHNV in European perch in widely separated river systems and impoundments suggested that EHNV was spread by translocation of live fish or bait by recreational fishers (Whittington *et al.,* 2010).

The route of infection is unknown. European perch and rainbow trout are susceptible to immersion exposure. The virus infects a range of cell types including hepatocytes, haematopoietic cells and endothelial cells in many organs (Reddacliff & Whittington, 1996). Virus is shed into water from infected tissues and carcasses as they disintegrate.

2.3.5. Environmental factors

Rainbow trout: Outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Damage to skin may provide a route of entry for EHNV. Outbreaks have been seen on farms at water temperatures ranging from 11 to 20°C (Whittington *et al.*, 1994; 1999). The incubation period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (Whittington & Reddacliff, 1995).

European perch: Natural epizootics of infection with EHNV affecting juvenile and adult European perch occur mostly in summer (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1994). It has been assumed that the disease in juvenile fish is related to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for outbreaks as juvenile fish feed in warm shallow waters on planktonic fauna, whereas adults feed on benthic invertebrates and larger prey in deeper cooler water (Whittington & Reddacliff, 1995). Experimentally, the incubation period ranged from 10 to 28 days at 12–18°C compared with 10–11 days at 19–21°C, and adult perch were refractory to infection at temperatures below 12°C (Whittington & Reddacliff, 1995). European stocks of European perch also displayed temperature-dependent susceptibility (Ariel & Bang Jensen, 2009).

2.3.6. Geographical distribution

Infection with EHNV has been reported from rainbow trout farms within two river catchments in New South Wales, Australia (Whittington *et al*., 2010). Infection with EHNV is endemic in south-eastern Australia, with a discontinuous distribution (Whittington *et al*., 2010).

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

Not available.

2.4.1. Vaccination

None available.

2.4.2. Chemotherapy including blocking agents

None available.

2.4.3. Immunostimulation

None available.

2.4.4. Breeding resistant strains

There has been no formal breeding programme for resistant strains of susceptible species. However, experimental trials using a bath exposure have shown that European perch from water bodies in New South Wales, Australia with previous EHNV infections showed lower mortality compared with European perch from neighbouring and distant water bodies in Australia that have no previous history of EHNV (Becker *et al.,* 2016).

2.4.5. Inactivation methods

EHNV is susceptible to 70% ethanol, 200 mg litre^{-1} sodium hypochlorite or heating to 60°C for 15 minutes (Langdon, 1989). Data for the inactivation of amphibian ranavirus may also be relevant: 150 mg/litre chlorhexidine and 200 mg/litre potassium peroxymonosulphate were effective after 1 minute contact time (Bryan *et al*., 2009). If it is first dried, EHNV in cell culture supernatant is resistant to heating to 60°C for 15 minutes (Whittington *et al.*, 2010).

2.4.6. Disinfection of eggs and larvae

Not tested.

2.4.7. General husbandry

Disease control in rainbow trout at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. Investigations on one rainbow trout farm indicated that ponds with high stocking rates and low water flow, and thus poorer water quality, may result in higher levels of clinical disease compared with ponds on the same farm with lower stocking rates and higher water flow (Whittington *et al.,* 1994). The mechanism of protection may be through maintenance of healthy integument (Whittington *et al.,* 1994).

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 which are most likely to be infected.

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species (e.g. rainbow trout and European perch) should be sampled preferentially. Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with EHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

3.2. Selection of organs or tissues

Liver, anterior kidney and spleen from individual fish are pooled (Jaramillo *et al.,* 2012).

3.3. Samples or tissues not suitable for pathogen detection

Inappropriate tissues include gonads, gonadal fluids, milt and ova, ~~since~~ because there is no evidence of reproductive tract infection.

3.4. Non-lethal sampling

~~No~~ Non-lethal samples (blood, fin, gill, integument or mucous) are unsuitable for testing ~~EHNV~~.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (*diseases of fish*).

3.5.2. Preservation of samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.~~ Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.2.5 of Chapter 2.3.0. *General information* (*diseases of fish*).

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

~~Tissue samples for histopathology should be fixed immediately after collection in 10% neutral buffered formalin. The recommended ratio of fixative to tissue is 10:1.~~ Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.3.0 *General information* (diseases of fish).

3.5.4. Samples for other tests

Not recommended for routine diagnostic testing.

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. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry or specimens can be pooled to provide the minimum amount of material needed for testing. ~~If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.~~

4. Diagnostic methods

The methods currently available for ~~identifying infection~~ pathogen detection that can be used in i) surveillance of apparently healthy ~~populations~~ animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

~~The designations used in the Table indicate:~~

Ratings ~~**against**~~ **for purposes of use.** For each recommended assay a qualitative rating ~~against~~ 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, ~~successful application by diagnostic laboratories,~~ availability, cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

~~Key:~~

+++ =	~~Most suitable~~ Methods ~~–~~are most suitable with desirable performance and operational characteristics.
++ =	~~Suitable~~ Method~~(s)~~ are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	~~Less suitable~~ Methods ~~–~~are suitable, but performance or operational characteristics may ~~significantly~~ limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

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	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology					++	++	++	1				
Cytopathology												
Cell culture	+++	+++	+++	~~2~~1	+++	+++	+++	~~2~~1	++	++	++	~~2~~1
Immunohistochemistry					+	+	+	1				
Real-time PCR	+++	+++	+++	~~2~~1	+++	+++	+++	2	++	++	++	~~2~~1
Conventional PCR	+	+	+	1	++	++	++	1	~~++~~	~~++~~	~~++~~	~~1~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	~~3~~1
In-situ hybridisation												
Bioassy												
LAMP												
Ab-ELISA			+	1								
Ag-ELISA	+	+	+	1	+	+	+	1				
Other antigen detection methods[3]												
Other method[3]												

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.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify 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

Light microscopy: routine methods can be used for tissue fixation, such as in 10% buffered neutral formalin, paraffin embedding, preparation of 4–10 µm sections and staining with H&E to demonstrate tissue necrosis and basophilic intracytoplasmic inclusion bodies. These inclusion bodies are indicative but not confirmatory for infection with EHNV. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (see below) to identify EHNV antigen associated with necrotic lesions.

Acute focal, multifocal or locally extensive coagulative or liquefactive necrosis of liver, haematopoietic kidney and spleen are commonly seen in routine haematoxylin and eosin (H&E)-stained sections of formalin-fixed material. A small number of basophilic intracytoplasmic inclusion bodies may be seen, particularly in areas immediately surrounding necrotic areas in the liver and kidney. Necrotic lesions may also be seen in heart, pancreas, gastrointestinal tract, gill and pseudobranch (Reddacliff & Whittington, 1996).

Affected tissues (e.g. kidney, liver and spleen) contain cells exhibiting necrosis. Cells contain conspicuous cytoplasmic inclusions that are rarefied areas of the cytoplasm in which the viruses are assembled. ~~Within the cytoplasm, aggregates (paracrystalline arrays) of large (175 nm ± 6 nm) nonenveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through the plasma membrane.~~ The nuclei of infected cells are frequently located peripherally and are distorted in shape.

4.3. Cell culture for isolation

4.3.1. Preparation of fish tissues for virus isolation

A simple method for preparation of fish tissues for cell culture and ELISA has been validated (Whittington & Steiner, 1993) (see sampling Section 3).

i) Freeze tubes containing tissues at –80°C until needed.

ii) Add 0.5 ml of homogenising medium (minimal essential medium Eagle, with Earle's salts with glutamine] [MEM] with 200 International Units [IU] ml^{-1} penicillin, 200 µg ml^{-1} streptomycin and 4 µg ml^{-1} amphotericin B) to each tube. Grind tissue to a fine mulch with a sterile fitted pestle.

iii) Add another 0.5 ml of homogenising medium to each tube and mix with a pestle.

iv) Add three sterile glass beads to each tube (3 mm diameter) and close the lid of the tube.

v) Vortex the suspension vigorously for 20–30 seconds and place at 4°C for 2 hours.

vi) Vortex the suspension again as above and centrifuge for 10 minutes at 2500 ***g*** in a benchtop microcentrifuge.

vii) Transfer the supernatant, now called clarified tissue homogenate, to a fresh sterile tube. Homogenates may be frozen at –80°C until required for virus isolation and ELISA.

4.3.2. Cell culture~~/artificial media~~

EHNV ~~grows~~ replicates well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (*epithelioma papulosum cyprini* [Cinkova *et al.,* 2010]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (Crane *et al.,* 2005). Incubation

temperatures of 20°C or 24°C result in higher titres than 15°C; and BF-2, EPC, or CHSE 214 incubated at 22°C and BF-2 EPC or CHSE-214 cells are recommended to maximise titres, which might be important for the detection of low numbers of viruses in fish tissues (Ariel *et al.,* 2009). BF-2 cells are preferred by the OIE Reference Laboratory with an incubation temperature of 22°C. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below (Section 4.7). The identity of viruses in cell culture is determined by immunostaining, ELISA, immuno-electron microscopy, PCR and amplicon sequencing.

4.3.3. Cell culture technical procedure

Samples: tissue homogenates.

Cells are cultured (in flasks, tubes or multi-well plates) with growth medium (MEM + 10% fetal calf bovine serum [FCBBS] with 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 µg ml^{-1} amphotericin B). The cells are incubated until almost confluent at 22°C, which can take up to 4 days depending on the seeding rate. Medium is changed to a maintenance medium (MEM with 2% FCBS and 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 µg ml^{-1} amphotericin B) on the day of inoculation. A 1/10 dilution using homogenising medium is made of single or pooled homogenates. Each culture is inoculated with 100 µl of sample per ml of culture medium. This represents a final 1/100 dilution of a 0.1 mg ml^{-1} tissue homogenate. A further 1/10 dilution is made representing a final 1/1000 dilution, and two cultures are inoculated. No adsorption step is used. As an alternative, two to three cultures can be inoculated directly with 10 µl undiluted homogenate per ml of culture medium. Note that a high rate of cell toxicity or contamination often accompanies the use of a large undiluted inoculum. The cultures are incubated at 22°C in an incubator for 6 days. Cultures are read at days 3 and day 6. Cultures are passed at least once to detect samples with low levels of virus. On day 6, the primary cultures (P1) are frozen overnight at –20°C, thawed, gently mixed and then the culture supernatant is inoculated onto fresh cells as before (P2), i.e. 100 µl P1 supernatant per ml culture medium. Remaining P1 supernatants are transferred to sterile 5 ml tubes and placed at 4°C for testing by ELISA or PCR or another means to confirm the cause of cytopathic effect (CPE) as EHNV. P2 is incubated as above, and a third pass is conducted if necessary.

4.3.4. Interpretation of results

CPE is well developed and consists of focal lysis surrounded by rounded granular cells. This change extends rapidly to involve the entire monolayer, which detaches and disintegrates. Cell cultures can be tested for EHNV DNA using real-time PCR and conventional PCR with sequence analysis as described in Section 4.4. Antigen can be detected using immunocytochemistry in cell cultures with polyclonal antibodies and protocol available from the reference laboratory.

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

4.4. Nucleic acid amplification

Although several conventional PCR or quantitative real-time PCR methods have been described for the detection of ranaviruses (Jaramillo *et al.,* 2012; Pallister *et al,* 2007; Stilwell *et al.,* 2018), EHNV can only be detected when these methods are combined with methods that specifically detect EHNV. none has been adequately validated according to OIE guidelines for primary detection of EHNV. However, identification of ranavirus at genus and species level is possible using several published PCR strategies.

Samples can be screened by real-time PCR, but as the assays described are not specific for EHNV, identification of EHNV by conventional PCR and amplicon sequencing must be undertaken on any samples screening positive by real-time PCR. For testing by conventional PCR, two PCR assays using MCP primers are used with amplicon sequencing required to differentiate EHNV from ECV, FV3 and BIV (Marsh *et al*., 2002). Alternatively, PCR of the DNA polymerase gene and neurofilament triplet H1-like protein genes can be used (Holopainen *et al*., 2011) (this method is not described in this chapter).

Samples: virus from cell culture or direct analysis of tissue homogenate.

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each sample should be tested in duplicate.

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked using optical density or by running a gel.

4.4.1. Real-time PCR

The ranavirus real-time screening protocol in use at the OIE Reference Laboratory is based on Pallister *et al.*, 2007. Alternative real-time PCR assays can be used according to published protocols for detection of the major capsid protein gene sequence of EHNV and other ranaviruses. The assay described by Jaramillo *et al.* (2012) uses SYBR Green detection chemistry and the assay described by Stilwell *et al.* (2018) detects multiple ranavirus species using hydrolysis probe detection chemistry.

Tissue samples can be homogenised by manual pestle grinding or by bead beating (Rimmer *et al.*, 2012). Commercially available nucleic acid extraction kits (e.g. spin columns, magnetic beads) may be used to extract DNA directly from tissues and from tissue homogenates and cell culture supernatants. Depending on the number of samples to be tested, in the OIE Reference Laboratory, nucleic acids are extracted with either the QIAamp Viral RNA Mini Kit (Qiagen) or MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. A negative extraction control, consisting of extraction reagents only, is included when test samples are extracted.

The ranavirus real-time screening protocol in use at the OIE Reference Laboratory, based on Pallister *et al.*, 2007 is as follows; Template (2 µl) is added to 23 µl reaction mixture containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM for each primer, 250 nM for probe, and molecular grade water. After 1 cycle of 50°C for 2 minutes and 95 °C for 10 minutes, PCR amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

Alternative real-time PCR assays can be used according to published protocols for detection of the major capsid protein gene sequence of EHNV and other ranaviruses. The assay described by Jaramillo *et al.* (2012) uses SYBR Green detection chemistry and the assay described by Stilwell *et al.* (2018) was designed to detect multiple ranavirus species using hydrolysis probe detection chemistry.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Table 4.4.1.1. Ranavirus primer and probe sequences

Primer	**Sequence (5'–3')**	**Reference**
RANA CON F RANA CON R **Probe** RANA CON Pr	5' CTC ATC GTT CTG GCC ATC A 3' 5' TCC CAT CGA GCC GTT CA 3' 5' 6FAM CAC AAC ATT ATC CGC ATC **MGB** 3'	Pallister *et al.*, 2007
Primer		
C1096 C1097	GAC TGA CCA ACG CCA GCC TTA ACG GCG GTG GTG TAC CCA GAG TTG TCG	Jaramillo *et al.*, 2012
Primer		
RanaF1 RanaR1 **Probe** RanaP1	CCA GCC TGG TGT ACG AAA ACA ACT GGG ATG GAG GTG GCA TA 6FAM TGG GAG TCG AGT ACT AC MGB	Stilwell *et al.*, 2018

Primer and probe sequences

Pathogen / target gene	**Primer/probe (5'–3')**	**Concentration**	**Cycling parameters**

Method 1 (Pallister *et al.*, 2007)			
Ranavirus	Fwd: RANA CON: CTC-ATC-GTT-CTG-GCC-ATC-A Rev: RANA CON: TCC-CAT-CGA-GCC-GTT-CA Probe: RANA CON Pr FAM-CAC-AAC-ATT-ATC-CGC-ATC-MGB	900 nM for each primer, 250 nM for probe	45 cycles of 95°C/15 sec; 60°C/60 sec

The ranavirus real-time screening protocol in use at the OIE Reference Laboratory, based on Pallister *et al.*, 2007. Alternative real-time PCR assays can be used according to published protocols for detection of the major capsid protein gene sequence of EHNV and other ranaviruses. The assay described by Jaramillo *et al.* (2012) uses SYBR Green detection chemistry and the assay described by Stilwell *et al.* (2018) detects multiple ranavirus species using hydrolysis probe detection chemistry.

Details of the controls to be run with each assay are set out in Section 5.5. of Chapter 2.2.1. of Section 2.2.

4.4.2. Conventional PCR

PCR and restriction endonuclease analysis (REA): technical procedure

Amplified product from PCR assay MCP-1 digested with PflM I enables differentiation of EHNV and BIV from FV3 and ECV. Amplified product from PCR assay MCP-2 digested with Hinc II, Acc I and Fnu4H I (individually) enables differentiation of EHNV and BIV from each other and from FV3 and ECV.

Preparation of reagents

EHNV-purified DNA and BIV-purified DNA PCR control reagents are supplied by the reference laboratory in freeze-dried form. Reconstitute using 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and allow the vial to stand at RT for 2 minutes. Mix the vial very gently. For routine use, as a PCR control, it is recommended that working stocks be prepared as a 1/10 dilution in TE buffer (pH 8.0). Aliquots of 250 µl should be stored at –20°C. Each aliquot is sufficient for at least 50 reactions (1 to 5 µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1, 321 bp), M153 and M154 (MCP-2, 625 bp) are supplied in working strength (100 ng µl^{-1}) and should be stored at –20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 4.4.2.1.

Table 4.4.2.1. MCP-1 and MCP-2 primer sequences

PCR assay	Primer	Sequence (5'–3')	Product size	Gene location
MCP-1	M151	AAC-CCG-GCT-TTC-GGG-CAG-CA	321 bp	266–586
	M152	CGG-GGC-GGG-GTT-GAT-GAG-AT		
MCP-2	M153	ATG-ACC-GTC-GCC-CTC-ATC-AC	625 bp	842–1466
	M154	CCA-TCG-AGC-CGT-TCA-TGA-TG		

PCR cocktail

Amplification reactions in a final volume of 50 µl (including 5 µl DNA sample) contain 2.5 µl (250 ng) of each working primer, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 5 µl of 10 × PCR buffer (66.6 mM Tris/HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg ml^{-1} BSA, 10 mM beta-mercaptoethanol) and 2 U Taq polymerase. Instructions on preparation of 10 × PCR buffer are included in Table 4.4.2.2.

Table 4.4.2.2. 10 × PCR buffer preparation

~~Ingredients~~	~~Amount~~	~~Final concentration in 50 µl PCR mix~~
~~Tris~~	~~4.050 g~~	~~66.6 mM~~
~~Ammonium sulphate~~	~~1.100 g~~	~~16.6 mM~~
~~BSA (albumin bovine fraction V fatty acid free)~~	~~0.825 g~~	~~1.65 mg ml^{-1}~~
~~Magnesium chloride~~	~~1.25 ml~~	~~2.5 mM~~
~~TE buffer (sterile)~~	~~50 ml~~	

~~NOTE: alternative commercial buffers may also be used.~~

~~Two negative controls are included, one comprising PCR cocktail only and the second containing 5 µl TE buffer.~~

~~The MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; a final extension of 72°C for 5 minutes, and cooling to 4°C.~~

~~NOTE: the annealing temperature may be increased to 60 or 62°C to reduce nonspecific amplification when the assay is used to test fish tissues.~~

~~PCR results are assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. EHNV PCR control DNA (1/10 working stock) should give a result similar in intensity to the 10–3 band in both cases.~~

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

Primer and probe sequences

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Product size MCP-1 is 321 bp and product size MCP-2 is 625 bp			
MCP-1 Gene location: 266-586 MCP-2 Gene location: 842-1466	M151: AAC-CCG-GCT-TTC-GGG-CAG-CA M152: CGG-GGC-GGG-GTT-GAT-GAG-AT M153: ATG-ACC-GTC-GCC-CTC-ATC-AC MI54: CCA-TCG-AGC-CGT-TCA-TGA-TG	250 ng of each primer	35 cycles of 50°C for 30 sec NOTE: the annealing temperature may be increased to 60 or 62°C to reduce non-specific amplification when the assay is used to test fish tissues.

4.4.3. Other nucleic acid amplification methods

Not applicable.

4.5. Amplicon sequencing

~~Amplicons generated using the MCP-1 and/or MCP-2 primers sets can be sequenced. Amplicons should be gel-purified and sequenced using both the forward and reverse primer. Consensus sequence, generated after analysis of the quality of the sequence chromatograms, can then be compared to reference sequences, for example by BlastN search of the NCBI database.~~

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published sequences.

4.6. *In-situ* hybridisation

Not applicable

4.7. Immunohistochemistry

Immunohistochemistry (immunoperoxidase stain)

Samples: formalin-fixed paraffin-embedded tissue sections.

Technical procedure

The following protocol is intended for the qualitative demonstration of EHNV antigens in formalin-fixed paraffin-embedded tissue sections (Reddacliff & Whittington, 1996). It assumes that antigens may have become cross linked and therefore includes a protease digestion step that may be omitted if unfixed samples are examined. A commercial kit (DAKO® LSAB K0679) with peroxidase-labelled streptavidin and a mixture of biotinylated anti-rabbit/anti-mouse/anti-goat immunoglobulins as link antibodies is used for staining. Other commercially supplied reagents are also used. For convenience these are also supplied by DAKO [15]. The primary affinity purified ~~rabbit~~ anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory.

i) Cut 5 µm sections and mount on SuperFrost® Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3). Mark around the section with a diamond pencil to limit the spread of reagents.

ii) Deparaffinise the section:

Preheat slides in a 60°C incubator for 30 minutes.

Place slides in a xylene bath and incubate for 5 minutes. Repeat once. Note that xylene replacements can be used without deleterious effects.

Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.

iii) Expose antigens using a protease treatment. Flood slide with proteinase K (5–7 $\mu g\ ml^{-1}$) and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020). Rinse slide by immersing three times in water. Place in a PBST bath for 5 minutes (PBS pH 7.2, 0.05% [v/v] Tween 20). Tap off the excess wash solution and carefully wipe around the section.

iv) Perform the immunostaining reaction using the Universal DAKO LSAB®+ Kit, Peroxidase (DakoCytomation Cat No. K0679). Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.

v) 3% hydrogen peroxide: cover the section and incubate for 5 minutes. Rinse gently with PBST and place in a fresh wash bath.

vi) Primary antibody (affinity purified rabbit anti-EHNV 1:/1500 Lot No. M708) and negative control reagent (non-immune rabbit serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.

vii) Biotin-labelled secondary link antibody: ~~Link:~~ cover the section and incubate for 15 minutes. Rinse slides.

viii) Streptavidin peroxidase: cover the section and incubate for 15 minutes. Rinse slides.

ix) Substrate–chromogen solution: cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.

x) Counterstain by placing slides in a bath of DAKO® Mayer's Haematoxylin for 1 minute (Lillie's Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.

xi) Mount and cover-slip samples with an aqueous-based mounting medium (DAKO® Faramount Aqueous Mounting Medium Cat. No. S3025).

15 Dako Cytomation California Inc., 6392 via Real, Carpinteria, CA 93013, USA, Tel.: (+1-805) 566 6655, Fax: (+1-805) 566 6688; Dako Cytomation Pty Ltd, Unit 4, 13 Lord Street, Botany, NSW 2019, Australia, Fax: (+61-2) 9316 4773; Visit http://www.dakosytomahon.com for links to other countries.

Interpretation of results

EHNV antigen appears as a brown stain in the areas surrounding degenerate and necrotic areas in parenchymal areas. There should be no staining with negative control rabbit serum on the same section.

Availability of test and reagents: antibody reagents and test protocols are available from the OIE Reference Laboratory.

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

An antigen ELISA for detection of EHNV and an EHNV antibody detection ELISA have been described (Whittington & Steiner, 1993). The same antibodies are suitable for immunohistochemistry on fixed tissues and for detection of ranavirus antigen in cell culture. Reagents and protocols are available from the reference laboratory. It should be noted that polyclonal antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses except Santee Cooper ranaviruses (Ahne *et al.,* 1998; Cinkova *et al.,* 2010; Hedrick *et al*., 1992; Hyatt *et al*., 2000).

4.10. Other methods

Neutralising antibodies have not been detected in fish or mammals exposed to EHNV. Indirect ELISA for detection of antibodies induced following exposure to EHNV has been described for rainbow trout and European perch (Whittington *et al*., 1994; 1999; Whittington & Reddacliff, 1995). The sensitivity and specificity of these assays in relation to a standard test are not known and interpretation of results is difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the most appropriate method of screening healthy fish populations for EHNV; however, the available methods are not specific for EHNV. Any real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish EHNV from other ranaviruses.

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 a suspect and confirmed case 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, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status [16]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. ~~Geographic~~ Hydrographical proximity to, or movement of animals or animal products or

[16] For example transboundary commodities.

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

i) EHNV-typical CPE in cell culture ~~Positive result for EHNV based on virus isolation in cell cultures~~

ii) Positive real-time or conventional PCR result

iii) Positive EHNV antigen ELISA

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with EHNV is considered to be confirmed if at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon;

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR and sequence analysis of the amplicon.

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

i) Histopathology consistent with EHNV;

ii) EHNV-typical CPE in cell cultures;

iii) Positive real-time or conventional PCR result.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with EHNV is considered to be confirmed if, in addition to the criteria in Section 6.2.1, at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon;

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR and sequence analysis of the amplicon.

~~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 EHNV are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with EHNV, 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
Real-time PCR	Diagnosis	Clinically diseased fish (multiple species) from disease outbreaks and experimental infections	Pool of kidney, liver and spleen from individual fish	European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*)	94.3%* (n= 105)	100% (n= 441)	Virus isolation in BF-2 cell culture	Jaramillo *et al.*, (2012)
Real-time PCR	Diagnosis	Clinically diseased fish (multiple species) from disease outbreaks and experimental infections	Pool of kidney, liver and spleen from individual fish	European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*)	95%* (n=106)	100% (n=80)	Virus isolation in BF-2 cell culture	Stilwell *et al.*, 2018

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study; PCR: = polymerase chain reaction. Note: these assays detect multiple ranaviruses in addition to EHNV that infect amphibian hosts. *A positive result requires characterisation using sequencing to confirm that the result indicates the presence of EHNV.

6.3.2. For surveillance of apparently healthy animals: not available

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

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*
* *

NB: There is an OIE Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV)
(please consult the OIE web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on infection with EHNV.
The OIE Reference Laboratory can supply purified EHNV DNA, heat killed EHNV antigen
and polyclonal antibodies against EHNV together with technical methods.
A fee is charged for the reagents to cover the costs of operating the laboratory.

NB: FIRST ADOPTED IN 1995 AS EPIZOOTIC HAEMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2018.

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[...]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

Family	Scientific name	Common name
Cyprinidae	*Abramis brama*	Bream
	Aristichthys nobilis	Bighead carp
	Carassius auratus	Goldfish
	Ctenopharyngodon idella	Grass carp
	Cyprinus carpio	Common carp (all varieties and subspecies)
	Danio rerio	Zebrafish
	Notemigonus crysoleucas	Golden shiner
	Pimephales promelas	Fathead minnow
	Percocypris pingi	Jinsha bass carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	Roach
Siluridae	*Silurus glanis*	Wels catfish

[...]

CHAPTER 2.4.2.

INFECTION WITH *BONAMIA EXITIOSA*

[...]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia exitiosa* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: Argentinean flat oyster (*Ostrea puelchana*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*), Olympia oyster (*Ostrea lurida*) and Suminoe oyster (*Magallana (syn. Crassostrea)* *ariakensis*).

2.2.1. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *B. exitiosa* according to Chapter 1.5 of the *Aquatic Code* are: dwarf oyster (*Ostrea stentina*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Pacific cupped oyster (*Magallana [syn. Crassostrea]* *gigas*) and Sydney rock oyster (*Saccostrea glomerata*).

[...]

CHAPTER 2.4.3.

INFECTION WITH *BONAMIA OSTREAE*

[. . .]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia ostrea* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: European flat oyster (*Ostrea edulis*), Chilean flat oyster (*Ostrea chilensis*), and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*).

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 infection with *B. ostreae* according to Chapter 1.5 of the *Aquatic Code* are: Argentinean flat oyster (*Ostrea puelchana*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: beadlet anemone (*Actina equina*), brittle star (*Ophiothrix fragilis*), European sea squirt (*Ascidiella aspersa*), grouped zooplankton and Pacific cupped oyster (*Magallana* [syn. *Crassostrea*] *gigas*).

[...]

Annex 30. Item 7.3.2. – Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*

CHAPTER 2.4.4.

INFECTION WITH *MARTEILIA REFRINGENS*

[...]

2.2. Host factors

2.2.1. Susceptible host species

~~*Oyster* species: *Ostrea edulis* (Grizel *et al*., 1974); and mussel species: *Mytilus* species including *M. edulis* (Le Roux *et al*., 2001) and *M. galloprovincialis* (López-Flores *et al*., 2004; Novoa *et al*., 2005; Robledo *et al*., 1995a; Villalba *et al*., 1993b).~~

~~Infection with *M. refringens* was demonstrated in the oyster *Ostrea stentina*, the clam species *Solen marginatus* (López-Flores *et al*., 2008a) and *Chamelea gallina* (López-Flores *et al*., 2008b) and the mussel *Xenostrobus securis* (Pascual *et al*., 2010).~~

~~Other *Ostrea* species including *O. chilensis, O. puelchana, O. angasi*, and *O. denselamellosa* were found to be infected with *Marteilia* sp. when deployed in an infected area (Berthe *et al*., 2004; Martin, 1993). However, in these cases, the parasite identification was not done at the molecular level.~~

~~In addition, different stages, including mature stages, of parasites looking like *M. refringens*, were observed by histology in cockles (*Cerastoderma edule*), clam species (*Ruditapes decussatus R. philippinarum*, *Tapes rhomboides*, *T. pullastra*, *Ensis minor, E. siliqua*), and oysters (*Crassostrea virginica*) among other bivalve species (Berthe *et al*., 2004; López-Flores *et al*., 2008b). In all these cases, parasite identification is uncertain.~~

~~Lastly, the copepod *Paracartia grani* was shown to be susceptible to *M. refringens* and this species could participate in the transmission of the parasites between bivalves (see 2.3.1)~~

Species that fulfil the criteria for listing as susceptible to infection with *Marteilia refringens* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: blue mussel (*Mytilus edulis*), dwarf oyster (*Ostrea stentina*), European flat oyster (*Ostrea edulis*), European razor clam (*Solen marginatus*), golden mussel (*Xenostrobus securis*), Mediterranean mussel (*Mytilus galloprovincialis*) and striped venus (*Chamelea gallina*).

Additionally, a copepod species (*Paracartia grani*) has been found to meet the criteria for listing as susceptible to infection with *Marteilia refringens* and is considered an intermediate host.

2.2.2. ~~Susceptible stages of the host~~ Species with incomplete evidence for susceptibility

~~Juveniles and older life stages are known to be susceptible (Grizel, 1985).~~

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *M.refringens* according to Chapter 1.5. of the *Aquatic Code* are: Chilean flat oyster (*Ostrea chilensis*), a copepod (*Paracartia latisetosa*) and Japanese flat oyster (*Ostrea denselamellosa*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Cortez oyster (*Crassostrea corteziensis*), grooved carpet shell (*Ruditapes decussatus*), Pacific cupped oyster (*Magallana* [syn. *Crassostrea]* *gigas*) and zooplankton (*Acartia discaudata, Centropages typicus*, *Euterpina acutifrons*, unidentified *Oithona sp*., *Penilia avirostris*).

[...]

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