Report of the Meeting of WOAH Aquatic Animal Health Standards Commission

Original: English (EN)

19 January and
15 to 22 February 2023
Hybrid

Introduction and Member contribution

This report presents the work of the Aquatic Animal Health Standards Commission (hereinafter "the Aquatic Animals Commission") who met on the 19 January, virtually, and in Paris, France from 15 to 22 February, 2023.

The WOAH 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 September 2022 report: Australia, Brazil, Canada, Chile, China (People's Republic of), Chinese Taipei, Germany, Ireland, Japan, New Zealand, Norway, Slovenia, Spain, Sweden, Switzerland, Thailand, the United Kingdom (UK), the United States of America (USA), Members of WOAH Americas Region, 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.

Annexes

Texts in Annexes 4 to 12 and 22 to 33 will be proposed for adoption at the 90th General Session in May 2023.

Texts in Annexes 13 to 15, 17 to 21 and 34 to 38 are presented for comment.

How to submit comments

The Aquatic Animals Commission strongly encourages Members and International Organisations with a Cooperative Agreement with WOAH to participate in the development of WOAH International Standards by submitting comments on relevant annexes of this report. All comments should be submitted to WOAH through the WOAH Delegates or from Organisations with which WOAH has a Cooperative Agreement.

The Commission wished to draw the attention of Members to those instances where an *ad hoc* Group has addressed a specific topic at the request of the Aquatic Animals Commission. In such cases, Members are encouraged to review these reports together with the report of the Commission. *Ad hoc* Group reports are no longer annexed to the Commission's report. Instead, they are available on the dedicated webpages on the WOAH website, e.g., *ad hoc* Group reports:

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

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

World Organisation for Animal Health
Founded as OIE

Standards Department
[ACC.Secretariat@woah.org]

12, rue de Prony
75017 Paris, France

T. +33 (0)1 44 15 18 88
F. +33 (0)1 42 67 09 87
woah@woah.org
www.woah.org

Comments should be presented within 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 '<u>double underline</u>'. 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 Secretariat by 3 July 2023 to be considered at the September 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 Aquatic Animals Commission noted the dates for its next meeting: 13 to 20 September 2023.

Table of Contents

1. Welcome 6

1.1. Deputy Director General, International Standards and Science 6

1.2. WOAH Director General 6

1.3. Updates from WOAH Headquarters 7

1.3.1. WOAH Specialist Commission reports 7

1.3.2. Pre-General Session 7

1.3.3. Use of the acronym 'WOAH' in the *Aquatic Code* and *Aquatic Manual* 7

2. Adoption of the agenda 8

3. Cooperation with Terrestrial Animal Health Standards Commission 8

4. Cooperation with Biological Standards Commission 9

4.1. Reference Centres: discussion on annual report templates and the use of data collected 9

4.2. Aquatic and Terrestrial Manuals: areas of common interest 9

4.2.1. Aquatic Animals Commission's table on polymerase chain reaction (PCR) parameters for consideration by the Biological Standards Commission 9

4.2.2. Updated *Terrestrial Manual* validation chapter 9

4.2.3. Addition of a new section to the disease-specific chapters to describe the rationale behind the selection of tests for different purposes given in Table 1 Test methods available and their purpose and an explanation for their score 9

4.2.4. Development of a template for validation reports for tests in the *Terrestrial Manual* 10

4.3. Work on the list of WOAH approved reference reagents 10

5. Work plan and priorities 10

6. Aquatic Animal Health Strategy 11

The WOAH Aquatic Animal Health Code 11

7. Texts that will be proposed for adoption in May 2023 11

7.1. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis) 11

7.2. Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus 12

7.3. Article 9.5.2 of Chapter 9.5. Infection with infectious myonecrosis virus 12

7.4. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus 13

7.5. New Chapter 10.X. Infection with tilapia lake virus 13

7.6. 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* 15

7.7. Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens* 16

7.8. Model Articles 11.X.9. - 11.X.14. for mollusc disease-specific chapters 16

8. Items for Member comments 17

8.1. Glossary definitions: 'Aquatic Animal Health Services', 'Competent Authority', and 'Veterinary Authority' 17

8.2. Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information 19
8.3. Article 1.3.1. of Chapter 1.3. Diseases listed by WOAH - Listing of Infection with all genogroups of the virus species infectious spleen and kidney necrosis virus 19
8.4. Safe commodities – Articles X.X.3. for disease-specific chapters 22
8.4.1. Articles 8.X.3. for amphibian disease-specific chapters 23
8.4.2. Articles 9.X.3. for crustacean disease-specific chapters 23
8.4.3. Articles 10.X.3. for fish disease-specific chapters 23
8.4.4. Articles 11.X.3. for mollusc disease-specific chapters 24
8.5. Article 11.5.1. and 11.5.2. of Chapter 11.5. Infection with *Perkinsus marinus* 24
9. Items for Member Information 25
9.1. Emerging diseases 25
9.1.1. Infection with carp edema virus 25
9.1.2. Covert mortality nodavirus (CMNV) 26
The WOAH Manual of Diagnostic Tests for Aquatic Animals 27
10. Texts that will be proposed for adoption in May 2023 30
10.1. Section 2.2. Disease of Crustaceans 30
10.1.1. Chapter 2.2.1. Acute hepatopancreatic necrosis disease 30
10.1.2. Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis) 31
10.1.3. Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus 32
10.1.4. Chapter 2.2.5. Infection with infectious myonecrosis virus 34
10.1.5. Chapter 2.2.7. Infection with Taura syndrome virus 35
10.1.6. Chapter 2.2.8. Infection with white spot syndrome virus 36
10.2. Section 2.3. Diseases of fish 38
10.2.1. Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome) 38
10.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus 39
10.2.3. Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus 41
10.3. Section 2.4. Diseases of molluscs 41
10.3.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa and* Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae* 41
10.3.2. Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens* 42
11. Items for Member comments 43
11.1. Section 2.2. Diseases of crustaceans 43
11.1.1. Chapter 2.2.0. General information: diseases of crustaceans 43
11.1.2. Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague) 44
11.1.3. Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease) 46
11.1.4. Chapter 2.2.9. Infection with yellow head virus genotype 1 46
11.2. Section 2.4. Diseases of molluscs 47
11.2.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *Perkinsus marinus* 47

11.3. Develop a mechanism to speed up the process of making updates to diagnostic methods in the *Aquatic Manual* available to Members quicker 48
12. *Ad hoc* Groups 48
12.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases 48
12.2. *Ad hoc* Group on Susceptibility of fish species to infection with WOAH listed diseases 48
12.3. *Ad hoc* Group on Susceptibility of crustacean species to infection with WOAH listed diseases .. 49
13. Reference centres or change of experts 49
13.1. Evaluation of applications for Reference Centres for aquatic animal health issues or change of experts 49
14. Any other business 50
14.1. Registration of Diagnostic Kits 50
14.1.1. WOAH Registry of Diagnostic kits 50
14.1.2. Innocreate Bioscience WSSV RP Rapid Test Kit 50
Annex 1. Item 2 – Adopted Agenda 51
Annex 2. Item 2 – List of Participants 55

List of Annexes

Annex 1. Item 2 – Adopted Agenda 51
Annex 2. Item 2 – List of Participants 55

1. Welcome

1.1. Deputy Director General, International Standards and Science

Dr Montserrat Arroyo, WOAH Deputy Director General, International Standards and Science (DDG ISS), 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 informed the Commission that the selection process for experts seeking nomination for election to WOAH Specialist Commissions will start with the Call for experts in July 2023 and that the elections will take place during the 91st General Session in May 2024. More information will be provided to the Delegates in due course.

Dr Arroyo informed the Commission that the 90th General Session will be held in a physical format only. She noted that this year, the programme will include an 'Animal Health Forum' with a focus on avian influenza that is aimed at promoting discussion among Delegates on this important global animal health issue.

Dr Arroyo informed the Commission that pre-General Session Commission webinars will be held again this year, as has been done in recent years. The Commission President, Dr Ernst, will deliver a presentation on the 20 April 2023 at 12:00-2:00 (CEST) on the new and revised chapters of the *Aquatic Code* and *Aquatic Manual* that will be presented for adoption. The webinar will have simultaneous interpretation into French and Spanish and will be recorded and uploaded onto the WOAH website.

Dr Arroyo informed the Commission that the new WOAH acronym will be applied in the 2023 versions of the *Aquatic Code* and *Aquatic Manual*.

Dr Arroyo provided an update on the progress and soon-to-be-published call for tenders to implement a new online navigation tool for WOAH Standards, and the work to promote the transparency of comments.

Dr Arroyo commended the Commission on the update to the Safe Commodities Assessments and that she anticipated Members will appreciate this additional guidance. Dr Arroyo informed the Commission that WOAH supports the use of consultants for specific technical pieces of work and that this practice should be maintained in the future.

Dr Arroyo acknowledged the improved harmonisation between Specialist Commissions, demonstrated by the Aquatic Animals Commission and Biological Standards Commission Bureau meeting and the increased coordination on harmonised items in the workplan with the Terrestrial Animal Health Standards Commission.

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 16 February and thanked its members for their support and commitment to achieving WOAH objectives.

Dr Eloit highlighted the relevance of the recently published UN Environment Program (UNEP) report, 'Bracing for Superbugs: Strengthening environmental action in the One Health response to antimicrobial resistance', to the Commission's workplan and the Aquatic Animal Health Strategy Activities, in relation to prudent and responsible use of antimicrobial agents. She highlighted that as a Quadripartite member, WOAH should ensure that it investigates how to address the recommendations outlined in the report, where relevant.

Dr Eloit updated the Commission on the progress of the review of the WOAH Science System and the evaluation of the benchmarking against other international organisations. Dr Eliot assured the Commission that she would keep them informed as the process progresses.

Dr Eloit highlighted the recently published WOAH Observatory annual report and indicated that it will help Members understand how the Observatory programme provides insight into the implementation of WOAH standards. The report contains recommendations for both WOAH and its Members to support the improvement of implementation of the standards. The Commission recognised the significant amount of information contained in the report and expressed interest in how the findings and recommendations will inform WOAH strategies. The Commission thanked Dr Eloit for these updates.

1.3. Updates from WOAH Headquarters

1.3.1. WOAH Specialist Commission reports

The Secretariats of the WOAH Specialist Commissions are always looking to improve the efficiency of the production and publication of their respective Specialist Commission reports whilst ensuring alignment, as relevant. The DDG ISS considered the proposals made by the Secretariat and agreed with the following changes to the publication of the Commission reports starting in February 2023:

1. All Specialist Commission reports will revert to a single report per Commission. (Note: the Scientific Commission for Animal Diseases report has always been produced as a single report);
2. Unofficial reports in English will no longer be published;
3. Specialist Commission reports will be published on the Delegates website (in Word format for Aquatic Animals Commission and Terrestrial Animal Health Standards Commission and PDF for Biological Standards Commission and Scientific Commission for Animal Diseases) and on the public website (all in PDF format) per language (i.e. English, French and Spanish) once final. A gap between the publication of the English version and the French and Spanish versions is unavoidable because the WOAH working language is English. However, WOAH endeavours to keep this period to a minimum;
4. The four Specialist Commission reports will be published in English at least two weeks prior to the pre-GS webinars.

1.3.2. Pre-General Session

1. Pre-General Session information webinars will be held every year for the Aquatic Animals Commission, Biological Standards Commission and Terrestrial Animal Health Standard Commission (with support from the Scientific Commission for Animal Diseases), in one time-zone only and recorded and uploaded onto the General Session website. These webinars will be presented by the President of the respective Commission and will focus on presenting information about new or revised standards that will be proposed for adoption at the General Session.

   NOTE: 2023 dates are: Biological Standards Commission - 18 April 2023; Terrestrial Animal Health Standards Commission - 19 April 2023; Aquatic Animals Commission - 20 April 2023. All webinars will be held between 12:00-2:00 pm CEST.

2. WOAH will no longer provide a mechanism for Members to submit pre-General Session positions, as was the case in 2021 and 2022 when General Sessions were held in a virtual or hybrid format. However, if Members wish to unofficially send pre-GS positions to assist the Presidents of the Specialist Commissions prepare their General Session reports, this can be done through email to the relevant Secretariat.

1.3.3. Use of the acronym 'WOAH' in the *Aquatic Code* and *Aquatic Manual*

Background

At the 89th General Session in May 2022, the World Assembly of Delegates adopted Resolution No. 10, recognising that the acronym OIE will be replaced by WOAH (and OMSA for French and Spanish) as part of a rebranding of the Organisation.

At the September 2022 meetings, the Specialist Commissions were informed by the WOAH DDG ISS that the new acronym would be introduced into WOAH Standards to replace OIE. The Commissions were informed that the relevant Secretariat would present an analysis and proposal to each specific Commission at its February 2023 meetings.

In addition, prior to its February 2023 meeting, the Aquatic Animals Commission received comments from several Members requesting to use the acronym 'WOAH' instead of 'OIE'.

February 2023 meeting

The Aquatic Animals Commission considered an analysis prepared by the Secretariat on the use of the acronym 'OIE' in the current edition of the *Aquatic Code* and discussed a proposed approach to replace OIE by WOAH. The Commission was informed that the Secretariats for the Specialist Commissions had worked collectively to ensure this amendment would be conducted in a consistent manner across all WOAH International Standards (i.e., the *Terrestrial Code*, the *Terrestrial Manual*, the *Aquatic Code*, and the *Aquatic Manual*).

The Commission agreed that the 'WOAH list' and 'listed diseases' (defined term in the Glossary) replace 'OIE list' and 'OIE listed diseases', respectively throughout the *Aquatic Code* and the *Aquatic Manual*. It also agreed that the title of Chapter 1.3. of the *Aquatic Code* be amended to 'Diseases listed by WOAH'.

The Commission noted that the term ''World Assembly of OIE Delegates' and 'World Assembly of Delegates' are both used in the *Aquatic Code*. It was agreed that only the 'World Assembly of Delegates' be used for consistency.

The Commission noted that the term 'OIE Organic Statutes' is referred to in the User's Guide and Chapter 1.1. of the *Aquatic Code*. It was agreed to replace this wording with 'Organic Statutes of the Office International des Epizooties' which is the formal title of the legal document.

The Commission agreed that in all other cases in both the *Aquatic Code* and *Aquatic Manual,* 'the OIE' will be replaced by 'WOAH' (or 'the WOAH' following WOAH's internal re-branding guidelines).

The Commission agreed that these amendments are editorial in nature and do not impact interpretation of standards of the *Aquatic Code* or the *Aquatic Manual*.

The Commission agreed to the proposal from the DG-ISS that these amendments be applied in the 2023 edition of the *Aquatic Code* and the *Aquatic Manual*.

The Commission wished to note that these changes, as relevant, have been made in all Annexes circulated in this report as silent changes, i.e. without strikeout/double underline as they are considered editorial.

2. Adoption of the agenda

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

3. Cooperation with Terrestrial Animal Health Standards Commission

The Secretariat of the Code Commission updated the Aquatic Animals Commission on progress made on the items that had been identified as of interest at a meeting of the Bureaus of the Code Commission and the Aquatic Animals Commission held in September 2022.

The Aquatic Animals Commission was informed that the Code Commission had reviewed the usage of the terms 'Veterinary Services', 'Competent Authority' and 'Veterinary Authority', throughout the *Terrestrial Code* and circulated them for comment in its February 2023 report.

The Aquatic Animals Commission was updated on the Code Commission's work on the revision of Chapters 5.4. to 5.7. and Chapter 6.10., and requested updates at future Commission meetings given the importance of ensuring alignment, as relevant, in the corresponding chapters in the *Aquatic Code*.

4. Cooperation with Biological Standards Commission

The Bureaus of the BSC and AAC met on 8 February, 2023 to discuss areas of common interest which are reported below.

4.1. Reference Centres: discussion on annual report templates and the use of data collected

At the Bureaus meeting, the two Bureaus discussed the updated annual report template used by the Reference Centres with the aim of improving the questions asked to receive clearer responses and improve the quality of the data collected. They agreed that the revised template, although an improvement, could be further improved by determining what outputs could be derived from the data collected, noting that such improvements might also provide benefits to the Reference Centres filling in the reports. The Bureaus agreed that in advance of the September 2023 meeting, the Biological Standards Commission will send a questionnaire to all the Reference Laboratories that includes questions on the usefulness of the annual report.

4.2. Aquatic and Terrestrial Manuals: areas of common interest

4.2.1. Aquatic Animals Commission's table on polymerase chain reaction (PCR) parameters for consideration by the Biological Standards Commission

The Bureau of the Biological Standards Commission was informed that the Aquatic Animals Commission had developed a table on PCR primer and probe sequences and cycling parameters so that critical information on PCR methods is presented in a uniform way in all the chapters of the *Aquatic Manual*. The Bureau of the Biological Standards Commission commented that presenting PCR parameters in tabular format is extremely useful and agreed to adopt this technique in the *Terrestrial Manual* chapters.

4.2.2. Updated *Terrestrial Manual* validation chapter

The Bureau of the Biological Standards Commission informed the Aquatic Animals Commission that Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases*,* of the *Terrestrial Manual* had been extensively revised and would be proposed for adoption at the General Session in May 2023. The Aquatic Animals Commission noted that the *Aquatic Manual* includes a similar chapter and that review of this chapter in the *Terrestrial Manual* could have implications for Chapter 1.1.2. Principals and method of validation of diagnostic assays for infectious diseases, of the *Aquatic Manual.* The Aquatic Animal Commission agreed to add revision of Chapter 1.1.2 to its forward work plan, and noted that it will take into account the revised *Terrestrial Manual* chapter, and provide any relevant feedback to the Biological Commission regarding Chapter 1.1.6. of the *Terrestrial Manual*.

4.2.3. Addition of a new section to the disease-specific chapters to describe the rationale behind the selection of tests for different purposes given in Table 1 Test methods available and their purpose and an explanation for their score

The Bureau of the Biological Standards Commission informed the Bureau of the Aquatic Animals Commission that it is working to add a new section to the disease-specific chapters of the *Terrestrial Manual* to describe the rationale behind the selection of tests for different purposes given in Table 1 *Test methods available and their purpose* and an explanation of their score. This will address

queries received from Members and provide justification for different tests. The work is in a pilot stage and the format needs to be finalised to give flexibility for the experts providing the justification.

The Bureau of the Aquatic Animals Commission noted that both Commissions are working to achieve similar outcomes by providing additional information on the fitness for purpose of particular assays. The Bureau of the Aquatic Animals Commission noted that is has a different approach in the *Aquatic Manual* Table 4.1. *WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*, which includes life stage, validation level and rating against purpose of use.

The Bureau of the Biological Standards Commission agreed that it would consider this approach as part of its work.

4.2.4. Development of a template for validation reports for tests in the *Terrestrial Manual*

The Bureau of the Biological Standards Commission informed the Bureau of the Aquatic Animals Commission that it had developed a template for the validation data for tests recommended in the *Terrestrial Manual*. Reference Laboratories would be invited to fill in the 'validation report' form, which would be made available in a repository on the website for anyone seeking the validation data available for the test. As a first step in a pilot scheme to test the template's suitability and usability, the document was shared with selected WOAH Reference Laboratories to complete and to provide their feedback.

The Bureau of the Aquatic Animals Commission reported that it has received comments from Reference Laboratories about the time it takes for new or changed methods to be included in the *Aquatic Manual* because the methods or validation information must be published in peer-reviewed articles. The Bureau of the Aquatic Animals Commission considered that the template developed by Biological Standards Commission has value in expediting the inclusion of new or changed assays in some scenarios and agreed to review it and provide feedback.

4.3. Work on the list of WOAH approved reference reagents

The Biological Standards Commission has a list of WOAH-approved International Standard Reagents available online and are planning to expand the list (https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-4).

Both Commissions considered this meeting to be very useful to identify and discuss areas of harmonisation.

5. Work plan and priorities

Comments were received from Australia, Canada, China (People's Rep. of), Norway, the UK, the USA, Members of the WOAH Americas Region, and the EU.

The Aquatic Animals Commission reviewed comments received and noted the support for the development of the new chapters in the workplan. The Commission considered progress to date on each of the four new draft chapters (Chapter 4.X. Emergency disease preparedness, Chapter 4.Y. Disease outbreak management, Chapter 5.X. Ornamental aquatic animals, Chapter 5.Y. Trade of Genetic Materials) and agreed to continue to progress work on all four chapters with the intent to present the four draft new chapters for comment in its September 2023 report.

The Commission agreed with a comment emphasising that, with regard to the work to revise *Terrestrial Code* Chapters 5.4. to 5.7., that an aligned approach should be taken with the Code Commission and explained that this work would be dealt with in liaison with the Code Commission.

The Commission agreed with a comment emphasising the need to ensure alignment of the review of usage of glossary definitions 'Aquatic Animal Health Services', 'Competent Authority', 'Veterinary Authority' and 'Veterinary Services' with the Code Commission and noted that the proposed changes in usage in the *Aquatic*

Code and *Terrestrial Code* have been aligned and will be presented for Member comment in both the Aquatic Animals Commission and Code Commission February 2023 reports (see item 8.1.)

The Commission reviewed responses on the questionnaire for the revision of Chapter 4.3. Application of Compartmentalisation, and thanked Members for their insights and experiences in the application of the standards on compartmentalisation. The Commission considered progress to date on the discussion paper on compartmentalisation and agreed to continue to progress work on the discussion paper with the intent to present it for comment in its September 2023 meeting report.

The Commission agreed that review and revision of Chapter 3.1. Quality of Aquatic Animal Health Services, should be considered in the Commission's forward work plan, to align with the corresponding chapter in the *Terrestrial Code* that was revised and adopted in 2022. The Commission agreed that the review and revision of Chapter 3.2. Communication, should also be considered for addition to the workplan, noting that it is not very clear and needs updating. These would need to be considered in the workplan of the next Commission, commencing from June 2024.

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 2023. The list of current and planned *ad hoc* Groups for 2023 are available on the WOAH website.

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

6. Aquatic Animal Health Strategy

The Coordinator of the Aquatic Animal Health Strategy provided an update on implementation of the Strategy. The Commission was informed of the key milestones and achievements over the last 12 months, the current status of activities and key priorities for 2023. The Strategy was launched in 2021 and 16 of the 23 activities are now underway. An annual workplan will be developed for 2023 to identify priorities and resource requirements, define timelines and assess possible obstacles to their successful implementation. This will ensure that the Strategy remains relevant and that its identified priorities are being realised. Achievements over the past year include dedicated resources for the Strategy, and progress on activities under all four of the strategy's objective.

The WOAH Aquatic Animal Health Code

7. Texts that will be proposed for adoption in May 2023

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

Comments were received from the UK and the EU.

Background

At its February 2022 meeting, the Aquatic Animals Commission agreed to amend Article 9.3.1. to ensure consistency with the listed disease name used in Article 1.1.3. of Chapter 1.3. Diseases listed by WOAH.

At its September 2022 meeting, the Commission agreed to further amend Article 9.3.1. to reflect the correct taxonomic classification.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 2.1.2., page 6); September 2022 report (Item 5.2., page 7).

February 2023 meeting

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

The revised Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis), is presented as Annex 4 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from Canada, Chinese Taipei, the UK 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 the latest update in the taxonomic classification of IHHNV and to ensure consistency with other disease-specific chapters.

The Commission also amended Article 9.4.2. to list the susceptible species alphabetically according to common name, to align with the agreed convention to be used for Articles X.X.2. of the *Aquatic Code*

At its September 2022 meeting, the Commission reviewed comments received and did not propose any additional amendments to Articles 9.4.1. and 9.4.2. noting that Members were supportive of the proposed changes.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 2.1.3., page 6); September 2022 report (Item 5.3., page 8).

February 2023 meeting

No comments were received on the revised Article 9.4.1.

The Commission agreed with a comment to amend the list of susceptible species in Article 9.4.2. in accordance with the convention to list susceptible species alphabetically by common name.

The revised Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus, are presented as Annex 5 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from the UK and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission agreed to amend Article 9.5.2. of Chapter 9.5. Infection with infectious myonecrosis virus to list the susceptible species alphabetically according to the common name, to align with the agreed convention to be used for Articles X.X.2. of the *Aquatic Code.*

Previous Commission reports where this item was discussed

September 2022 report (Item 5.4., page 8).

February 2023 meeting

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

The revised Article 9.5.2 of Chapter 9.5. Infection with infectious myonecrosis virus, is presented as Annex 6 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from Canada, China (People's Rep. of), Chinese Taipei, New Zealand, the UK and the EU.

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 WOAH listed diseases, assessments of new or reassessments of existing susceptible species will need to be undertaken. The Commission has encouraged Members to provide any new scientific evidence on susceptibility to the Commission for assessment.

In response to a Member comment providing new evidence on susceptibility, in preparation for its September 2022 meeting, the Commission requested that the *ad hoc* Group on Susceptibility of fish species to WOAH listed diseases assess the susceptibility of Jinsha bass carp (*Percocypris pingi*) to infection with spring viraemia of carp virus (SVCV). The *ad hoc* Group applied the criteria in Chapter 1.5., Criteria for listing species as susceptible to infection with a specific pathogen, for susceptibility of Jinsha bass carp (*P. pingi*) to infection with SVCV.

At its September 2022 meeting, the Commission reviewed the assessment undertaken by the *ad hoc* Group and agreed to include Jinsha bass carp (*P. pingi*) in the list of susceptible species in Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus.

Previous Commission reports where this item was discussed

September 2022 report (Item 5.5., page 8).

February 2023 meeting

The Commission agreed with a comment that the English common name for *P. pingi* should be 'Jingsha barbel carp' rather than 'Jingsha bass carp' as the taxonomy of the species indicates the species is a barbel carp and revised the common name for *P. pingi* in Article 10.9.2.

The Commission noted that in Article 10.9.2. the common names for the susceptible species did not follow the convention used in the *Aquatic Code;* to only use a capital letter for proper nouns. The Commission agreed to amend, where relevant, in this article as well as other instances where this occurs in the 2023 version of the *Aquatic Code*.

The revised Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus, is presented as Annex 7 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from China (People's Republic of), New Zealand, the UK, the USA and the EU.

Background

At its September 2022 meeting, the Commission reviewed the new draft Chapter 10.X. Infection with tilapia lake virus (TiLV), and agreed to place the susceptible species in Article 10.X.2. 'under study' pending assessments 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 1. a) 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 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.

Previous Commission reports where this item was discussed

September 2022 report (Item 5.6., page 9).

February 2023 meeting

Article 10.X.3.

The Commission agreed to amend the recommended time/temperature treatment required for the inactivation of infection with TiLV and to remove '(under study)' at the end of point 2, to align with the newly updated safe commodity assessments (see item 8.4.).

Article 10.X.5.

In the first sentence, the Commission did not agree to change 'within' to 'between' as countries and zones are plural within the sentence.

The Commission considered a request for clarity on how six months was chosen as the default period for basic biosecurity conditions, in point 1. of Article 10.X.5. and point 1. of Article 10.X.6. The Commission noted that the default periods for basic biosecurity conditions and targeted surveillance presented in Chapter 1.4., were applied for all WOAH listed diseases, including infection with TiLV. The default periods would apply until an assessment of the periods for basic biosecurity conditions and targeted surveillance against the criteria in Chapter 1.4. could be completed for each listed disease. The Commission noted that it had requested expert advice to undertake these assessments and once reviewed by the Commission, changes will be proposed to disease-specific chapters, where applicable.

In point 3., the Commission agreed with a comment that the wording could be considered ambiguous and added 'and have been in place' after 'continuously met' for clarity. The Commission also agreed to add this wording to point 3. of Article 10.X.6. and point 1. of Article 10.X.7. to ensure consistency throughout the chapter. The Commission noted that as these articles are harmonised across all disease-specific chapters, these changes once adopted, will be applied to all disease-specific chapters.

In point 4. b), the Commission did not agree with a comment to add 'aquaculture establishments' after 'infected populations' as eradication of all infected populations would be required in order to re-claim freedom at the level of country.

In the final paragraph of point 4., the Commission amended the wording for clarity and to ensure the appropriate reference for actions that should be achieved prior to declaring a new free zone located outside the infected and protected zones. The Commission also agreed to add a new final paragraph with the same wording as point 4. of Article 10.X.5. to ensure consistency between country and zone freedom. The Commission noted that as these articles are harmonised across all disease-specific chapters, these changes once adopted, will be applied to all disease-specific chapters.

Article 10.X.7

In point 2. c), the Commission agreed that the default period for targeted surveillance to return to compartment freedom should better align with Article 1.4.14. of Chapter 1.4. Aquatic animal disease

surveillance. The Commission revised the wording and noted that as these articles are harmonised across all disease-specific chapters, these changes once adopted, will be applied to all disease-specific chapters.

Article 10.X.11. and Article 10.X.14.

The Commission did not agree to add 'If the aquatic animals or aquatic animal products are intended for human consumption only, then additional testing or surveillance should not be required since the pathogen is not zoonotic' after the final sentence in Article 10.X.11. and after the final sentence in point 1. of Article 10.X.14. as the intent of the article is to indicate that pathway controls may need to be considered to prevent disease introduction through unintended use of the aquatic animal product within a country. The Commission also noted that Article 10.X.11. relates to sanitary safety rather than food safety.

In response to a comment, the Commission recognised that it can be difficult to find resources for disinfectants for all listed diseases but noted that the *Aquatic Code* is not the appropriate place to provide such guidance. The Commission referred Members to other resources on disinfection and suggested the Australian Department of Agriculture, Fisheries, and Forestry AQUAVETPLAN, Decontamination Manual as a starting point.

The revised new Chapter 10.X. Infection with tilapia lake virus, is presented as Annex 8 and will be proposed for adoption at the 90th General Session in May 2023.

7.6. 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*

Comments were received from the UK and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission considered the report of the *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases and noted that the *ad hoc* Group had recommended new nomenclature for some *Crassostrea* species.

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*' wherever they are used in the *Aquatic Code* and *Aquatic Manual.*

Previous Commission reports where this item was discussed

Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa* – September 2022 report (Item 5.7., page 9).

Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae* - September 2022 report (Item 5.7., page 9).

February 2023 meeting

The Commission noted that the common name in FAOTERM for *Magallana* [Syn. *Crassostrea*] *ariakensis* was recently changed to Ariake cupped oyster and agreed to amend 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* to reflect this change. The Commission reminded Members that the agreed source for common names of listed susceptible species is FAOTERM.

The Commission also agreed to amend Articles 11.2.2. and 11.3.2. to list the susceptible species alphabetically according to the common name, to align with the agreed convention to be used for Article X.X.2. of the *Aquatic Code.*

The revised Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa,* and revised Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*, are presented as Annex 9 and Annex 10, respectively, and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission considered the report of the *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases who undertook assessments of susceptible species for infection with *Marteilia refringens* against the criteria presented in Chapter 1.5., Criteria for listing species as susceptible to infection with a specific pathogen.

The Commission agreed to amend the list of susceptible species in Article 11.4.2. in line with the recommendations of the *ad hoc* Group, except for the copepod (*Paracartia grani*). The Commission noted that although the copepod (*Paracartia grani*) met the criteria for listing as susceptible to infection with *M. refringens* it agreed not to include it in Article 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens,* of the *Aquatic Code* because the Commission did not consider that this species was relevant to trade in molluscs or mollusc products. However, the Commission did agree 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*.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 4.1., page 17); September 2022 report (Item 5.8., page 10).

February 2023 meeting

The Commission considered a comment requesting that the scope of the chapter clearly include *Marteilia refringens* types O and M. The Commission noted that the chapter applies to both types and agreed to add '(including O and M types)' after 'the pathogenic agent *M. refringens*' in Article 11.4.1. to ensure consistency with Section 1. of Chapter 2.4.4. Infection with *Marteilia refringens,* in the *Aquatic Manual.*

In Article 11.4.2., the Commission agreed with a comment and corrected the common for *Chamelea gallina*, to 'striped venus clam'.

The revised Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*, are presented as Annex 11 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from Canada, New Zealand, the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission noted that some amendments that had been previously made to *Aquatic Code* sections 8, 9 and 10 (diseases of amphibians, crustaceans and fish) had not been systematically applied to the relevant articles of chapters in Section 11 (diseases of molluscs). The Commission therefore agreed to amend Articles 11.X.9. to 11.X.14. with the horizontal changes previously applied to other disease-specific chapters. The Commission agreed that the amendments noted in the model Articles 11.X.9. to 11.X.14. will be applied to all disease-specific chapters in Section 11. once the proposed amendments for 11.X.9. to 11.X.14. are adopted.

Previous Commission reports where this item was discussed

February 2018 report (Item 1.11., page 13); September 2022 report (Item 5.9., page 11).

February 2023 meeting

The Commission noted some additional changes were required in these model articles to ensure alignment between all disease-specific chapters and that upon adoption, all disease-specific chapters would be reviewed to ensure harmonised wording throughout the *Aquatic Code*.

Article 11.X.10.

In point 1. b) of Article 11.X.10., the Commission did not agree with a comment to add 'and disposed of in a biosecure manner in accordance with Chapter 4.8. or' after 'are killed' as this requirement for biosecure disposal is addressed in point 1. c).

The Commission did not agree to add 'receiving' after 'original' in point 1. b) as it was not considered to be an improvement.

Article 11.X.11.

In point 2. or point 3. of Article 11.X.11. and point 2 of Article 11.X.12., the Commission did not agree to add a cross reference to Chapter 4.1. as these points specifically refer to inactivation of pathogens and biosecure disposal, and not overall biosecurity. Chapter 4.1. Biosecurity in aquaculture establishments, was deemed too broad for inclusion as a cross reference within these points.

In response to a comment on point 2. of Article 11.X.11., referred Members to other resources on disinfection (see item 7.5.)

Similarly to Chapter 10.X. Infection with TiLV, the Commission did not agree to add 'If the aquatic animals or aquatic animal products are intended for human consumption only, then additional testing or surveillance should not be required since the pathogen is not zoonotic' after the final sentence in Article 11.X.11., after the final sentence in point 1 and final sentence of point 2., of Article 11.X.14. (see Item 7.5.).

Article 11.X.13.

In Article 11.X.13., in response to a comment about inclusion of guidance for aquatic animal products imported for use in laboratories for use as feed for zoo animals or tissues or cells for research, the Commission agreed that additional amendments were not necessary as this is addressed in Article 11.X.12.

The Commission reminded Members that once adopted, the model Articles 11.X.9. to 11.X.14. will be applied to all disease-specific chapters in Section 11. The revised model Articles 11.X.9. to 11.X.14. are presented as Annex 12 and will be proposed for adoption at the 90th General Session in May 2023.

8. Items for Member comments

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

Background

At the 89th General Session, in May 2022, revised Glossary definitions for 'Aquatic Animal Health Services', 'Competent Authority' and 'Veterinary Authority' in the *Aquatic Code* were adopted. The revised Glossary definitions for 'Competent Authority', 'Veterinary Authority' and 'Veterinary Services' for the *Terrestrial Code* were also adopted in May 2022. The revision of these definitions had been done in coordination with the Terrestrial Animal Health Standards Commission and both Commissions agreed to

coordinate work to revise the use of these definitions in the *Aquatic Code* and *Terrestrial Code*, respectively, to ensure consistency when relevant.

At its September 2022 meeting, the Aquatic Animal Commission and the Terrestrial Animal Health Standards Commission agreed to coordinate work to revise the use of these definitions in the *Aquatic Code* and *Terrestrial Code*, respectively, to ensure consistency, when relevant.

Previous Commission reports where this item was discussed

September 2022 report (Item 6.1., page 12).

February 2023 meeting

At its February 2023 meeting, the Aquatic Animals Commission reviewed every occurrence of 'Aquatic Animal Health Services', 'Competent Authority' and 'Veterinary Authority' in the *Aquatic Code* and agreed to the following:

- To change 'Aquatic Animal Health Services' in some usages to 'Competent Authority' to reflect the appropriate responsibility for oversight of the aquaculture industry including compartments and issuance of international aquatic animal health certificates. For example, in Article 3.1.2. of Chapter 3.1. Quality of Aquatic Animal Health Services, to change 'Aquatic Animal Health Services' to 'Competent Authority' as this usage relates to the issuance of international aquatic animal health certificates. This change ensures consistency throughout the *Aquatic Code* with respect to the responsible authority for issuance of international aquatic animal health certificates.

- To change 'Competent Authority' to 'Veterinary Authority' when it relates to the notification of disease. This differentiated role for a Veterinary Authority, including disease notification requirements and demonstration of compliance with international standards for international trade or for disease free status, was taken into consideration when developing the glossary definition.

- The removal of 'Veterinary Services' throughout the *Aquatic Code* as this term is no longer relevant within the *Aquatic Code*.

- In point B.5. of the User's Guide, the Commission agreed to add 'Aquatic Animal Health Services and the' before 'Competent Authorities' to ensure alignment with the proposed changes in User's Guide of the *Terrestrial Code*.

- Despite the use of some incorrect usages of these definitions in Chapter 3.2. Communication, the Commission agreed not to make any changes as it considered that correcting the usage could not be addressed without thorough revision of the chapter. Consequently, the Commission agreed to add review of Chapter 3.2. to its forward work plan, including the usage of 'Aquatic Animal Health Services' throughout the chapter to reflect the revised definitions, However, in the second paragraph of Article 3.2.1., the Commission agreed to delete 'Communication between the Aquatic Animal Health Services, and Veterinary Services (particularly where Aquatic Animal Health Services are separate, and independent of Veterinary Services) is especially important.' as it agreed that this text was not necessary.

The Aquatic Animals Commission wished to note that the proposed amendments for the use of 'Competent Authority' 'Veterinary Authority' and 'Veterinary Services' in the *Terrestrial Code* are being circulated in the February 2023 report of the Code Commission. The Commission encouraged Members to consider both reports to when commenting given the previous work done to ensure alignment between the two Codes, as relevant.

The proposed amendments to 'Aquatic Animal Health Services', 'Competent Authority' and 'Veterinary Authority' are presented as Annex 13 for comments.

8.2. Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information

Background

At its February 2019 meeting, the Terrestrial Code Commission agreed to remove Article 1.1.5. as it considered that the information was addressed in Chapter 1.6. Procedures for official recognition of animal health status, endorsement of an official control programme, and publication of a self-declaration of animal health status, by WOAH. The amendment of Chapter 1.1. of the *Terrestrial Code,* removing Article 1.1.5., was adopted in May 2021.

February 2023 meeting

The Commission agreed that the requirements included in Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information, were now addressed in the recently revised and adopted Chapter 1.4. Aquatic animal disease surveillance. The Commission agreed to delete Article 1.1.5. to remove duplication within the *Aquatic Code* and to ensure alignment with Chapter 1.1. Notification of diseases and provision of epidemiological information, of the *Terrestrial Code*.

The revised Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information, is presented as Annex 14 for comments.

8.3. Article 1.3.1. of Chapter 1.3. Diseases listed by WOAH - Listing of Infection with all genogroups of the virus species infectious spleen and kidney necrosis virus

Comments were received from Australia, Canada, China (People's Republic of), Chinese Taipei, Japan, Thailand, the UK, the USA, Members of the WOAH Americas Region, the EU, and a joint statement from Brazil, Canada, Chile, New Zealand and the USA.

Background

At its February 2022 meeting, the Aquatic Animals Commission noted that other viruses in the Genus *Megalocytivirus*, in addition to red sea bream iridovirus (RSIV), may cause significant disease in fish. These viruses include two other genogroups of the species infectious spleen and kidney necrosis virus (ISKNV) – the genogroup turbot reddish body iridovirus (TRBIV) and the genogroup ISKNV. The genogroups ISKNV and TRBIV are not included in the scope of Chapter 10.8. Infection with red sea bream iridovirus of the *Aquatic Code*.

The Commission noted that if the genogroups ISKNV and TRBIV were to be listed (in addition to RSIV), the viruses would first need to be assessed against the criteria in Chapter 1.2. Criteria for listing aquatic animal diseases. The Commission requested the *ad hoc* Group to assess the susceptible species for the three genogroups of the virus species ISKNV (i.e. RSIV, ISKNV and TRBIV) to assist in informing an assessment for listing.

At its September 2022 meeting, the Commission considered the *ad hoc* Group's interim report and noted the advice that it was not always possible to distinguish the susceptible species by genogroup (i.e. susceptibility to infection with either RSIV, ISKNV or TRBIV).

The Commission assessed the virus species infectious spleen and kidney necrosis virus (ISKNV species), including its three genogroups RSIV, ISKNV and TRBIV, against criteria in Chapter 1.2. Criteria for listing aquatic animal diseases. The Commission agreed that the species ISKNV, including 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. Consequently, the Commission proposed that the name of the listed disease should be changed to "infection with infectious spleen and kidney necrosis virus (ISKNV)" and would be defined to include the three genogroups of the species ISKNV (i.e. ISKNV, RSIV and TRBIV) but would not include scale drop disease virus (SDDV), the other recognised species of *Megalocytivirus*.

Previous Commission reports where this item was discussed

February 2022 (Part B, Item 3.1.2.3., page 13); September 2022 (Item 5.1., page 7).

February 2023 meeting

The Commission reiterated that the proposal for listing is to amend the name of the listed disease from infection with RSIV to infection with the virus species infectious spleen and kidney necrosis virus (ISKNV). This proposal would maintain the RSIV genogroup as a listed disease and would also include the ISKNV genogroup and the TRBIV genogroup. The Commission noted that while several Members supported the proposed change, a number did not.

The Commission noted that the nomenclature of megalocytiviruses does cause some confusion. This is because "ISKNV" is the name of the virus species recognised by the ICTV and is also the name of one of the three recognised genogroups within the virus species. The Commission reviewed and updated the assessment of infection with the virus species ISKNV against the criteria in Chapter 1.2, Criteria for listing aquatic animal disease, to ensure clarity and consistency of terminology throughout with respect to the viral species ISKNV and the genogroup ISKNV.

The Commission considered comments proposing that the name of the listing be "infection with *Megalocytivirus*". The Commission noted that the genus *Megalocytivirus* includes two recognised species, ISKNV and scale drop disease virus (SDDV). SDDV is not under consideration for listing and so the proposed disease name would be problematic as it would need to be defined to include the species ISKNV but exclude SDDV. The Commission proposed to alter the disease name in Article 1.3.1. to "Infection with all genogroups of the virus species ISKNV" to provide clarity on the scope of the listed disease. This is a similar approach to that used for Infection with infectious salmon anaemia virus which is included in Chapter 1.3. Diseases listed by WOAH, as "Infection with HPR-deleted or HPR0 infectious salmon anaemia virus".

The Commission referred Members to the April and November/December 2022 report of the *ad hoc* Group for susceptibility of fish species to WOAH listed diseases for details of the assessments conducted for susceptibility to infection with ISKNV species. The *ad hoc* Group report outlines the susceptible species for infection with the virus species ISKNV and where possible each genogroup. The *ad hoc* group report will assist Members in determining the scope of application of standards for infection with the virus species ISKNV. The Commission agreed to delay amendments to Article 10.8.2. of Chapter 10.8. Infection with red sea bream iridovirus, based on the recommendations of the *ad hoc* Group report, until the proposed listing of infection with the virus species ISKNV has been considered by members. The *ad hoc* Group report can be found on the WOAH website (https://www.woah.org/en/what-we-do/standards/standard-setting-process/ad-hoc-groups/#ui-id-3)

The Commission reviewed comments received and concluded that the information provided in the assessment of infection with the virus species ISKNV against the criteria in Chapter 1.2., Criteria for listing aquatic animal disease was robust and reiterated that the assessment supported the listing of all genogroups, including RSIV, ISKNV, and TRBIV. The Commission noted that there was strong rationale for this listing at the level of the virus species as the three genogroups have overlapping susceptible species, similar epidemiology, and similar diagnostic methods, as was highlighted in the *ad hoc* group report.

The Commission noted that most of the comments received centred on three points: the widespread nature of the ISKNV genogroup, validation of diagnostic tests for the TRBIV genogroup and the merits of listing. The Commission agreed to respond collectively to these three points rather than to individual comments.

1) The widespread nature of the ISKNV genogroup

Several comments questioned how Criterion No. 2. could be met considering the widespread nature of the ISKNV genogroup, especially in ornamental fish. The Commission noted that Criterion

2 requires that at least one country may be able to declare country or zone freedom based on the provisions of Chapter 1.4. The Commission noted that some countries have basic biosecurity conditions in place for all genogroups of the virus species ISKNV and have conducted targeted surveillance. The Commission agreed that it was confident that at least one Member would be able to claim freedom from ISKNV species.

The Commission noted some comments that without listing (and availability of the associated WOAH standards), the design of existing surveillance programs may not address all known susceptible species and the true geographic distribution of the disease being assessed may not be known. The Commission noted that such an argument could apply to any new listing, and for that reason the criterion is prospective i.e. "…at least one country may…". The Commission agreed that it was confident that at least one Member would be able to claim freedom from infection with all genogroups of the virus species ISKNV.

2) Validation of diagnostic tests for the TRBIV genogroup

The Commission agreed with comments that there are some limitations on the validation of diagnostic tests for detection of the TRBIV genogroup due to the availability of TRBIV-infected tissues. However, the Commission noted that there are a variety of methods that are inclusive of all three genogroups (Kawato *et al*., 2021a, Koda *et al*., 2023 and Kim *et al*., 2022). Kawato *et al*., 2021a, showed that three of four real time PCR assays tested were able to detect RSIV, ISKNV and TRBIV genogroups; however, for TRBIV this was determined using synthesized plasmids and not tissue samples.

The Commission supported a comment requesting that specific work be undertaken to conduct an inter-laboratory comparison to assess the performance of diagnostic methods inclusive of RSIV, ISKNV and TRBIV genotypes. However, the Commission noted that such work would be constrained by availability of TRBIV-positive tissues. The Commission requested that Members who have access to TRBIV-positive tissues advise WOAH so that additional validation of available methods might be conducted.

In conclusion, the Commission agreed that there are sufficient diagnostic tools available to detect the species ISKNV, inclusive of its three genogroups, and to construct appropriate case definitions. Further diagnostic accuracy studies are warranted, in particular using TRBIV-infected tissues, however, this is not an impediment to this criterion being met.

3) Merits of listing

The Commission agreed that overall, the assessment of infection with ISKNV species against the criteria for listing an aquatic animal disease demonstrated that all relevant criteria had been met to support its listing. In addition, the Commission wished to highlight that the *ad hoc* Group had advised that identifying susceptible species to the level of the specific ISKNV-genogroup was not always possible and that the three genogroups present with similar clinical signs, histopathology and epidemiology. The Commission also noted that the proposed listing is consistent with other listed diseases for which the pathogenic agent may be comprised of different genotypes or strains.

The Commission considered some comments that listing of the virus species ISKNV would create logistical and resource issues, particularly related to the susceptible species that are traded internationally as ornamental fish. The Commission noted that if standards are applied correctly, any trade measures for the virus species ISKNV would be of significance only for trade between countries of different disease status. The Commission also noted that the standards are intended to support trade by providing a set of common sanitary measures.

The Commission wished to remind Members that one of the recommendations at the Global Conference on Aquatic Animal Health held in Chile in April 2019, based on Member requests, was additional guidance in the *Aquatic Code* for trade of ornamental aquatic animals. The ISKNV genogroup is a key pathogen of concern in ornamental fish species traded internationally, supporting the proposed listing. The Commission noted that while the virus does not, in all cases,

cause significant morbidity and mortality in ornamental fish species, the Commission noted that the ISKNV genogroup impacts other important aquaculture species (e.g. Nile tilapia (*Oreochromis niloticus*), barramundi (*Lates calcarifer*), largemouth bass (*Micropterus salmoides*), orange-spotted grouper (*Epinephelus coioides*), Malabar grouper (*Epinephelus malabaricus*), mandarin fish (*Siniperca chuatsi))* that are traded internationally.

The revised assessment of infection with all genogroups of the virus species infectious spleen and kidney necrosis virus against the criteria in Chapter 1.2, Criteria for listing aquatic animal diseases, is presented as Annex 16 for information.

The revised Article 1.3.1. of Chapter 1.3. Diseases listed by WOAH, is presented as Annex 15 for comments.

8.4. Safe commodities – Articles X.X.3. for disease-specific chapters

Background

At its September 2020 meeting, the Aquatic Animals Commission reviewed Article X.X.3. of all disease-specific chapters to address comments that the recommended time/temperature treatments in these articles represented different levels of thermal treatment and that some were not commercially feasible as they would diminish product quality.

Between September 2020 and February 2022, the Commission circulated proposed amendments to Articles X.X.3. in all disease-specific chapters of the *Aquatic Code* to reflect this revised approach. In May 2022, the proposed amendments to Articles 9.X.3. and 10.X.3. were adopted.

At its February 2022 meeting, the Commission noted that the assessments previously undertaken against 'Criteria to assess the safety of aquatic animal products imported (or transited) for any purpose regardless of the disease X status of the exporting country, zone or compartment (as described in Article 1.4.1.) needed to be reviewed based on any new evidence on thermal stability, and requested that a consultant be contracted to undertake this review.

February 2023 meeting

The Commission reviewed the Safe commodity assessments that had been conducted for all aquatic animal products listed in Article X.X.3. for all the disease-specific chapters to apply the new approach and new scientific information, where relevant. The Commission wished to acknowledge the tremendous amount of work required to undertake this review.

The Commission agreed with all of the recommended amendments to the time/temperature for inactivation identified in the assessments.

The Commission encouraged researchers doing work on inactivation of pathogens to apply a range of time/temperatures combinations to ensure that there is sufficient information to develop z-values as this would support Members in determining equivalent time/temperatures for inactivation of pathogenic agents.

The Commission requested that the Safe commodity assessments for WOAH listed aquatic animal diseases, currently available on the WOAH website, be updated with the revised assessments. In the meantime, the updated Safe Commodities assessments will be published on the WOAH website for information. Members are encouraged to refer to these assessments when reviewing the proposed changes to Articles X.X.3. being presented for comments (see items 8.4.1. to 8.4.4.). The Safe commodities assessments can be found on the WOAH website at: https://www.woah.org/en/what-we-do/standards/standard-setting-process/aquatic-animals-commission/#ui-id-4

8.4.1. Articles 8.X.3. for amphibian disease-specific chapters

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

Background

At its February 2022 meeting, the Commission amended Articles 8.X.3. to align with amendments recently adopted in Articles 9.X.3. and 10.X.3. regarding a revised approach to time/temperature treatments and circulated these for comment.

Previous Commission reports where this item was discussed

September 2020 (Item 4.7., page 10); February 2021 (Part B: Item 1.4., page 8); September 2021 (Item 5.1.5., page 24); February 2022 report (Part B: Item 2.1.1.1., page 5).

February 2023

The Commission considered the revised Safe commodity assessments for products listed in Articles 8.X.3. and amended these articles accordingly.

In point 2., the Commission agreed with a comment that mechanically dried amphibian products are a subset of aquatic animal products that have been subjected to a heat treatment and proposed to delete this point to remove duplication.

The Commission agreed to delete mechanically dried products from all Articles X.X.3., where relevant, for alignment across disease-specific chapters

The revised Articles 8.1.3., 8.2.3. and 8.3.3. are presented in Annex 17 in track changes and clean versions, respectively, for comments.

8.4.2. Articles 9.X.3. for crustacean disease-specific chapters

February 2023

The Commission considered the revised Safe commodity assessments for products listed in Articles 9.X.3. and amended these articles accordingly.

The Commission agreed to present only those Articles 9.X.3. where the time/temperature treatments had changed as a result of the updated Safe commodities assessments. The Commission noted that Articles 9.X.3. did not previously include mechanically dried crustacean products so there are no other harmonised changes to all crustacean disease-specific chapters.

The revised Articles 9.3.3., 9.5.3., 9.6.3., 9.7.3. and 9.10.3. are presented in Annex 18 in track changes and clean versions, respectively, for comments.

8.4.3. Articles 10.X.3. for fish disease-specific chapters

February 2023

The Commission considered the revised Safe commodity assessments for products listed in Articles 10.X.3. and amended these articles accordingly.

In point 2., the Commission agreed that mechanically dried eviscerated fish products are a subset of aquatic animal products that have been subjected to a heat treatment and proposed to delete this point to remove duplication.

The Commission agreed to delete mechanically dried products from all Articles X.X.3., where relevant, for alignment across disease-specific chapters.

The revised Articles 10.1.3., 10.2.3., 10.3.3., 10.4.3., 10.5.3., 10.6.3., 10.7.3., 10.8.3., 10.9.3. and 10.10.3. are presented as Annex 19 in track changes and clean versions, respectively, for comments.

8.4.4. Articles 11.X.3. for mollusc disease-specific chapters

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

Background

At its February 2022 meeting, the Commission amended Articles 11.X.3. to align with amendments recently adopted in Articles 9.X.3. and 10.X.3. regarding a revised approach to time/temperature treatments and circulated these for comment.

Previous Commission reports where this item was discussed

September 2020 (Item 4.7., page 10); February 2021 (Part B: Item 1.4., page 8); September 2021 (Item 5.1.5., page 24); February 2022 report (Part B: Item 2.1.1.2., page 5).

February 2023

The Commission considered the revised Safe commodity assessments for products listed in Articles 11.X.3. and amended these articles accordingly.

In point 2. of Article 11.1.3. the Commission agreed that mechanically dried abalone products are a subset of aquatic animal products that have been subjected to a heat treatment. The Commission agreed to delete mechanically dried products from all Articles X.X.3., where relevant, for alignment across disease-specific chapters.

The Commission noted that the updated Safe commodities assessment evaluated the inactivation time/temperatures for all mollusc pathogens. Consequently, in Article 11.2.3. of Chapter 11.2. Infection with *Bonamia exitiosa* and Article 11.3.3. of Chapter 11.3. Infection with *Bonamia ostreae*, the Commission agreed to add a new point 1. to include aquatic animal products that have been subjected to a heat treatment as safe commodities to align with other disease-specific chapters.

The revised Articles 11.1.3., 11.2.3., 11.3.3., 11.4.3., 11.5.3., 11.6.3. and 11.7.3. are presented as Annex 20, in track changes and clean versions, respectively, for comments.

8.5. Article 11.5.1. and 11.5.2. of Chapter 11.5. Infection with *Perkinsus marinus*

Background

The *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases met in November-December 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 the assessments for susceptibility of mollusc species to infection with *Perkinsus marinus*.

February 2023 meeting

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

The Commission agreed to amend the list of susceptible species in Article 11.5.2. in line with recommendations of the *ad hoc* Group, i.e.

- Two species currently listed in Article 11.5.2., American cupped oyster (*Crassostrea virginica*) and Ariake cupped oyster (*Magallana* [Syn. *Crassostrea*] *ariakensis*), were assessed to meet the criteria for listing as susceptible to infection with *P. marinus* and are therefore proposed to remain in Article 11.5.2.

- Two new susceptible species, Cortez oyster (*Crassostrea corteziensis*) and palmate oyster (*Saccostrea palmula*) were assessed to meet the criteria for listing as susceptible to infection with *P. marinus* and are therefore proposed to be added to Article 11.5.2.

- Four species currently listed in Article 11.5.2., Baltic clam (*Macoma balthica*), northern quahog (*Mercenaria mercenaria*), Pacific cupped oyster (*Magallana* [Syn. *Crassostrea*] *gigas*), and soft shell clam (*Mya arenaria*), were assessed and did not meet the criteria for listing as a susceptible species to infection with *P. marinus* and are therefore proposed to be deleted from Article 11.5.2.

Relevant sections of Chapter 2.4.4. Infection with *Perkinsus marinus*, in the *Aquatic Manual* were also amended in line with the recommendations of the *ad hoc* Group (see item 11.2.1.).

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

The Commission also amended Article 11.5.1. to ensure consistency with the approach taken in other mollusc disease-specific chapters.

The revised Articles 11.5.1. and 11.5.2. of Chapter 11.5. Infection with *Perkinsus marinus*, are presented as Annex 21 for comments.

9. Items for Member Information

9.1. Emerging diseases

Background

A standing agenda item for every meeting of the Aquatic Animals Commission is to review scientific information on emerging diseases to determine whether the disease should be considered as an emerging disease by WOAH Members or whether any other actions are warranted. The Commission also considers requests made from other sources such as WOAH Members, experts and Reference Centres.

9.1.1. Infection with carp edema virus

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 WOAH 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 2021 and September 2021 meetings, the Commission reviewed Member comments and new scientific evidence and agreed it met the WOAH definition of an 'emerging disease' whilst noting 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 more reports of detections and mortalities caused by infection with CEV are published every year. The Commission recognised that there was still uncertainty as to the impact on production

associated with infection with CEV and the extent of the spread globally, particularly within Europe and agreed it met the WOAH definition of an 'emerging disease'.

At its September 2022 meeting, the Commission reviewed new scientific evidence and noted that since its February 2022 meeting there have been several more outbreaks reported in the Asia Pacific region. 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 as to whether the criteria for listing (Chapter 1.2.) should be applied or if it should no longer be considered as an emerging disease.

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 (Item 6.2.1., page 12).

February 2023

The Commission noted that only one comment was received in response to its request for provision of relevant information on infection with CEV to inform the Commission's consideration as to whether the criteria for listing should be applied or if it should no longer be considered as an emerging disease.

The Commission reviewed the evidence provided and noted that CEV may be a regionally important disease, however, there appears to be differences between strains and levels of virulence.

The Commission agreed that at this time infection with CEV did not warrant an assessment against the criteria for listing and noted that there did not seem to be interest from Members for standards to guide management of infection with CEV. In addition, there have also been no reports of CEV as an emerging disease through WOAH WAHIS by Members since the Commission considered it an emerging disease in February 2020.

The Commission agreed that infection with CEV no longer met the definition of 'emerging disease'. The Commission noted that if the situation for infection with CEV was to change and new scientific evidence was provided, a review against the criteria for listing could be re-visited in the future.

The Commission noted that individual Members that wished to implement measures for infection with CEV, could do so based on a risk analysis.

The Commission reviewed the disease card for infection with CEV and agreed with a comment to add salt treatment as a possible control measure. The Commission informed Members that the revised disease card for infection with CEV will remain available on the WOAH website at: https://www.woah.org/en/what-we-do/standards/standard-setting-process/aquatic-animals-commission/#ui-id-4.

9.1.2. Covert mortality nodavirus (CMNV)

No Member comments received.

Background

At its September 2022 meeting, the Aquatic Animals Commission considered scientific information available on covert mortality nodavirus (CMNV) and 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*.

Previous Commission reports where this item was discussed

September 2022 (Item 6.2.2., page 13).

February 2023

No Member comments were received.

The Commission reviewed scientific information for infection with Covert mortality nodavirus (CMNV) and agreed that infection with CMNV continues to meet 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 encouraged Members to investigate mortality and morbidity events in the range of aquatic animal species affected, emphasising that a better understanding of the virus is essential for efforts to control its possible spread and impacts on aquatic animal populations.

The Commission wished to inform 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/standards/standard-setting-process/aquatic-animals-commission/#ui-id-4.

The WOAH Manual of Diagnostic Tests for Aquatic Animals

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).

The Commission reflected on how best to report on amendments to the *Aquatic Manual* so that Members can more easily see and understand the Commission's decisions in response to comments. The Commission decided to use a table format for this report to indicate the location in the chapter, summarise the comment and give the decision. The Commission would welcome Member feedback on this new reporting format.

Some Members had submitted the same comments for all the *Aquatic Manual* chapters circulated with the September report. The Aquatic Animals Commission addressed these comments as follows:

Section/ paragraph	Comment	Decision
2.3.6. Geographical distribution	Change the heading to "Geographic" distribution	Disagree: "geographical" is the correct term in British English, which is used by WOAH

Section/ paragraph	Comment	Decision
3.5.2. Preservation of samples for molecular detection	Provide a reference to support the assertion that freezing samples is acceptable	Add the following sentence to the general information chapters: "Surveillance samples for demonstration of freedom from disease should be collected, stored and tested in a time frame to minimise sample degradation and maximise the likelihood that the analyte would be detected if present. ~~should not be frozen for a long period of time~~." Also add the following text to all the crustacean disease chapters: "Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans)."
4.4. Nucleic acid amplification, paragraph: Extraction of nucleic acids	Clarify the text	~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.
4.4.1. Real-time PCR and 4.4.2. Conventional PCR: tables on primer and probe sequences and PCR cycling parameters	Clarify the text in the headings	Replace "product" size by "amplicon" size; delete "probes" from the conventional PCR table; for nested PCR the sequences to be labelled "primary" and "nested" rather than "inner" and outer".
4.5. Amplicon sequencing	Clarify the text	The size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

Section/ paragraph	Comment	Decision
6. Corroborative diagnostic criteria	Remove the requirement for all suspect positive tests to be sent to the appropriate WOAH Reference Laboratory: no need if the country has the capacity to confirm the suspect positive test. The positive finding will be reported to WOAH through the WAHIS reporting system. Also there may not be a specific WOAH Reference Laboratory for the disease in question. The requirement is inconsistent with the *Terrestrial Manual.*	Delete: "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." Illustrate that the paragraph is concerned with diagnostic capability through the following changes to the next sentence: "If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free." For diseases for which there are no WOAH Reference Laboratories, the following will be added: "there are currently no WOAH Reference Laboratories designated for this disease"
6. Corroborative diagnostic criteria	What is the Commission's position with regards to eDNA?	The Commission's position on eDNA methods is outlined in their discussion paper: The use of environmental DNA methods for detection of WOAH listed aquatic animal diseases. Currently eDNA methods are recommended for only one listed disease (*Gyrodactylus salaris*).
6.1. Apparently healthy animals or animals of unknown health status	Replace "hydrographical proximity" with "hydrographic linkage"	Maintain "hydrographical" proximity as it is the correct term
6.1.2. Definition of confirmed case in apparently healthy animals and 6.2.2. Definition of confirmed case in clinically affected animals	Bioassay is not specific enough to be used as a confirmatory test	Agree. A positive bioassay result indicates suspicion of infection, but requires another method such as an agent identification test for confirmation of infection. Bioassay will be removed from all definitions of a confirmed case.

Section/ paragraph	Comment	Decision
6.3. Diagnostic sensitivity and specificity for diagnostic tests, Table 6.3.2. For surveillance of apparently healthy animals	Remove Table 6.3.2 until data are available	Maintain Table 6.3.2 but add “no data are currently available” to the text in the first paragraph of the Section The Commission has previously agreed to maintain empty tables in Section 6 to highlight the lack of diagnostic performance data.

The amendments described above will be applied to all chapters that will be proposed for adoption in May 2023 or circulated for comment, as well as the template, where appropriate.

10. Texts that will be proposed for adoption in May 2023

10.1. Section 2.2. Disease of Crustaceans

10.1.1. Chapter 2.2.1. Acute hepatopancreatic necrosis disease

Comments were received from Australia, Canada, China (People’s Republic of), Chinese Taipei, New Zealand, the UK, 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.

At its September 2022 meeting, the Commission amended the proposed chapter after considering Member comments. The Commission agreed to review all published information on non-*Vibrio* parahaemolyticus species that have been associated with AHPND in consultation with the two WOAH Reference Laboratories.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.1., page 9); September 2022 report (Item 7.1.2., page 15).

February 2023

Section/ paragraph	Comment	Decision
1. Scope	Delete the last sentence as other *Vibrio* species carrying pathogenic plasmids and causing the death of prawns have been found	Agree. The Commission will continue to seek the advice of the Reference Laboratories to monitor advances in the scientific evidence that might warrant a change to the scope of the chapter
2.3.2. Clinical signs, including behavioural changes	Clarify the text as sinking to the bottom of the tank is a behavioural change and not a clinical sign	Agree and amended the text accordingly
3.2. Selection of organs or tissues	Add the stomach as a suitable organ for sampling	Disagree as no reference was provided with the comment

Section/ paragraph	Comment	Decision
Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals	Replace “cell culture” with “isolation” as the AHPND is infection with strains of *Vibrio parahaemolyticus* that contain a pathogenic plasmid, which does not require cell culture	Agree and added it for presumptive diagnosis of clinically affected animals as it is described in the chapter
		Deleted bioassay for confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis (see the table in the introduction to the *Aquatic Manual* section of this report above)
4.2. Histopathology and cytopathology	Rewrite the Section as it is inconsistent with other chapters and add more recent references such as Ananda Raja *et al.* (2017)	Disagree: for AHPND is it important to describe the phases of the disease. Ananda Raja *et al.* does not describe AHPND pathology, it describes the pathology of *V. parahaemolyticus* that does not cause AHPND. Histopathological changes caused by non-AHPND causing *V. parahaemolyticus* is quite different. Added some more details to the text.
4.4. Nucleic acid amplification	Loop-mediated amplification protocol (LAMP) is mentioned in Table 4.1 and is used for corroborative diagnosis in Section 6. Corroborative diagnostic criteria, but no detailed method is given in Section 4.4.	Agree: a new Section 4.3 *Isothermal loop-mediated amplification protocol* has been added including a of the parameters as for the PCR protocols
4.4.2. Conventional PCR, Table of the cycling parameters	Merge methods 7 as 8 as they are two steps in the same nested PCR	Agree. Also delete methods 1 and 2 as improved methods have been developed
6.1.1. Definition of suspect case in apparently healthy animals	Add a positive result by isolation and by bioassay	Disagree: it is not likely that these tests would be used in apparently healthy animals
6.2.1. Definition of suspect case in clinically affected animals	Add a positive result by isolation and by bioassay	Agree

The revised Chapter 2.2.1. Acute hepatopancreatic necrosis disease, is presented as Annex 22 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from Australia, Canada, China (People’s Republic of), New Zealand, the UK, the USA and the EU.

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.

At its September 2022 meeting, the Commission amended the proposed chapter after considering Member comments.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.2., page 10); September 2022 report (Item 7.1.4., page 17).

February 2023

Section/ paragraph	Comment	Decision
2.3.1. Mortality, morbidity and prevalence	Clarify that the incubation period and severity of the disease depend to some extent on the size or age	Add “with juveniles always being the most severely affected”
2.3.1. Mortality, morbidity and prevalence	Delete the text: no practical significance to compare mortalities between HNP-affected and non-NHP-affected broodstock	Agree
3.2. Selection of organs or tissues	Clarify the purpose of selecting the hepatopancreas as the preferred sample	Disagree: the sentence is clear as written
Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals	Delete bioassay for confirmatory diagnosis of a suspect result as a positive result from bioassay indicates suspicion of infection but must be confirmed using another method	Agree, and consequently deleted it from section 6.2.2 Definition of a confirmed case in clinically affected animals (see table with harmonised changes for all chapters above)
4.2. Histopathology and cytopathology	Add that molecular methods are recommended for “screening population for infection with” *H. penaei* to be consistent with the *Aquatic Code*	Agree
4.4.1. Real-time PCR and 4.4.2. Conventional PCR: tables on primer and probe sequences and PCR cycling parameters	Add the name of the gene to the Tables	Agree: also as the title of the column is “pathogen/target gene”, so added the pathogen. Changed “Flg” to “flagella hook gene” in the text when relevant

The revised Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis), is presented as Annex 23 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from Australia, Canada, China (People's Republic of), Chinese Taipei, New Zealand, the UK, 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.

At its September 2022 meeting, the Commission amended the proposed chapter after considering Member comments.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.1.3., page 10); September 2022 report (Item 7.1.5., page 18).

February 2023

Section/ paragraph	Comment	Decision
2.1.1. Aetiological agent	Add a statement that IHHNV genotypes in Ecuador and Peru were found to be within a separate lineage of IHHNV type 2 genotypes circulating within these countries	Agree
2.2.2. Species with incomplete evidence for susceptibility	Check if the five finfish species that were deleted in the text are supposed to be listed in the table	They are: the rule is that if there are more than 10 species, they should be listed in table format. As there were more than 10, all the species originally listed in the text have now been incorporated into the table
	Add a legend to the table.	Disagree, none needed
2.3. Disease pattern	Rearrange the information in Sections 2.3.1.–2.3.3. to adopt a different approach to describing disease pattern in terms of acute or chronic infection, prevalence, transmission, experimental infection etc.	Disagree: the approach followed here has been in practice for a long time, is used in all the crustacean disease chapters, and there is no added value to the proposed rearrangement
2.3.1. Mortality, morbidity and prevalence	Delete the statement on the clinical picture of IHHNV in Peru and Ecuador as it is misleading implying that the genotype is non-virulent	Disagree: the statement represents the findings of the referenced study accurately
3.1. Selection of populations and individual specimens	Add a sentence on the specimens suitable for testing for infection with IHHNV	Agree

Section/ paragraph	Comment	Decision
	Add “using the tests in Table 4.1.” to the end of the second sentence because detection limits depend on tests and surveillance strategy	Disagree: the purpose of this Section is to describe which samples are best for demonstrating free status; all the tests rated in Table 4.1. are recommended
3.4. Non-lethal sampling	Provide references or data on the diagnostic parameters when using haemolymph or pleopods taken from non-lethal sampling as a method for screening for IHHNV in apparently healthy populations	Disagree: diagnostic performance extends beyond the scope of this Section, which is tissue type selection. Agree to include this statement: If non-lethal sample types are used, the diagnostic performance of the method for a specific purpose of use should be considered.
3.5.1. Samples for pathogen isolation	Change the heading from “Samples for pathogen isolation” to Samples for bioassay” and delete mention of pathogen isolation in the first sentence as IHHNV does not grow *in vitro*	Agree
Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals	Add bioassay for presumptive diagnosis of clinically affected fish as it is described in the chapter and is suitable for IHHNV diagnosis	Agree: added it to the table for this purpose and consequently added to Section 6.2.1. Definition of suspect case in clinically affected animals
Table 4.4.2.1. Recommended primer sets for conventional PCR detection of IHHNV	Correct the sequences given for the forward and reverse primers in method 2	Agree
6.2.2. Definition of confirmed case in clinically affected animals	Histopathology should be one of the diagnostic methods listed in Section 6.2.2.	Disagree: histopathology alone is not sufficient to confirm a positive case

The revised Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus, is presented as Annex 24 and will be proposed for adoption at the 90th General Session in May 2023.

10.1.4. Chapter 2.2.5. Infection with infectious myonecrosis virus

Comments were received from Canada, China (People’s Republic of), New Zealand, the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.2.5. Infection with infectious myonecrosis virus, which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.1.6., page 19).

February 2023

Section/ paragraph	Comment	Decision
2.1.1. Aetiological agent		Clarified that IMNV is tentatively assigned to the family *Totiviridae*, and updated the references to this statement
3.1. Selection of populations and individual specimens	Add “using the tests in Table 4.1.” to the end of the second sentence because detection limits depend on tests and surveillance strategy	Disagree: the purpose of this Section is to describe which samples are best for demonstrating free status; all the tests rated in Table 4.1. are recommended
3.4. Non-lethal sampling	Provide references or data on the diagnostic parameters when using haemolymph or pleopods taken from non-lethal sampling as a method for screening for IMNV in apparently healthy populations	Disagree: diagnostic performance extends beyond the scope of this Section, which is tissue type selection. Added a statement To consider the impact on diagnostic performance.
6.1.1. Definition of suspect case in apparently healthy animals	Delete histopathology from the definition as it cannot be recommended for apparently healthy animals	Agree
6.1.2. Definition of confirmed case in apparently healthy animals	Delete *in-situ* hybridisation from the definition as it is not sufficiently specific	Agree
6.2.2. Definition of confirmed case in clinically affected animals	Add histopathology to the definition	Disagree, not specific enough to confirm a positive case

The revised Chapter 2.2.5. Infection with infectious myonecrosis virus, is presented as Annex 25 and will be proposed for adoption at the 90th General Session in May 2023..

10.1.5. Chapter 2.2.7. Infection with Taura syndrome virus

Comments were received from Australia, Canada, China (People’s Republic of), New Zealand, the UK, the USA and the EU.

Background

At its 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 experts and reformatted using the new disease chapter template.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.1.7., page 20).

February 2023

Section/ paragraph	Comment	Decision
3.1. Selection of populations and individual specimens	Add “using the tests in Table 4.1” to the end of the second sentence because detection limits depend on tests and surveillance strategy	Disagree: the purpose of this Section is to describe which samples are best for detecting TSV; all the tests rated in Table 4.1. are recommended. Deleted the text referring to certification of freedom from infection with TSV from the last sentence as not useful.
3.4. Non-lethal sampling	Provide references or data on the diagnostic parameters when using haemolymph or pleopods taken from non-lethal sampling as a method for screening for TS viruses in apparently healthy populations	Disagree: diagnostic performance is beyond the scope of this Section, which is about tissue type selection. Agree to include this statement: If non-lethal sample types are used, the diagnostic performance of the method for a specific purpose of use should be considered.
3.5.1 Samples for pathogen isolation	Replace the paragraph with “not available” as cell culture cannot be used for TSV isolation	Changed “pathogen isolation” to “bioassay” in the title and text so that the section now covers material for bioassay
4.2.1. Acute phase of Taura syndrome	Delete the first sentence as it is not relevant to histopathology and cytopathology	Disagree: the comment is incorrect – necrosis is observed in histological sections
4.2.3. Chronic phase of infection with Taura syndrome virus	Delete the first sentence as it is not relevant to histopathology and cytopathology	Disagree: the sentence introduces the text on histological findings
6.1.1. Definition of suspect case in apparently healthy animals	Delete histopathology from the definition as it cannot be recommended for apparently healthy animals	Agree but kept it in Table 4.1 as it is a useful method to detect infection with TSV in the acute and chronic phases of infection
6.1.2. Definition of confirmed case in apparently healthy animals	Delete *in-situ* hybridisation from the definition as it is not sufficiently specific to confirm a case in this population	Agree
6.2.2. Definition of confirmed case in clinically affected animals	Add histopathology to the definition	Disagree, not specific enough to confirm a positive case

The revised Chapter 2.2.7. Infection with taura syndrome virus, is presented as Annex 26 and will be presented for adoption at the 90th General Session in May 2023.

10.1.6. Chapter 2.2.8. Infection with white spot syndrome virus

Comments were received from Australia, Canada, China (People's Republic of), New Zealand, the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission reviewed Chapter 2.2.8. Infection with white spot syndrome virus (WSSV), which had been updated by the WOAH Reference Laboratory experts and reformatted using the new disease chapter template. The Commission noted that the assessments completed by the *ad hoc* Group on susceptibility of crustacean species to WOAH 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. The Commission noted that the current adopted text in Section 2.2.1. of Chapter 2.2.8. of the *Aquatic Manual* will not be revised until this review is completed.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.1.8., page 20).

February 2023

The Commission noted that the scientific literature on susceptibility of crustaceans to infection with WSSV has not been assessed since the last meeting of the *ad hoc* Group on susceptibility of crustacean species to WOAH listed diseases in June 2016. The Commission noted that the application of criteria in Chapter 1.5. Criteria for listing species as susceptible to infection with a specific pathogen, may result in additional species meeting the criteria for susceptibility and impact the application of criteria in Article 1.5.9. The Commission noted that the *ad hoc* Group has been reconvened to complete the assessments for the remaining WOAH listed diseases and requested that the *ad hoc* Group update the assessments of infection with WSSV to ensure that the list of susceptible species is based on current literature. Once the *ad hoc* Group has completed this work, the Commission would apply Article 1.5.9. of Chapter 1.5. to amend Article 9.8.2. of the *Aquatic Code* and Sections 2.2.1. and 2.2.2. of the *Aquatic Manual*.

Section/ paragraph	Comment	Decision
3.4. Non-lethal sampling	Add a reference to Section 6. Corroborative diagnostic criteria for sample size determination when selecting animals from either apparently healthy or clinically affected populations	Disagree: not necessary or appropriate to include sample size here. There are multiple issues that need to be considered in any survey design, including expected prevalence, confidence, etc. Agree to include this statement: If non-lethal sample types are used, the diagnostic performance of the method for a specific purpose of use should be considered.
4.4.1. Real-time PCR	Add the GenBank accession number and correct the cycling parameters	Agree
6.1.2. Definition of confirmed case in apparently healthy animals	Delete *in-situ* hybridisation from the definition as it is not sufficiently specific to confirm a case in this population	Agree, but deleted due to sensitivity in this population

Section/ paragraph	Comment	Decision
6.2.2. Definition of confirmed case in clinically affected animals	Add histopathology to the definition	Disagree, not specific enough to confirm a positive case

The revised Chapter 2.2.8. Infection with white spot syndrome virus, is presented as Annex 27 and will be presented for adoption at the 90th General Session in May 2023.

10.2. Section 2.3. Diseases of fish

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

Comments were received from Australia, China (People's Republic of), Chinese Taipei, New Zealand, the UK, 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 and reformatted using the new disease chapter template.

At its September 2022 meeting, the Commission amended the proposed chapter after considering Member comments.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 3.1.2.1., page 10); September 2022 report (Item 7.2.1., page 21).

February 2023

Section/ paragraph	Comment	Decision
2.4.6. Disinfection of eggs and larvae: first sentence	Delete the text and replace with "Not applicable" because fish eggs and larvae are not infected by *A. invadans*	Amended the text to state: "There are no published protocols for *A. invadans* disinfection."
4.1. Observation for clinical signs	Include a description of clinical signs as it is included in Table 4.1 as a recommended method for targeted surveillance	Agree: added this new Section and consequently amended Section 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations
4.4.2. Conventional PCR, Diagnostic PCR techniques	Add details of specificity and sensitivity of the three PCR primers and protocols to facilitate the user's selection	These details are in the publications and the table of PCR cycling parameters. Added a referenced sentence on cross reactivity of one of the PCR assays with *A. frigidophilus*
4.6. In-situ hybridisation		Shortened the description of the procedure as it included unnecessary details

Section/ paragraph	Comment	Decision
5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations	As examination of target populations for gross signs is the test for targeted surveillance to declare freedom from infection with *A. invadans,* this should be stated here	Agree: replaced the existing text with the proposed statement and cross referenced to the new Section 4.1. Observation for clinical signs

The revised Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), is presented as Annex 28 and will be proposed for adoption at the 90th General Session in May 2023.

10.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus

Comments were received from Australia, Canada, China (People's Republic of), New Zealand, Thailand, the UK, 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.

At its February and September 2022 meetings, the Commission amended the proposed chapter after considering Member comments.

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 report (Item 7.2.2., page 22).

February 2023

Section/ paragraph	Comment	Decision
2.2.1. Susceptible host species and 2.2.2. Species with incomplete evidence for susceptibility	Each of the species should be referenced	The references are included in the report of the *ad hoc* Group on susceptibility of fish species to WOAH listed diseases
2.2.3. Likelihood of infection by species, host life stage, population or sub-populations	Delete 'other' in the sentence as there are no descriptions of infection of eggs or early life stages of any fish species	Agree
2.2.5. Aquatic animal reservoirs of infection	Add a sentence on sporadic outbreaks observed in EHNV-endemic areas and clarify why only rainbow trout and European perch are specifically referenced	Based on feedback from the Reference Laboratory expert deleted all the existing text and replaced with 'None known"

Section/ paragraph	Comment	Decision
2.3.1. Mortality, morbidity and prevalence	Add a sentence that European perch from distinct geographical areas have demonstrated susceptibility to EHN under experimental conditions	Agree
2.3.6. Geographical distribution	Expand the sentence on EHNV in Australia by including the published findings on sporadic outbreaks involving small numbers of European perch	Agree
3.1. Selection of populations and individual specimens, point i)	Delete 'rainbow trout' as European perch are significantly more susceptible to EHNV	Clarified that European perch should be sampled where available, otherwise rainbow trout or the other susceptible species listed in Section 2.2.1.
3.1., point ii)	Replace "lots of population" with "epidemiological units"	Agree
3.1., point iii)	Proposed some minor text changes so that the sentence covers sampling during disease outbreaks that occur outside a fish farm environment	Agree
3.4. Non-lethal sampling	Add a reference to support the statement or delete the text	Replaced the text with "Not applicable"
4. Diagnostic methods, first paragraph	Delete "surveillance of" in point i)	Disagree: the text reflects the title of columns A, B, C in the Table 4.1.
Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals	Reduce the ratings for cell culture for all three purposes as it may be appropriate for surveillance of apparently healthy animals during/after an EHNV outbreak but there is no evidence that this is the case for general EHNV surveillance	Agree in consultation with the Reference Laboratory: cell culture is used less often since the advent of molecular tests and is slow and labour intensive
4.3.2. Cell culture	Amend the heading to "Cell lines for virus isolation" as the Section only describes the cell lines used to incubate EHNV	Agree
	Delete the last sentence as it refers to the identity of viruses in cell culture and is irrelevant to this section	Agree: the sentence was moved to Section 4.3.4. Interpretation of results
4.4.1. Real-time PCR, table of PCR cycling parameters	Reinstall the real-time PCRs described by Jaramillo *et al.* (2012) and Stilwell *et al.* (2018) based on the original text and references	Agree: added both methods and accompanying details

Section/ paragraph	Comment	Decision
4.9. Antibody- or antigen-based detection methods	Move the sentence on the indirect ELISA from Section 4.10. Other methods to this Section as it fits better	Agree
4.10. Other methods	Delete the first sentence as it contradicts published literature	Agree

The revised Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, is presented as Annex 29 and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from China (People's Republic of), Chinese Taipei, the UK and the EU.

Background

At its 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.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.2.3., page 23).

February 2023

Section/ paragraph	Comment	Decision
2.2.1.	The common name for *Percocypris pingi* should be "Jingsha barbel carp" as the taxonomic status of this fish is: Cyprinidae, Subbarbinae, Genus of barbel carp.	"Jingsha bass carp" was changed to "Jingsha barbel carp" (see item 7.4.)
2.2.1.	The common names for the susceptible species do not follow the convention used in the *Aquatic Code;* to only use a capital letter for proper nouns.	The common names that did not begin with proper nouns were changed to lower case letters. Any other instances where this occurs will be amended in the 2023 version of the *Aquatic Manual.*

The revised Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus, is presented as Annex 30 and will be proposed for adoption at the 90th General Session in May 2023.

10.3. Section 2.4. Diseases of molluscs

10.3.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa and* Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae*

Comments were received from the UK and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission agreed to amend 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 new nomenclature of the Suminoe oyster and Pacific cupped oyster (see item 7.6.)

Previous Commission reports where this item was discussed

Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa* - February 2022 report (Part B: Item 7.3.1., page 23).

Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae* - February 2022 report (Part B: Item 7.3.1., page 23).

February 2023

Chapter/ Section	Comment	Decision
Chapter 2.4.2., Section 2.2.1. and Chapter 2.4.3., Section 2.2.1.	The common name in FAOTERM for *Magallana* (Syn. *Crassostrea*) *ariakensis* was recently changed to Ariake cupped oyster	The common name for *Magallana* (Syn. *Crassostrea*) *ariakensis* was changed to Ariake cupped oyster (see item 7.6.)

The revised Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa,* and Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae*, are presented as Annex 31 and Annex 32, respectively and will be proposed for adoption at the 90th General Session in May 2023.

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

Comments were received from the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission considered the report of the *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases who undertook assessments of susceptible species for infection with *Marteilia refringens* against the criteria presented in Chapter 1.5., Criteria for listing species as susceptible to infection with a specific pathogen. The Commission agreed to amend 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, except for the copepod (*Paracartia grani*). The Commission agreed to add a new paragraph in Section 2.2.1. to reflect the unique situation with the risk associated with the copepod (*Paracartia grani*) as an intermediate host.

Previous Commission reports where this item was discussed

February 2022 report (Part B: Item 7.3.1., page 23).

February 2023

Section/ paragraph	Comment	Decision
2.2.1.	The common name for *Chamelea gallina* should have the word “clam” inserted at the end	“striped venus” was changed to “striped venus clam” (see item 7.7.)

The revised Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*, is presented as Annex 33 and will be proposed for adoption at the 90th General Session in May 2023.

11. Items for Member comments

11.1. Section 2.2. Diseases of crustaceans

11.1.1. Chapter 2.2.0. General information: diseases of crustaceans

Comments were received from Australia, Canada, China (People's Republic of), Thailand, the UK, the USA and the EU.

Background

At its September 2022 meeting, the Aquatic Animals Commission amended Chapter 2.2.0. General Information (diseases of crustaceans), in consultation with the crustacean disease Reference Laboratory experts.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.1.1., page 15).

February 2023

Section/ paragraph	Comment	Decision
General comment on cross referencing of chapters in the *Aquatic Manual*	Cross referencing of chapters should be consistent throughout the *Aquatic Manual*	Agree: the style is to give the chapter number and title in full, in italics, the first time it is mentioned; just the chapter number is used for all subsequent references to it within the chapter. As the general information chapters have distinct sections, the same rule applies but adapted: in full the first time mentioned in each section, subsequently just the chapter number
A.1.2. Specifications according to crustacean populations	Replace "lots" with "epidemiological units"	Agree
	Clarify the articles i) to iv), which refer to sampling diseased animals only while the opening paragraph of Section 1.2 makes reference to various reasons for sampling	Disagree: the introductory paragraph refers to the design of a surveillance system for demonstrating disease-free status for a country, zone or compartment so choosing diseased animals would be appropriate under such circumstances. Amended the Section to align with Chapter 2.3.0 General information (diseases of fish)
A.1.3. Specifications according to clinical status	Add information on sampling apparently healthy populations for disease freedom or surveillance.	Disagree: the proposal has already been covered in Section 1.2.
	Delete last sentence as the information is already in the previous paragraph	Agree to delete the last sentence

Section/ paragraph	Comment	Decision
A.2.2.2. Virus isolation	Delete the text and replace with "Not applicable"	Agree: *Macrobrachium rosenbergii* nodavirus can be isolated using insect cell lines or SSN-1 but is not a recommended method. Explanatory sentence included.
A.2.3. Bacteriological examination	Remove pathogen-specific information from general crustacean chapter to be consistent with approach in general fish chapter.	Disagree: amended the text to clarify that though not routinely used for listed diseases, bacteriological examination may be used for some
B.1.3.2. Virus production	Clarify the text as it states that infection of known susceptible host species is the preferred method for virus production for experimental purposes	Agree: "for experimental purposes" to the title of the Section
B.5.Techniques	Delete recommending 50 to 150 post larvae suitable for pooling as it conflicts with the recommendations in most disease specific chapters	Agree
B.5.5. Use of molecular and antibody-based techniques for confirmatory testing and diagnosis	Delete the third paragraph on PCR protocols as it does not align with the disease-specific chapters, and should reflect the current approach on use of PCR	Disagree: the paragraph includes important information on what can go wrong with PCR that is not given elsewhere. The paragraph is also included in chapter 3.3.0. It is not related to diagnostic performance and does not conflict with the disease-specific chapters
B.5.5.1. Sample preparation and types		Added a new point vi) on fixed tissues for *in-situ* hybridisation as it was missing
B.5.5.3. Nucleic acid extraction	Add text to indicate that the suitability of extraction kits should be confirmed	Disagree as this is covered in the following sentence

The revised Chapter 2.2.0. General information (diseases of crustaceans), is presented as Annex 34 for comments.

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

Comments were received from Australia, Canada, China (People's Republic of), New Zealand, Thailand, the UK, the USA and the EU.

Background

At its 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 experts and reformatted using the new disease chapter template.

Previous Commission reports where this item was discussed

September 2022 report (Item 7.1.3., page 17).

February 2023

Section/ paragraph	Comment	Decision
Throughout the chapter	Delete the term “highly susceptible” before crayfish species” as this term is inconsistent with the definition of susceptible species in accordance with Chapter 1.5. of the *Aquatic Code*	Agree: reworded to “species that are prone to development of clinical disease”
2.1.1. Aetiological agent	Five groups (A–E) of *A. astaci* are described, but no information on group E is provided	Agree: added a sentence and reference on a species that has been shown to be a carrier of group E
2.2.1. Susceptible host species	Keep the list “under study” as the *ad hoc* Group on susceptibility of crustacean species to WOAH listed diseases has not yet completed the work on this disease	Aligned the text with the *Aquatic Code* and included a note that the *ad hoc* Group assessment is not yet completed
2.2.2. Species with incomplete evidence for susceptibility	Keep the list “under study” as the *ad hoc* Group on susceptibility of crustacean species to WOAH listed diseases has not yet completed the work on this disease	Agree
2.2.3. Likelihood of infection by species, host life stage, population or sub-populations	Delete the text: the information in this section needs to describe any difference in infection between species, if applicable, predilection to infection by life stage. If there is no information then the section should indicate not applicable	Re-worded the text to clarify differences in expression of clinical disease and mortality. Added new text containing some of the information that had been deleted from Section 2.2.1.
2.2.5. Aquatic animal reservoirs of infection	Re-write to better explain statement about “colonisation of habitats” remove incorrect use of the term “susceptibility” and clarify the terms “carrier”, “animal reservoir” and “vector” for this section	Agree: reworded the text on colonisation, replaced “carriers” with “reservoirs” and deleted “highly susceptible”
2.2.6. Vectors	Move the text to Section 2.3.4. *Modes of transmission and life cycle* as it fits better there	Agree: added the text to the end of the section as the fourth paragraph. Also moved the first two paragraphs to after the third paragraph
2.3.6. Geographical distribution	Clarify the text to ensure that information provided to Members is supported by evidence	Agree: deleted the text on potentially infected geographical areas
2.4.2. Chemotherapy including blocking agents	Simplify the text by deleting a redundant clause	Agree

Section/ paragraph	Comment	Decision
2.4.3. Immunostimulation	Simplify the text by deleting a redundant clause	Agree
2.4.4. Breeding resistant strains	Delete text on possible selection for resistance in North American crayfish as it is inaccurate	Agree
2.4.7. General husbandry	Delete all the text as the information is inconsistent with other chapters and is not specific to crayfish plague	Disagree: the text is not inconsistent with other chapters and provides useful information
4.4.1. Real-time PCR, first paragraph following the table of PCR cycling parameters	Delete "which is equivalent to less than one *A. astaci* genome" as it adds confusion to the statement on the detection limit of the method	Agree

The revised Chapter 2.2.2. Infection with *Aphanomyces astaci* (crayfish plague), is presented as Annex 35 for comments.

11.1.3. Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

February 2023

The Aquatic Animals Commission reviewed Chapter 2.2.6. *Infection with Macrobrachium rosenbergii* nodavirus (white tail disease [WTD]), which had been updated by the WOAH Reference Laboratory expert and a Commission member, and reformatted using the new disease chapter template.

The main amendments include:

Section/ paragraph	Change
2.1.1. Aetiological agent	Updated the information in this Section to clearly distinguish the description of the two viruses associated with WTD
2.2.3 Vectors	Expanded this Section to include text and a reference to insect vectors
Table 4.1.	Completed Table 4.1. and aligned with the case definitions in Section 6.
4.2. Histopathology and cytopathology	Added a description of histopathological features and references
4.6. In-situ hybridisation	Added text and a reference
6. Corroborative diagnostic criteria	Revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals
7. References	Updated the references

The revised Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease), is presented as Annex 36 for comments.

11.1.4. Chapter 2.2.9. Infection with yellow head virus genotype 1

February 2023

The Aquatic Animals Commission reviewed Chapter 2.2.9. Infection with yellow head virus genotype 1 [YHD]), which had been updated by the WOAH Reference Laboratory expert and reformatted using the new disease chapter template.

The main amendments include:

Section/ paragraph	Change
2.1.1. Aetiological agent	Removed information on genotypes other than YHV1
Table 4.1.	Completed Table 4.1. and aligned with the case definitions in Section 6.
4.4.2. Conventional RT-PCR	Updated the text: YHV methods are very complicated due to the different genotypes and the need to discriminate them, therefore described some of the particulars of application of the methods that would not otherwise be reflected in the tables
4.6. In-situ hybridisation	Streamlined the description of the test as there are references for it
4.8. Bioassay	Streamlined the description of the test as there are references for it
5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations	Clarified that the nested RT-PCR is recommended. The Commission is aware that a specific real-time PCR method has been validated and publication is pending.
6. Corroborative diagnostic criteria	Revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals
7. References	Updated the references

The revised Chapter 2.2.6. Infection with yellow head virus genotype 1, is presented as Annex 37 for comments.

11.2. Section 2.4. Diseases of molluscs

11.2.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *Perkinsus marinus*

Background

The *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases met in November-December 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 the assessments for susceptibility of mollusc species to Infection with *Perkinsus marinus*.

February 2023 meeting

The Aquatic Animals Commission amended Sections 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *P. marinus*, in line with the recommendations of the *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases (see Item 8.5.).

The revised Section 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *Perkinsus marinus*, is presented as Annex 38 for comments.

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

Background

At its September 2022 meeting, the Aquatic Animals Commission identified two situations relating to the timely dissemination of important new information on diagnostic tests in the *Aquatic Manual*. The first was 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 could be immediately added to the chapter detailing the nature of the problem and providing instructions on how to manage it.

The second issue was that of including new diagnostic tests in the *Aquatic Manual*. The Commission was informed that the Biological Standards Commission was working to develop a template of the validation data that would be requested of applicants wishing to have their tests included in the Terrestrial Manual. The Commission agreed to discuss the template further at its February 2023 meeting, in regards to its suitability and applicability to the *Aquatic Manual*.

Previous Commission reports where this item was discussed

February 2022 (Item 3.3.1., page 16); September 2022 (Item 8.3., page 25)

February 2023

The Aquatic Animals Commission discussed the template and amendments to streamline it and to make it applicable to the *Aquatic Manual,* for example to replace the seven intended fitness for purpose of a diagnostic test in the *Terrestrial Manual* with the three purposes given in the *Aquatic Manual*. The Biological Standards Commission had decided that the template should be used as a 'validation report' form for tests recommended in the *Terrestrial Manual.*

The Aquatic Animals Commission stressed the importance of validating diagnostic tests for aquatic animal diseases. It considered that publication of diagnostic accuracy studies in peer-reviewed journals was preferable; however in some instances, this validation report form could provide a mechanism for incorporation of new or revised methods pre-publication. The Commission identified a WOAH Reference Laboratory expert who is in the final stages of developing a new PCR method. Once the changes have been made to the template, it will be provided to the expert and the expert's feedback will be reviewed at the next meeting.

12. *Ad hoc* Groups

12.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases

The *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases met in November/December 2022 to complete the assessments for susceptibility of mollusc species to infection with *Perkinsus marinus* (see Items 8.5. and 11.2.1.).

The Commission was informed that the *ad hoc* Group is planning to meet in June 2023 to progress its work assessing species susceptible to infection with *Perkinsus olseni*.

The report of the *ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases is available on the WOAH Website at: https://www.woah.org/en/what-we-do/standards/standard-setting-process/ad-hoc-groups/#ui-id-3

12.2. *Ad hoc* Group on Susceptibility of fish species to infection with WOAH listed diseases

The *ad hoc* Group on Susceptibility of fish species to infection with WOAH listed diseases met in April and November/December 2022 to complete the assessments for susceptibility of fish species to infection

with red sea bream iridovirus genogroup, infectious spleen and kidney necrosis virus genogroup, turbot reddish body iridovirus genogroup and infectious spleen and kidney necrosis virus species (see Item 8.3.).

The Commission was informed that the *ad hoc* Group is planning to meet in April 2023 to progress its work assessing species susceptible to infection with tilapia lake virus.

The report of the *ad hoc* Group on susceptibility of fish species to infection with WOAH listed diseases is available on the WOAH Website at: https://www.woah.org/en/what-we-do/standards/standard-setting-process/ad-hoc-groups/#ui-id-3

12.3. *Ad hoc* Group on Susceptibility of crustacean species to infection with WOAH listed diseases

The Commission was informed that the *ad hoc* Group on Susceptibility of crustacean species to infection with WOAH listed diseases would be reconvened to apply the criteria in Chapter 1.5., for listing species as susceptible to infection with a specific pathogen to WOAH listed diseases, for diseases listed since it last met (in 2016). The *ad hoc* Group plans to meet in March 2023, to conduct assessments for susceptibility of crustacean species to infection with decapod iridescent virus 1.

13. Reference centres or change of experts

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

The Aquatic Animals Commission reviewed an application for a WOAH Collaborating Centre for Economics of Animal Health. The Commission was impressed with this strong application, which is linked to the other WOAH Collaborating Centre is this field and the WOAH-led project on the Global Burden of Animal Disease (GBADs). The Commission was pleased that aquatics was one of the central targeted areas of activity. The Commission fully endorsed the application and recommended its acceptance:

WOAH *Collaborating Centre for Economics of Animal Health in the Americas Region*

Department of Agricultural Economics, Kansas State University, UNITED STATES OF AMERICA
Tel.: (+1-785 532.35.25)
E-mail: dpendell@ksu.edu
Website: http://www.ksu.edu/
Designated Contact Point: Dustin L. Pendell.

This multi-national WOAH Collaborating Centre will include participation from the following institutions:

Department of Economics, Business and Sociology (ESALQ/USP), University of São Paulo, BRAZIL
and
Faculty of Agronomy and Veterinary Medicine, University of Brasília, BRAZIL
Tel: (+55-19) 34.29.44.44 and (+55-61) 992.09.06.66
E-mail: shgdmira@usp.br / vitorspg@unb.br
Web site: www.esalq.usp.br / www.unb.br
Designated Contact Points: Dr Silvia Helena Galvão de Miranda / Prof. Vitor Salvador Picão Gonçalves.

Department of Business, Economics and Rural Development, Faculty of Veterinary Medicine and Husbandry, Universidad Nacional Autonoma De México, MEXICO
Tel: (+1-52) 56.22.59.05
E-mail: jldf@fmvz.unam.mx
Web site: www.fmvz.unam.mx
Designated Contact Point: Prof. José Luis Dávalos Flores.

School of Economic Sciences, Paul G. Allen School for Global Health, Washington State University, UNITED STATES OF AMERICA
Tel: (+1-509) 335.85.97

E-mail: tl_marsh@wsu.edu
Web site: www.wsu.edu
Designated Contact Point: Prof. Thomas L. Marsh.

14. Any other business

14.1. Registration of Diagnostic Kits

14.1.1. WOAH Registry of Diagnostic kits

The Commission was informed about potential ways to increase the value WOAH can provide to Members in the field of diagnostics kits. After twenty years of existence, and only 14 kits being part of WOAH's registry, an internal consultation was carried out with key stakeholders in the field, internal and external, yielding three options worth exploring:

- Explore mechanisms that could be implemented for facilitating regulatory harmonisation of diagnostic kits.
- Explore value of setting minimum criteria needed for reliable Registration of Diagnostic Kits, facilitating accessibility to Members, regardless of their regulatory capacity.
- Streamline recognition and alignment of WOAH Reference Laboratories with WOAH Diagnostic Kit activities.

This might lead to a renewed role for the Secretariat for Registration of Diagnostic Kits. While the aforementioned leads are explored, no new dossiers will be considered. Only those currently under evaluation or potential renewals will be processed, as well as exceptional cases linked to an emergency situation for animal health

14.1.2. Innocreate Bioscience WSSV RP Rapid Test Kit

The Aquatic Animals Commission was informed that the evaluation of the new dossier for the Innocreate Bioscience WSSV RP Rapid Test Kit (Innocreate Bioscience Co., Ltd) had been completed. After consideration of the final report of the WOAH Expert Review Panel, the Commission endorsed the Panel's recommendation to approve the kit to be added to the WOAH Register of Diagnostic kits, validated as fit for purpose (qualitative detection kit for Whispovirus, White Spot Syndrome Virus (WSSV) infection in shrimps). This lateral flow immunoassay device is designed for the following purposes:

- Field based confirmatory diagnosis of clinical cases (includes confirmation of suspect cases and a positive screening test).
- Estimate the prevalence of infection to facilitate risk analysis in production system shrimp farms to aid in management practices. IMPORTANT - the test kit should not be used to estimate prevalence in broodstock or post larvae shrimp for risk analysis prior to translocation to other farms or across borders).
- For use in conjunction with other tests or diagnostic procedures, as an aid in the diagnosis or other clinical or epidemiological assessments.

The Validation Studies Abstract drafted by the manufacturer and approved by the Expert Review Panel, was endorsed by the Commission and is presented as Annex 39.

The addition of 'Innocreate Bioscience WSSV RP Rapid Test Kit, (Innocreate Bioscience Co., Ltd)' to the WOAH Register of Diagnostic kits validated as fit for purpose, will be proposed for adoption at the 90th General Session in May 2023.

_____

…/Annexes

Annex 1. Item 2 – Adopted Agenda

MEETING OF THE WOAH AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Hybrid, 19 January and 15 to 22 February 2023

_____

1. Welcome from the Deputy Director General
2. Adoption of the agenda
3. Meeting with the Director General
4. Cooperation with Terrestrial Animal Health Standards Commission
   - 4.1. Update on the revised Chapter 6.10. Responsible and prudent use of antimicrobial agents in veterinary medicine of the *Terrestrial Code*
   - 4.2. Update on the *ad hoc* Group on the revision of Chapters 5.4. to 5.7. of the *Terrestrial Code*
   - 4.3. Glossary definitions: 'Aquatic Animal Health Services', 'Competent Authority' and 'Veterinary Authority'
   - 4.4. Chapter 4.3. Application of Compartmentalisation
5. Cooperation with Biological Standards Commission
   - 5.1. Reference Centres: discussion on report templates, SOPS and use of data collected
   - 5.2. Aquatic and Terrestrial Manuals: areas of common interest
     - 5.2.1. AAC's table on PCR parameters for consideration by BSC
     - 5.2.2. Updated validation chapter
     - 5.2.3. Addition of a new section to the disease-specific chapters to describe the rationale behind the selection of tests for different purposes given in Table 1 Test methods available and their purpose and an explanation for their score.
     - 5.2.4. Development of a template for validation reports for tests in the Terrestrial Manual
   - 5.3. Work on the list of WOAH approved reference reagents
6. Work plan of the Aquatic Animals Commission
7. Aquatic Animal Health Strategy
8. *Aquatic Code*
   - 8.1. Texts that will be proposed for adoption in May 2023
     - 8.1.1. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)
     - 8.1.2. Article 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus
     - 8.1.3. Article 9.5.2 of Chapter 9.5. Infection with infectious myonecrosis virus.
     - 8.1.4. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus
     - 8.1.5. New Chapter 10.X. Infection with tilapia lake virus

8.1.6. Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*

8.1.7. Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*

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

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

8.2. Items for consideration

8.2.1. Change of OIE to WOAH in the *Aquatic Code*

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

8.2.3. Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information

8.2.4. Chapter 1.3. Diseases listed by WOAH

8.2.4.1. Infection with infectious spleen and kidney necrosis virus

8.2.5. Chapter 4.3. Application of compartmentalisation

8.2.6. New Chapter 4.X. Emergency disease preparedness

8.2.7. New Chapter 4.Y. Disease outbreak management

8.2.8. New Chapter 5.X. Ornamental aquatic animals

8.2.9. New Chapter 5.Y. Trade of genetic materials

8.2.10. Safe commodities – Articles X.X.3. for disease-specific chapters

8.2.10.1. Safe commodities consultant report.

8.2.10.2. Revised Articles 8.X.3. for amphibian disease-specific chapters

8.2.10.3. Revised Articles 9.X.3. for crustacean disease-specific chapters

8.2.10.4. Revised Articles 10.X.3. for fish disease-specific chapters

8.2.10.5. Revised Articles 11.X.3. for mollusc disease-specific chapters

8.2.11. Model Articles X.X.4.-X.X.8.

8.2.12. Assessment of default periods in Articles X.X.4.-X.X.8. for disease-specific chapters

8.2.13. Article 9.8.2. of Chapter 9.8. Infection with white spot syndrome virus

8.2.14. Article 11.5.1. and 11.5.2. of Chapter 11.5. Infection with *Perkinsus marinus*

8.2.15. Consideration of emerging diseases

8.2.15.1. Infection with carp edema virus (CEV)

8.2.15.2. Covert mortality nodavirus (CMNV) in zebrafish

8.2.16. Wildlife Strategy Consultancies

9. Aquatic Manual

9.1. Texts that will be proposed for adoption in May 2023

9.1.1. Section 2.2. Diseases of crustaceans

9.1.1.1. Chapter 2.2.1. Acute hepatopancreatic necrosis disease

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

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

9.1.2. Section 2.3. Diseases of fish

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

9.1.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus

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

9.1.3. Section 2.4. Diseases of molluscs

9.1.3.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*

9.1.3.2. Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae*

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

9.2. Items for consideration

9.2.1. Change of OIE to WOAH in the *Aquatic Manual*

9.2.2. Chapter 1.1.2. Principals and methods of validation of diagnostic assays for infectious diseases

9.2.3. Section 2.2. Diseases of crustaceans

9.2.3.1. Chapter 2.2.0. General information: diseases of crustaceans

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

9.2.3.3. Chapter 2.2.5. Infection with infectious myonecrosis virus

9.2.3.4. Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

9.2.3.5. Chapter 2.2.7. Infection with taura syndrome virus

9.2.3.6. Chapter 2.2.8. Infection with white spot syndrome virus

9.2.3.7. Chapter 2.2.9. Infection with yellow head virus genotype 1

9.2.4. Section 2.4. Diseases of molluscs

9.2.4.1. Section 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *Perkinsus marinus*

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

10. *Ad hoc* Groups

10.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases

10.2. *Ad hoc* Group on Susceptibility of fish species to infection with WOAH listed diseases

10.3. *Ad hoc* Group on Susceptibility of mollusc species to infection with WOAH listed diseases

11. Reference Centres or change of experts

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

12. Other issues

12.1. For discussion

12.1.1. Registration of Diagnostic Kits

12.1.2. Self-declarations of freedom

12.1.3. Commission reports – presenting annexes separate to the report text.

12.2. For information

12.2.1. Global Burden of Animal Diseases (GBADs) – present Aquatic Projects

13. Meeting Review

14. Next Meeting: 13-20 September 2023

Annex 2. Item 2 – List of Participants

MEETING OF THE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Hybrid, 19 January and 15 to 22 February 2023

_____

MEMBERS OF THE COMMISSION

Dr Ingo Ernst
(President)
Director Aquatic Pest and Health Policy,
Department of Agriculture, Fisheries and Forestry,
Canberra,
AUSTRALIA

Dr Alicia Gallardo Lagno
(Vice-President)
Senior advisor FARMAVET,
University of Chile,
La Pintana,
CHILE

Dr Prof. Hong Liu
(member)
Deputy Director,
Animal and Plant Inspection and Quarantine Technical Centre,
Shenzhen City,
CHINA (People's Rep of)

Dr Fiona Geoghegan
(Vice-President)
Legislative Officer,
European Commission,
DG SANTE
Brussels,
BELGIUM

Dr Kevin William Christison
(member)
Specialist Scientist,
Department of Forestry, Fisheries and the Environment,
Vlaeberg,
SOUTH AFRICA

Dr Espen Rimstad
(member)
Professor in Virology,
Norwegian University of Life Sciences
Ås,
NORWAY

OTHER PARTICIPANTS

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

Prof Edmund Peeler
Epidemiologist
Aquatic Pests and Pathogens,
Barrack Road, Weymouth
Dorset, DT4 8UB
UNITED KINGDOM

WOAH HEADQUARTERS

Dr Gillian Mylrea
Head of Department
Standards Department

Dr Kathleen Frisch
Scientific Coordinator for Aquatic Animal Health
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 5. – Work plan and priorities

WORK PLAN FOR THE AQUATIC ANIMALS COMMISSION

(including provisional timelines for commenting and adoption)

Aquatic Code					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Monitor emerging diseases and consider any required actions	On-going				
Glossary definitions: 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services'	Reviewed usage in the *Aquatic Code* and present amendments for comments		Review comments (1st round)	Review comments (2nd round)	Propose for adoption
Chapter 1.3. Diseases listed by WOAH – Listing of infection with infectious spleen and kidney necrosis virus species	Reviewed comments (1st round)		Review comments (2nd round)	Review Member comments (3rd round)	Propose for adoption
SOP for Self Declaration of freedom	Drafted a template for Member guidance for submission of a self-declaration of freedom	Upload onto WOAH website prior to the General Session			
Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information	Reviewed and amended Article 1.1.5 and presented for comments		Review comments (1st round)	Review comments (2nd round)	Propose for adoption
Chapter 4.3. Application of Compartmentalisation	Reviewed Member responses to questionnaire and initiate working on draft discussion paper		Present discussion paper for comment	Review responses to discussion paper	
Chapter 4.X. New draft chapter on Emergency disease preparedness	Continued to work on draft Chapter 4.X.		Present draft chapter for comment	Review comments (1st round)	
Chapter 4.Y. New draft chapter on Disease outbreak management	Continued to work on draft Chapter 4.Y.		Present draft chapter for comment	Review comments (1st round)	
Chapters 5.6. – 5.9.	Reviewed TCC *ad hoc* Group report		Review TCC *ad hoc* Group report		
Chapter 5.X. Trade in ornamental aquatic animals	Reviewed draft outline for chapter		Present draft chapter for comment	Review comments (1st round)	

Aquatic Code					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Chapter 5.Y. Trade in genetic materials	Reviewed draft outline for chapter		Present draft chapter for comment	Review comments (1st round)	
Chapter 6.2. Principals for responsible and prudent use of the antimicrobial agents in aquatic animal	Received update from TCC on the on-going revision to *Terrestrial Code* Chapter 6.10.		Update from TCC on the on-going revision to *Terrestrial Code* Chapter 6.10.	Update from TCC on the on-going revision to *Terrestrial Code* Chapter 6.10.	
Susceptible Species Assessment of new species/evidence for previously assessed diseases as necessary	On-going				
Safe commodities – disease specific chapters Articles 8.X.3. – Amphibian Articles 9.X.3. – Crustacean Articles 10.X.3. – Fish Articles 10.X.3. – Mollusc	Amphibian: Reviewed Member comments (1st round), amend articles based on safe commodity assessment		Review comments (2nd round)	Review comments (3rd round)	Present for adoption
	Crustacean: amended articles based on safe commodity assessment and present for Member comment		Review comments (1st round)	Review comments (2nd round)	Present for adoption
	Fish: amended articles based on safe commodity assessment and present for Member comment		Review comments (1st round)	Review comments (2nd round)	Present for adoption
	Mollusc: Reviewed Member comments (1st round), amended articles based on safe commodity assessment		Review comments (2nd round)	Review comments (3rd round)	Present for adoption
Assessment of default periods in Articles X.X.4.-X.X.8. for disease-specific chapters			Present assessment of default periods for comment	Review comments (1st round)	
Susceptible Species – Crustacean diseases –	Re-convened *ad hoc* Group:		Infection with DIV1: Review *ad hoc* Group	Review comments (1st round)	Present for adoption

Aquatic Code					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Articles 9.X.1. and 9.X.2. for: **– Infection with decapod iridescent virus** **– Infection with white spot syndrome virus** **– Infection with *Aphanomyces astaci* (Crayfish plague)** **– review new evidence for previously assessed diseases**	Next meeting March 2023		report and present amended articles for comment		
				Infection with WSSV: Review *ad hoc* Group report and present amended articles for comment	
Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)	Reviewed 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)	Reviewed comments (2nd round)	Propose for adoption			
Article 9.5.2. of Chapter 9.5. Infection with infectious myonecrosis virus (IMNV)	Reviewed 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)**	Infection with RSIV: Reviewed *ad hoc* Group report				
	Infection with TiLV: Next *ad hoc* Group meeting planned for April 2023		Infection with TiLV: Review *ad hoc* Group report and present amended articles for comment	Review comments (1st round)	Propose for adoption
				Infection with EUS: Review interim *ad hoc* Group report	
Articles 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus	Reviewed comments (1st round)	Propose for adoption			
Chapter 10.X. Infection with tilapia lake virus	Reviewed comments (1st round)	Propose for adoption			
Susceptible species – Mollusc diseases – Articles 11.X.1. and 11.X.2. for:	*Marteilia refringens* : Reviewed comments (1st round)	Propose for adoption			

Aquatic Code					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
– **Infection with *Marteilia refringens*** – **Infection with *Perkinsus marinus*** – **Infection with *Perkinsus olseni*** – **Infection with *Xenohaliotis californiensis***	*Perkinsus marinus*: Reviewed *ad hoc* Group report and present amended articles for comment		Review Comments (1st round)	Review Comments (2nd round)	Propose for adoption
			Perkinsus olseni: Review interim *ad hoc* Group report	*Perkinsus olseni*: Review *ad hoc* Group report and present amended articles for comment	
Susceptible species – Articles 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*	Reviewed comments (1st round)	Propose for adoption			
Susceptible species – Articles 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*	Reviewed comments (1st round)	Propose for adoption			
Model articles 11.X.9. – 11.X.14.: Harmonisation with other disease-specific chapters	Reviewed comments (1st round)	Propose for adoption			

Aquatic Manual					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Chapter 2.2.0. General provisions – Crustaceans	Reviewed comments (1st round)		Review comments (2nd round)	Review comments (3rd round)	Propose for adoption
Chapter 2.2.1. Acute hepatopancreatic necrosis disease	Reviewed comments (2nd round)	Propose for adoption			
Chapter 2.2.2. Infection with *Aphanomyces astaci* (Crayfish plague)	Reviewed comments (1st round)		Review comments (2nd round)	Review comments (3rd round)	Propose for adoption
Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)	Reviewed comments (2nd round)	Propose for adoption			
Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus	Reviewed comments (2nd round)	Propose for adoption			
Chapter 2.2.5. Infection with infectious myonecrosis virus	Reviewed comments (1st round)	Propose for adoption			

Aquatic Manual					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Chapter 2.2.6. Infection with *Macrobrachium rosenbergii nodavirus* (white tail disease)	Reviewed further updated draft and presented for comments		Review comments (1st round)	Review comments (2nd round)	Propose for adoption
Chapter 2.2.7. Infection with taura syndrome virus	Reviewed comments (1st round)	Propose for adoption			
Chapter 2.2.8. Infection with white spot syndrome virus	Reviewed comments (1st round)	Propose for adoption			
Chapter 2.2.X. Infection with decapod iridescent virus 1			Review updated draft and present for Member comments	Review comments (1st round)	
Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)	Reviewed comments (2nd round)	Propose for adoption			
Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus	Reviewed comments (3rd round)	Propose for adoption			
Sections 2.2.2. of Chapter 2.3.9. Infection with spring viraemia of carp virus	Reviewed Comments (1st round)	Propose for adoption			
Chapter 2.3.X. Infection with tilapia lake virus			Review updated draft and present for Member comments	Review comments (1st round)	
Chapter 2.4.0. General Information			Review updated draft and present for comments	Review comments (1st round)	
Chapter 2.4.1. Infection with abalone herpes virus			Review updated draft and present for comments	Review comments (1st round)	
Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens*	Reviewed comments (1st round)	Propose for adoption			
Section 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*	Reviewed comments (1st round)	Propose for adoption			
Chapter 2.4.3. Infection with *Bonamia ostreae*			Review updated draft and present for comments	Review comments (1st round)	
Section 2.2.2. of Chapter 2.4.2. Infection with *Bonamia ostreae*	Reviewed comments (1st round)	Propose for adoption			

Aquatic Manual					
Chapter/Subject	**Status**				
	February 2023	**May GS 2023**	**September 2023**	**February 2024**	**May GS 2024**
Chapter 2.4.7. Infection with *Xenohaliotis californiensis*			Review updated draft and present for comments		

Annex 4. Item 7.1. – Article 9.3.1. of Chapter 9.3. Infection with NHP

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 5. Item 7.2. – Articles 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*) and yellowleg shrimp (*Penaeus californiensis*).

[…]

Annex 6. Item 7.3. – 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 7. Item 7.4. – 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*	~~B~~bream
	Aristichthys nobilis	~~B~~bighead carp
	Carassius auratus	~~G~~goldfish
	Ctenopharyngodon idella	~~G~~grass carp
	Cyprinus carpio	~~C~~common carp (all varieties and subspecies)
	Danio rerio	~~Z~~zebrafish
	Notemigonus crysoleucas	~~G~~golden shiner
	Pimephales promelas	~~F~~fathead minnow
	Percocypris pingi	Jinsha barbel ~~bass~~ carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	~~R~~roach
Siluridae	*Silurus glanis*	Wels catfish

[…]

Annex 8. Item 7.5. – Chapter 10.X. Infection with TiLV

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*, C*ompetent Authorities* should not require any *sanitary measures* related to TiLV, regardless of the infection with ~~TILV~~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~~60°C for at least ~~five~~120 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~~60°C for at least ~~five~~120 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~~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~~TILV~~

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

As described in Article 1.4.~~X~~4., a Member Country may make a self-declaration of freedom from infection with TiLV~~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~~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~~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~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place 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~~TILV~~ but the following conditions have been met:

   a) on detection of TiLV~~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~~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~~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~~ and farmed *susceptible species* without detection of TiLV~~TILV~~; or

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

In the meantime, the part of the country outside the infected zone and protection zones ~~part or all of the country, apart from the *infected* and *protection zones,*~~ may be declared a free *zone* in accordance with Article 1.4.4. provided ~~that such a part meets the conditions in point 2 of Article 10.X.6.~~points 4. a) to c) have been achieved.

Article 10.X.6.

Zone free from infection with TiLV~~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~~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~~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~~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~~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~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place 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~~TILV~~ in the *zone* but the following conditions have been met:

   a) on detection of TiLV~~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~~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~~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~~TILV~~.

In the meantime, a part of the *zone* outside the *infected zone* and *protection zone* may be declared a new free *zone* in accordance with Article 1.4.4. provided points 4. a) to c) have been achieved.

Article 10.X.7.

Compartment free from infection with TiLV~~TILV~~

As described in Article 1.4.X., a Member Country may make a self-declaration of freedom from infection with TiLV~~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~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place 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~~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~~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~~ one survey for infection with TiLV has been completed at least [six months] after restocking (as described in Article 1.4.14.) without detection of the pathogen~~TILV~~.

Article 10.X.8.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TiLV~~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~~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~~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~~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~~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~~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~~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~~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~~THLV~~.

b) In the importing *country*:

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

ii) test the F-0 population for TiLV~~THLV~~ 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~~THLV~~, and sample and test for TiLV~~THLV~~ in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter X.X.6. of the *Aquatic Manual*;

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

vi) if TiLV~~THLV~~ 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.~~7~~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~~THLV~~

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~~THLV~~ 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~~THLV~~ 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~~THLV~~

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~~THLV~~, 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~~THLV~~ 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~~THLV~~ 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~~THLV~~

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~~THLV~~, 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~~THLV~~ 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~~THLV~~ 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~~THLV~~ status of the exporting country, zone or compartment

1) [*Competent Authorities* should not require any conditions related to TiLV~~THLV~~, regardless of the infection with TiLV~~THLV~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation ~~(~~or transit~~)~~ of the following ~~commodities~~ *aquatic animal products* 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~~THLV~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Annex 9. Item 7.6. – Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*

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*), Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*), and Olympia oyster (*Ostrea lurida*) and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*).

[…]

Annex 10. Item 7.6. – Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*

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.: Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~European flat oyster (*Ostrea edulis*)~~, Chilean flat oyster (*Ostrea chilensis*) and European flat oyster (*Ostrea edulis*)~~Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~.

[…]

Annex 11. Item 7.7. – Article 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*

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* (including O and M types) 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 clam (*Chamelea gallina*). ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

[…]

Annex 12. Item 7.8. – Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters

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 or~~and~~ 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 a 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.~~4~~5., 11.X.~~5~~6. or 11.X.7. (as applicable) and 11.X.~~6~~8., the place of production of the *aquatic animals* or~~and~~ *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 products*~~*commodities*~~ ~~referred to~~listed 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~~inactivate [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* for a duration sufficient for, and 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 sample and test for [Pathogen X] in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter 2.4.X. of the *Aquatic Manual*;

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 in accordance with Chapter 4.8.

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 a 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~~14., 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* or containment facilities ~~for slaughter and processing~~until processed into one of the products referred to in point 1 of Article 11.X.3. 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~~ to ~~that~~ ensure~~s~~ 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.~~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 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.~~13~~14.

Importation ~~for transit~~ of ~~aquatic animals and~~ aquatic animal products for retail trade for human consumption regardless of the infection with [Pathogen X] status of the exporting country, zone or compartment~~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 *aquatic animal products*~~*commodities*~~ that~~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 13. Item 8.1. Glossary definitions: 'Aquatic Animal Health Services', 'Competent Authority' and 'Veterinary Authority'

Article	2022 *Aquatic Code* Page Number	Usage
User's guide B.5.	vii	5) The standards in the chapters of Section 3 are designed for the establishment, maintenance and evaluation of Aquatic Animal Health Services, including communication. These standards are intended to assist the Aquatic Animal Health Services and the Competent Authorities of Member Countries to meet their objectives of improving aquatic animal health and the welfare of farmed fish, as well as to establish and maintain confidence in their international aquatic animal health certificates.
User's guide: C.8.	ix	8) International aquatic animal health certificates An international aquatic animal health certificate is an official document that the Competent Authority of the exporting country issues in accordance with Chapter 5.1. and Chapter 5.2. It lists aquatic animal health requirements for the exported commodity. The quality of the exporting country's Aquatic Animal Health Services is essential in providing assurances to trading partners regarding the safety of exported aquatic animal products. This includes the relevant ~~Aquatic Animal Health Services'~~Competent Authority's ethical approach to the provision of international health certificates and the Veterinary Authority's history in meeting their notification obligations.
Glossary	xiv	***NOTIFICATION*** means the procedure by which: a) the ~~*Competent Authority*~~*Veterinary Authority* informs the *Headquarters*, b) the *Headquarters* inform ~~*Competent Authority*~~ the *Veterinary Authority* of Member Countries of the occurrence of a *disease* in accordance with the provisions of Chapter 1.1.
Article 1.1.1.	1	For the purposes of the *Aquatic Code* and in terms of Articles 5, 9 and 10 of the Organic Statutes of the Office International des Epizooties, Member Countries shall recognise the right of the *Headquarters* to communicate directly with the ~~*Competent Authority*~~*Veterinary Authority* of its *territory* or *territories*. All *notifications* and all information sent by WOAH to the ~~*Competent Authority*~~ *Veterinary Authority* shall be regarded as having been sent to the country concerned and all *notifications* and all information sent to WOAH by the ~~*Competent Authority*~~ *Veterinary Authority* shall be regarded as having been sent by the countryconcerned.
Article 1.1.3. paragraph 1	2	The ~~*Competent Authority*~~*Veterinary Authority* shall, under the responsibility of the Delegate, send to the *Headquarters*:
Article 1.1.4. paragraph 1	2	~~*Competent Authority*~~*Veterinary Authority* shall, under the responsibility of the Delegate, send to the *Headquarters*:
Article 1.1.5. point 1	2	The ~~*Competent Authority*~~ *Veterinary Authority* of a country in which an *infected zone* or *compartment* is located shall inform the *Headquarters* when this country, *zone* or *compartment* becomes free from the *disease*.
Article 1.1.5. point 3	2	The ~~*Competent Authority*~~ *Veterinary Authority* of a Member Country which establishes one or several *free zones* or *free compartments* shall inform the *Headquarters*, giving necessary details, including the criteria on which the free status is based, the requirements for maintaining the status and indicating clearly the location of the *zones* or *compartments* on a map of the territory of the Member Country.
Article 3.1.2. point 7 paragraph 3	32	*Competent Authorities*~~*Aquatic Animal Health Services*~~ should define and document the responsibilities and structure of the organisation (in particular the chain of command) in charge of issuing *international aquatic animal health certificates*.

Article 3.1.2. point 10	33	10. Information, complaints and appeals The relevant *Competent Authority*~~*Aquatic Animal Health Services*~~ should undertake to reply to requests from ~~*Aquatic Animal Health Services*~~ *the Competent Authority* of other Member Countries ~~or any other authority~~, in particular ensuring that any requests for information, complaints or appeals that are presented are dealt with in a timely manner. A record should be maintained of all complaints and appeals and of the relevant action taken by the *Competent Authority* ~~*Aquatic Animal Health Services*~~.
Article 3.1.5. paragraph 4	34	The expert(s) facilitate(s) the evaluation of the *Aquatic Animal Health Services* of the Member Country using the WOAH *Performance of* ~~*Veterinary Services and/or*~~ *Aquatic Animal Health Services* (WOAH *PVS Tool*- Aquatic). The expert(s) produce(s) a report in consultation with the ~~Veterinary Services~~*Aquatic Animal Health Services* of the Member Country.
Article 3.2.1. paragraph 2	35	The recognition of communication as a discipline of the *Aquatic Animal Health Services* and its incorporation within it is critical for their operations. The integration of aquatic animal health and communication expertises is essential for effective communication. ~~Communication between the *Aquatic Animal Health Services,* and Veterinary Services (particularly where *Aquatic Animal Health Services* are separate, and independent of Veterinary Services) is especially important.~~
Article 4.2.3. point 1	50	1) The extent of a *zone* should be established by the ~~*Aquatic Animal Health Service*~~*Competent Authority* on the basis of the definition of *zone* and made public through official channels.
Article 4.2.3. point 3	50	3) The factors defining a *compartment* should be established by the ~~*Aquatic Animal Health Service*~~*Competent Authority* on the basis of relevant criteria such as management and husbandry practices related to *biosecurity*, and made public through official channels.
Article 4.2.3. point 6	50	6) For a *compartment*, the *biosecurity plan* should describe the partnership between the relevant enterprise/industry and the ~~*Aquatic Animal Health Service*~~ *Competent Authority*, and their respective responsibilities, including the procedures for oversight of the operation of the *compartment* by the ~~*Aquatic Animal Health Service*~~ *Competent Authority*.
Article 5.3.4. point 2) a	85	a) infrastructure including the legislative base (e.g. *aquatic animal* health law) and administrative systems (e.g. organisation of ~~Veterinary Service~~s ~~or *Aquatic Animal Health Services*~~*Competent Authority*);
Article 5.3.7. point 1) d i	88	i) an evaluation of the *exporting country*'s ~~Veterinary Services or~~ *Aquatic Animal Health Services*;
Article 5.3.7. point 2) e i	88	ii) an evaluation of the *exporting country*'s ~~Veterinary Services or~~ *Aquatic Animal Health Services*;

Annex 14. Item 8.2. – Article 1.1.5. of Chapter 1.1. Notification of diseases and provision of epidemiological information

CHAPTER 1.1.

NOTIFICATION OF DISEASES, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

[…]

~~Article 1.1.5.~~

~~1) The *Competent Authority* of a country in which an *infected zone* or *compartment* is located shall inform the *Headquarters* when this country, *zone* or *compartment* becomes free from the *disease*.~~

~~2) A country, *zone* or *compartment* may be considered to have regained freedom from a specific *disease* when all relevant conditions given in the *Aquatic Code* have been fulfilled.~~

~~3) The *Competent Authority* of a Member Country which establishes one or several *free zones* or *free compartments* shall inform the *Headquarters*, giving necessary details, including the criteria on which the free status is based, the requirements for maintaining the status and indicating clearly the location of the *zones* or *compartments* on a map of the territory of the Member Country.~~

Article 1.1.~~6~~5.

1) Although Member Countries are only required to notify *listed diseases* and *emerging diseases*, they are encouraged to provide WOAH with other important *aquatic animal* health information.

2) The *Headquarters* shall communicate by email or through the interface of WAHIS to *Competent Authorities* all *notifications* received as provided in Articles 1.1.2. to 1.1.~~5~~4. and other relevant information.

[…]

Annex 15. Item 8.3. – Article 1.3.1. of Chapter 1.3. Diseases listed by WOAH – Listing of infectious spleen and kidney necrosis virus species

CHAPTER 1.3.

DISEASES LISTED BY WOAH

[…]

Article 1.3.1.

The following *diseases* of fish are *listed diseases*:

– 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 all genogroups of the virus species *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 16. Item 8.3. – Assessment of infection with all genogroup of the virus species infectious spleen and kidney necrosis virus for listing in the *Aquatic Code*

ASSESSMENT OF INFECTION WITH ALL GENOGROUPS OF THE VIRUS SPECIES 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 all genogroups of the virus species ISKNV”. Infection with all genogroups of the virus species 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 all genogroups of the viral species 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 WOAH 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; Hick *et al*., 2016).

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 the ISKNV species. When used within this document, "ISKNV genogroup" refers to the genogroup ISKNV and "the species ISKNV" is used whenever referring to the species ISKNV.

Infection with 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 ISKNV genogroup 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 genogroup has been detected in archival ornamental fish samples from as early as 1996 (Go *et al*., 2006; Go *et al*., 2016; Becker *et al*., 2022). 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 genogroup was analysed and found to be genetically similar to RSIV (He *et al*., 2001). ISKNV genogroup has been detected from numerous freshwater fish species, including many associated with ornamental fish trade (see review by Johan & Zainathan, 2020; Becker *et al*., 2022). This genogroup has been reported from numerous species of ornamental fish that have been traded internationally (see Rimmer *et al*., 2015). ISKNV genogroup 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; Fusianto *et al*., 2021).

The TRBIV genogroup 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

[1] RSIV was included in the *Aquatic Code* prior to 2003 as an "other disease of significance".
[2] Note that the species listed as susceptible to infection with RSIV in accordance with Chapter 1.5. of the *Aquatic Code* has not been revised based on the recommendations of the *ad hoc* Group.

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 the ISKNV and TRBIV genogroups, 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

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 genogroup 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; Rimmer *et al*., 2015) and as such may act as carriers of the virus. ISKNV genogroup has also been detected in important farmed species for human consumption that are traded internationally, such as tilapia (Ramírez-Paredes *et al*., 2020). ISKNV genogroup 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 genogroup 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 genogroup and may be able to demonstrate freedom. Further, PCR assays used in surveillance for RSIV would also detect ISKNV genogroup, providing evidence of freedom from ISKNV genogroup.

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.

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 some PCR assays for RSIV (and some other methods, e.g. histopathology), are inclusive of ISKNV genogroup, the case definitions could be easily adapted to include ISKNV genogroup. 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 genogroups. Kim *et al.* (2022) reported on the performance of a real-time PCR assay with inclusivity for RSIV, ISKNV and TRBIV genogroups. 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 genogroup has been associated with numerous cases of disease in ornamental fish (see review by Johan & Zainathan, 2020; Becker *et al*., 2022). ISKNV genogroup 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

[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.

(e.g. Figueiredo *et al*., 2021; Ramírez-Paredes *et al*., 2021) and groupers (e.g. Chao *et al.*, 2004; Huang *et al.*, 2020; Fusianto *et al*., 2021).

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 genogroups in wild fish populations and their consequences such as morbidity, mortality or ecological impacts. ISKNV genogroup 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.

References

ARMSTRONG, R. & FERGUSON, H. (1989). Systemic viral disease of the chromide cichlid Etroplus maculatus. *Diseases of Aquatic Organanisms,* **7**, 155-157.

ANDERSON, I.G., PRIOR, H.C., RODWELL, B.J. & HARRIS, G.O. (1993). Iridovirus-like virions in imported dwarf gourami (*Colisa lalia*) with systemic amoebiasis. *Australian Veterinary Journal,* **70(2)**, 66-67.

BECKER, J.A., FUSIANTO, C., HICK, P.M. (2022). Infection with Megalocytivirus in Ornamental Fish. In: Aquaculture Pathophysiology, Pharmacology and Toxicology (F. Kibenge, R.S. Chong, B. Baldisserotto, eds), Elsevier. (Currently IN REVIEW).

CHAO, C.B., CHEN, C.Y., LAI, Y.Y., LIN, C.S. & HUANG, H.T. (2004). Histological, ultrastructural, and in situ hybridization study on enlarged cells in the grouper *Epinephelus* hybrids infected with grouper iridovirus in Taiwan (TGIV). *Diseases of Aquatic Organisms*, **58**, 127–142.

CHEN, X.H., LIN, K.B. & WANG, X.W. (2003). Outbreaks of an iridovirus disease in maricultured large yellow croaker, *Larimichthys crocea* (Richardson), in China. *Journal of Fish Diseases*, **26**, 615-619.

CHINCHAR, V.R., HICK, P., INCE, I.A., JANCOVICH, J.K., MARSCHANG, R., QIN, Q., SUBRAMANIAM, K., WALTZEK, T.B., WHITTINGTON, R., WILLIAMS, T. & ZHANG, Q. (2017). ICTV Report Consortium ICTV Virus Taxonomy Profile: *Iridoviridae*. *Journal of Geneneral Virology,* **98**, 890–891.

CHINCHAR, V.G., HICK, P.H., HUANG, J., INCE, I.A., JANCOVICH, J.K., MARSCHANG, R., QIN, Q., SUBRAMANIAM, K., WALTZEK, T.B., WHITTINGTON, R., WILLIAMS, T. & ZHANG, Q. (2020) ICTV Virus Taxonomy Profile: *Iridoviridae*, *Journal of General Virology*, **98**, 890-891.

DO, J.W., CHA, S.J., KIM, J.S., AN, E.J., LEE, N.S., CHOI, H.J., LEE, C.H., PARK, M.S., KIM, J.W., KIM, Y.C. & PARK, J.W. (2005). Phylogenetic analysis of the major capsid protein gene of iridovirus isolates from cultured flounders *Paralichthys olivaceus* in Korea. *Diseases of Aquatic Organisms,* **64**, 193–200.

DONG, H.T., JITRAKORN, S., KAYANSAMRUAJ, P., PIRARATE, N., RODKHUM, C., RATTANAROJPONG, T., SENAPIN, S., SAKSMERPROME, V. (2017). Infectious spleen and kidney necrosis disease (ISKND) outbreaks in farmed barramundi (*Lates calcarifer*) in Vietnam. *Fish & Shellfish Immunology*, **68**, 65-73.

FIGUEIREDO, H.C.P., TAVARES, G.C., DORELLA, F.A., ROSA, J.C.C., MARCELINO, S.A.C., PIEREZAN, F. & PEREIRA, F.L. (2022). First report of infectious spleen and kidney necrosis virus in Nile tilapia in Brazil. *Transboundary and Emerging Diseases*, 69(5), 3008-3015.

FUSIANTO, C., HICK, P.M., HERLAMBANG, A., WHITTINGTON, R.J. & BECKER, J.A. (2021). Outbreak investigation attributes Infectious spleen and kidney necrosis virus as a necessary cause of a mortality epidemic in farmed grouper (*Epinephelus* spp.) in Bali, Indonesia. *Aquaculture Reports* **20**, 100723.

GIRISHA, S.K., PUNEETH, T.G., NITHIN, M.S., NAVEEN KUMAR, B.T., AJAY, S.K., VINAY, T.N. & RAMESH, K.S. (2020). Red sea bream iridovirus disease (RSIVD) outbreak in Asian seabass (*Lates calcarifer*) cultured in open estuarine cages along the west coast of India: first report. *Aquaculture*, **520**, 734712.

GO, J., WALTZEK, T.B., SUBRAMANIAM, K., YUN, S.C., GROFF, J.M., ANDERSON, I.G., CHONG, R., SHIRLEY, I., SCHUH, J.C.L., HANDLINGER, J.H., TWEEDIE, A. & WHITTINGTON, R.J. (2016). Complete genome analysis of the mandarin fish infectious spleen and kidney necrosis iridovirus. *Virology*, **291**, 126–139.

GO, J. & WHITTINGTON, R. (2006). Experimental transmission and virulence of a megalocytivirus (Family Iridoviridae) of dwarf gourami (*Colisa lalia*) from Asia in Murray cod (*Maccullochella peeli peeli*) in Australia. *Aquaculture*, **258**, 140-149.

GO, J. & WHITTINGTON, R.J. (2019). Experimental transmission of Infectious Spleen and Kidney Necrosis Virus (ISKNV) from freshwater ornamental fish to silver sweep *Scorpis lineolata*, an Australian marine fish. *Diseases of Aquatic Organisms*, **137(1)**, 1-21.

HE, J.G., DENG, M., WENG, S.P., LI, Z., ZHOU, S.Y., LONG, Q.X., WANG, X.Z. & CHAN, S.M. (2001). Complete genome analysis of the mandarin fish infectious spleen and kidney necrosis iridovirus. *Virology*, **291**, 126–139, doi: 10.1006/viro.2001.1208.

HE, J.G., ZENG, K., WENG, S.P. & CHAN, S.M. (2000). Systemic disease caused by an iridovirus-like agent in cultured mandarin fish *Siniperca chuatsi* (Basillewsky), in China, *Journal of Fish Diseases*, **23**, 219–222.

HE, J.G., ZENG, K., WENG, S.P. & CHAN, S.M. (2002), Experimental transmission, pathogenicity and physical-chemical properties of infectious spleen and kidney necrosis virus (ISKNV), *Aquaculture*, **204**, 11–24. doi: 10.1016/S0044-8486(01)00639-1.

HICK, P.M., BECKER, J.A. & WHITTINGTON, R.J. (2016). Iridoviruses of fish In: Aquaculture Virology (F. Kibenge and M. Godoy, eds), Elsevier, London, UK, 127-152.

HUANG, S.M., TU, C., TSENG, C.H., HUANG, C.C., CHOU, C.C., KUO, H.C. & CHANG, S.K. (2011). Genetic analysis of fish iridoviruses isolated in Taiwan during 2001-2009. *Archives of Virol*ogy, **156**, 1505-1515.

HUANG, Y., CAI, S., JIAN, J., LUI, G., & XU, L. (2020). Co-infection of infectious spleen and kidney necrosis virus and *Francisella* sp. in farmed pearl gentian grouper (♀*Epinephelus fuscoguttatus* ×♂*E. lanceolatus*) in China — A case report. *Aquaculture*, **526**, 735409.

INOUYE, K., YAMANO, K., MAENO, Y., NAKAJIMA, K., MATSUOKA, M., WADA, Y. & SORIMACHI, M. (1992). Iridovirus infection of cultured red sea bream, *Pagrus major*, *Fish Pathology*, **27**, 19–27.

JEONG, J.B., KIM, H.Y., JUN, L.J., LYU, J.H., PARK, N.G., KIM, J.K.& JEONG, H.D. (2008a). Outbreaks and risks of infectious spleen and kidney necrosis virus diseases in freshwater ornamental fishes, *Diseases of Aquatic Organisms*, **78**, 209–215. doi: 10.3354/dao01879.

JEONG, J., CHO, H., JUN, L., HONG, S., CHUNG, J. & JEONG, H. (2008b). Transmission of Iridovirus from freshwater ornamental fish (pearl gourami) to marine (rock bream). *Diseases of Aquatic Organisms*, **82(1)**, 27-36.

JOHAN, C.A.C.& ZAINATHAN, S.C. (2020). Megalocytiviruses in ornamental fish: A review. *Veterinary World*, **13**, 2565–2577.

JUNG, S.J., OH,M.J.. (2000). Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula, *Journal of Fish Diseases*, **23**, 223–226. doi: 10.1046/j.1365-2761.2000.00212.x.

KERDDEE, P., DINH-HUNG, N., THANH DONG, H., HIRONO, I., SOONTARA, C., AREECHON, N., SRISAPOOME, P. & KAYANSAMRUAJ, P. (2021). Molecular evidence for homologous strains of infectious spleen and kidney necrosis virus (ISKNV) genotype I infecting inland freshwater cultured Asian sea bass (*Lates calcarifer*) in Thailand. *Archives of Virology,* **166**, 3061–3074.

KIM, K.H., CHOI, K.M., KANG, G., WOO, W.S., SOHN, M.Y., SON, H.J., YUN, D., KIM, D.H.& PARK, C.I. (2022). Development and Validation of a Quantitative Polymerase Chain Reaction Assay for the Detection of Red Sea Bream Iridovirus. *Fishes*, **7**, 236. https://doi.org/ 10.3390/fishes7050236

KODA, S.A., SUBRAMANIAM, K., HICK, P.M., HALL, E., WALTZEK, T.B. & BECKER, J.A. (2023). Partial validation of a TaqMan quantitative polymerase chain reaction for the detection of the three genotypes of *Infectious spleen and kidney necrosis virus*. *PloS ONE*, **18(2)**:e0281292.

KODA, S.A., SUBRAMANIAM, K., FLOYD-FRANCIS, R., YANONG, R.P., FRASCA, S., GROFF, J.M., POPOV, V.L., FRASER, W.A., YAN, A., MOHAN, S. & WALTZEK, T.B. (2018). Phylogenomic characterization of two novel members of the genus Megalocytivirus from archived ornamental fish samples. *Diseases of Aquatic Organisms*, **130(1)**, 11-24.

KURITA, J., NAKAJIMA, K., (2012), Megalocytiviruses, *Viruses*, **4(4)**, 521-538.

KAWATO, Y., CUMMINS, D.M., VALDETER, S., MOHR, P., ITO, T., MIZUNO, K., KAWAKAMI, H., WILLIAMS, L.M., CRANE, M.ST.J. & MOODY, N.J.G. (2021). Development of New Real-time PCR Assays for Detecting *Megalocytivirus* Across Multiple Genotypes. *Fish Pathology*, **56 (4)**, 177-186. doi.org/10.3147/jsfp.56.177

LAJIMIN, S., RAZAK, A.A., DENIL, D. J., RANSANGAN, J., ABDUL WAHID, M.E. & SADE, A. (2015). First detection of Megalocytivirus (*Iridoviridae*) in trash fish used for aquaculture feed in Sabah, Malaysia. *Int. J. of Aquatic Science*, 6(1): 54-66.

NAKAJIMA, K., MAENO, Y., HONDA, A., YOKOYAMA, K., TOORIYAMA, T. & MANABE, S. (1999). Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test, *Diseases of Aquatic Organisms*, **36(1)**, 73-5.

NI, S.Z., WANG, Y.J., HU, J. B., SHI, J., XU, Y., ZHOU, S.M., LI, J.J., HONG, B.H. & QIAN, D. (2021). Identification, histopathology, and phylogenetic analysis of an iridovirus from cultivated silver pomfret in Zhejiang Province, East China. *Aquaculture,* **530**,735619.

RAMÍREZ-PAREDES, J.G., PALEY, R.K., HUNT, W., FEIST, S.W., STONE, D.M., FIELD, T.R., HAYDON, D.J., ZIDDAH, P.A., NKANSA, M., GUILDER, J., GRAY, J., DUODU, S., PECKU, E.K., AWUNI,. J.A., WALLIS, T.S. & VERNER-JEFFREYS, D.W. (2021). First detection of infectious spleen and kidney necrosis virus (ISKNV) associated with massive mortalities in farmed tilapia in Africa. *Transboundry Emerging Diseases,* **68**, 1550–1563. https://doi.org/10.1111/tbed.13825

RIMMER A.E., BECKER J.A., TWEEDIE A., LINTERMANS M., LANDOS M. & WHITTINGTON R.J. (2015). Detection of dwarf gourami iridovirus (Infectious spleen and kidney necrosis virus) in populations of ornamental fish prior to and after importation into Australia, with the first evidence of infection in domestically farmed Platy (*Xiphophorus maculatus*). Preventive Veterinary Medicine, **122**, 181-194.

SHI, C.Y., WANG, Y.G., YANG, S.L., HUANG, J. & WANG, Q.Y. (2004). The first report of an iridovirus-like agent infection in farmed turbot, *Scophthalmus maximus*, in China. *Aquaculture*, **236**, 11-15. https://doi.org/10.1016/j.aquaculture.2003.11.007

SONG, J-Y., KITAMURA, S-I., JUNG, S-J., MIYADAI, T., TANAKA, S., FUKUDA, Y., KIM, S-R. & OH, M-J. (2008). Genetic variation and geographic distribution of megalocytiviruses. *Journal of Microbiology*, **46**, 29-33.

SUBRAMANIAM, K., SHARIFF, M., OMAR, A.R., HAIR-BEJO, M. & ONG, B.L. (2014). Detection and molecular characterisation of infectious spleen and kidney necrosis virus from major ornamental fish breeding states in peninsular Malaysia, *Journal of Fish Diseases*, **37**, 609–618, https://doi.org/10.1111/jfd.12152

SUBRAMANIAM, K., GOTESMAN, M., SMITH, C.E., STECKLER, N.K., KELLEY, K.L,, GROFF, J.M. & WALTZEK, T.B. (2016). *Megalocytivirus* infection in cultured Nile tilapia *Oreochromis niloticus*. *Diseases of Aquatic Organisms,* **119**, 253-258. https://doi.org/10.3354/dao02985

SUMITHRA, T.G., KRUPESHA SHARMA, S.R., NEELIMA, L., DHANUTHA, N.R., JOSHY, A., ANUSREE, V.N., GAYATHRI, S., RAGHU, R.K., PRAVEEN, N.D., THOMAS, S. & RAJESH, K.M. (2022). Red sea bream iridovirus infection in cage farmed Asian sea bass (*Lates calcarifer*): Insights into the pathology, epizootiology, and genetic diversity. *Aquaculture*, **548**, 737571. https://doi.org/10.1016/j.aquaculture.2021.737571

SWAMINATHAN, T.R., JOHNY, T.K., NITHIANANTHAM, S.R., SUDHAGAR, A., PRADHAN, P.K., SULUMANE RAMACHANDRA, K.S., NAIR, R.R., & SOOD, N. (2022). A natural outbreak of infectious spleen and kidney necrosis virus (ISKNV) threatens wild pearlspot, *Etroplus suratensis* in Peechi Dam in the Western Ghats biodiversity hotspot, India. *Transboundary and Emerging Diseases*, **69(5)**, 1595-1605. https://doi.org/10.1111/tbed.14494

TSAI, J.M., HUANG, S.L. & YANG, C.D. (2020). PCR Detection and Phylogenetic Analysis of *Megalocytivirus* Isolates in Farmed Giant Sea Perch *Lates calcarifer* in Southern Taiwan. Viruses, **12(6)**, 681. https://doi.org/10.3390/v12060681

WANG, Y.Q., LÜ, L., WENG, S.P., HUANG, J.N., CHAN, S.M.& HE, J.G. (2007). Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis viruslike (ISKNV-like) virus. A*rchives of Virology*, **152**, 763–773.

Annex 17. Item 8.4.1. – Articles 8.X.3. for Amphibian disease-specific chapters

(TRACK CHANGES VERSION)

CHAPTER 8.1.

INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

[…]

Article 8.1.3.

Measures for the ~~i~~importation or transit of aquatic animal products for any purpose regardless of the infection with *B. dendrobatidis* status of the exporting country, zone or compartment

~~1.~~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* ~~conditions~~ related to *B. dendrobatidis*, regardless of the infection with *B. dendrobatidis* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.1.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. dendrobatidis*;

   ~~a) heat-sterilised hermetically-sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

   ~~b) cooked amphibian products that have been subjected to heat treatment at 100°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

   ~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

   ~~d)~~2) ~~mechanically dried amphibian products~~ that have been subjected to ~~(~~i.e. a heat treatment sufficient to attain a core temperature ~~of at least 100~~60°C ~~for at least five~~30 minutes, ~~or~~ a~~any time/temperature equivalent that has been demonstrated to inactivates *B. dendrobatidis*);~~

   ~~e)~~32) amphibian skin leather.

~~2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.1.2., other than those referred to in point 1 of Article 8.1.3., *Competent Authorities* should require the conditions prescribed in Articles 8.1.7. to 8.1.12. relevant to the infection with *B. dendrobatidis* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.1.2. but which could reasonably be expected to pose a *risk* of transmission of *B. dendrobatidis*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of thisanalysis.~~

[…]

———————

(CLEAN VERSION)

CHAPTER 8.1.

INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

[…]

Article 8.1.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. dendrobatidis* 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 *B. dendrobatidis*, regardless of the infection with *B. dendrobatidis* 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 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. dendrobatidis*;

2) amphibian skin leather.

[…]

(TRACK CHANGES VERSION)

CHAPTER 8.2

INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

[…]

Article 8.2.3.

Measures for the ~~i~~importation or transit of aquatic animal products for any purpose regardless of the infection with *B. salamandrivorans* status of the exporting country, zone or compartment

~~1)~~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 authorizing the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* ~~conditions~~ related to *B. salamandrivorans*, regardless of the infection with *B. salamandrivorans* status of the *exporting country*, *zone* or *compartment*~~, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.2.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least ~~5~~ five minutes, or a time/temperature equivalent that inactivates *B. salamandrivorans*;

   ~~a) heat sterilised hermetically sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

   ~~b) cooked amphibian products that have been subjected to heat treatment at 100°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

   ~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

   ~~d)~~2) ~~mechanically dried amphibian products~~ that have been subjected ~~to (i.e. a heat~~ treatment sufficient to attain a core ~~temperature of at least 10060°C for at least five30 minutes, or a~~ any time/temperature equivalent ~~that has been demonstrated to inactivates *B. salamandrivorans;*~~

   ~~e)~~ 32)amphibian skin leather.

~~2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.2.2., other than those referred to in point 1 of Article 8.2.3., *Competent Authorities* should require the conditions prescribed in Articles 8.2.7. to 8.2.12. relevant to the infection with *B. salamandrivorans* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.2.2. but which could reasonably be expected to pose a *risk* of transmission of *B. salamandrivorans*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of thisanalysis.~~

[…]

(CLEAN VERSION)

CHAPTER 8.2.

INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

[…]

Article 8.2.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. salamandrivorans* 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 authorizing the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to *B. salamandrivorans*, regardless of the infection with *B. salamandrivorans* 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 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. salamandrivorans*;

2) amphibian skin leather.

[…]

(TRACK CHANGES VERSION)

CHAPTER 8.3.

INFECTION WITH RANAVIRUS SPECIES

[…]

Article 8.3.3.

Measures for the ~~i~~importation or transit of aquatic animal products for any purpose regardless of the infection with *Ranavirus* species 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* ~~conditions~~ related to *Ranavirus* species, regardless of the infection with *Ranavirus* species status of the *exporting country*, *zone* or *compartment*~~:, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.3.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~65~~60°C for at least 30 minutes, or a time/temperature equivalent that inactivates *Ranavirus* species~~:~~.

~~a) heat sterilised hermetically sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~b) cooked amphibian products that have been subjected to heat treatment at 65°C for at least 30 minutes (or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~d~~2) ~~mechanically dried amphibian products that have been subjected to (i.e. a heat treatment sufficient to attain a core temperature of at least 100~~65°C for at least 30 minutes, or ~~a~~any time/temperature equivalent that has been demonstrated to inactivate~~s~~ *Ranavirus* species~~).~~

~~2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.3.2., other than those referred to in point 1 of Article 8.3.3., *Competent Authorities* should require the conditions prescribed in Articles 8.3.7. to 8.3.12. relevant to the infection with *Ranavirus* species status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.3.2. but which could reasonably be expected to pose a *risk* of transmission of *Ranavirus* species, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis.~~

[…]

(CLEAN VERSION)

CHAPTER 8.3.

INFECTION WITH RANAVIRUS SPECIES

[…]

Article 8.3.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *Ranavirus* species 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 *Ranavirus* species, regardless of the infection with *Ranavirus* species 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 60°C for at least 30 minutes, or a time/temperature equivalent that inactivates *Ranavirus* species.

[…]

Annex 18. Item 8.4.2.– Articles 9.X.3. for Crustacean disease-specific chapters

CHAPTER 9.3.

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

[…]

Article 9.3.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the *H. penaei* 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 *H. penaei,* regardless of the infection with *H. penaei* 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 ~~63~~95°C for at least ~~30~~five minutes, or a time/temperature equivalent inactivates *H. penaei*;

2) crustacean *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~63~~95°C for at least ~~30~~five minutes, or a time/temperature equivalent that inactivates *H. penaei*;

3) crustacean oil;

4) chemically extracted chitin.

[…]

CHAPTER 9.5.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

[…]

Article 9.5.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with IMNV 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 IMNV, regardless of the infection with IMNV 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 ~~60~~75°C for at least ~~60~~five minutes, or a time/temperature equivalent that inactivates IMNV;

2) crustacean *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~60~~75°C for at least ~~60~~five minutes, or a time/temperature equivalent that inactivates IMNV;

3) crustacean oil;

4) chemically extracted chitin.

[…]

_____

CHAPTER 9.6.

INFECTION WITH *MACROBRACHIUM ROSENBERGII* NODAVIRUS (WHITE TAIL DISEASE)

[…]

Article 9.6.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with MrNV 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 MrNV, regardless of the infection with MrNV 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 ~~60~~50°C for at least ~~60~~50 minutes, or a time/temperature equivalent that inactivates MrNV;

2) crustacean *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~60~~50°C for at least ~~60~~50 minutes, or a time/temperature equivalent that inactivates MrNV;

3) crustacean oil;

4) chemically extracted chitin.

[…]

CHAPTER 9.7.

INFECTION WITH TAURA SYNDROME VIRUS

[…]

Article 9.7.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with TSV 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 TSV, regardless of the infection with TSV 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 70°C for at least ~~30~~108 minutes, or a time/temperature equivalent that inactivates TSV;

2) crustacean *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 70°C for at least ~~30~~108 minutes, or a time/temperature equivalent that inactivates TSV;

3) crustacean oil;

4) chemically extracted chitin.

[…]

CHAPTER 9.10.

INFECTION WITH DECAPOD IRIDESCENT VIRUS 1

[…]

Article 9.10.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with DIV1 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 DIV1, regardless of the infection with DIV1 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~~80°C for at least 30 minutes, or a time/temperature equivalent that inactivates DIV1;

2) crustacean *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~56~~80°C for at least 30 minutes, or a time/temperature equivalent that inactivates DIV1;

3) ~~crayfish~~crustacean oil;

4) chemically extracted chitin.~~] (under study).~~

[…]

Annex 19. Item 8.4.3. – Articles 10.X.3. for Fish disease-specific chapters

CHAPTER 10.1.

INFECTION WITH EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS

[…]

Article 10.1.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with EHNV 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 EHNV, regardless of the EHNV 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 60°C for at least 15 minutes, or a time/temperature equivalent that inactivates EHNV;

~~2) mechanically dried eviscerated fish that has been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least 15 minutes, or a time/temperature equivalent that inactivates EHNV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least 15 minutes, or a time/temperature equivalent that inactivates EHNV;

~~4~~3) fish oil;

~~5~~4) fish skin leather.

[…]

CHAPTER 10.2.

INFECTION WITH *APHANOMYCES INVADANS* (EPIZOOTIC ULCERATIVE SYNDROME)

[…]

Article 10.2.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *A. invadans* 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 *A. invadans*, regardless of the infection with *A. invadans* 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 ~~60~~100°C for at least ~~five~~one minute~~s~~, or a time/temperature equivalent that inactivates *A. invadans*;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *A. invadans*;~~

~~3~~2) fish meal that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~60~~100°C for at least ~~five~~one minute~~s~~, or a time/temperature equivalent that inactivates *A. invadans*;

~~4~~3) fish oil;

~~5~~4) frozen eviscerated fish;

~~6~~5) frozen fish fillets or steaks.

[…]

CHAPTER 10.3.

INFECTION WITH *GYRODACTYLUS SALARIS*

[…]

Article 10.3.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *G. salaris* 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 *G. salaris*, regardless of the *G. salaris* status of the *exporting country*, *zone* or *compartment*:

1) *aquatic animal products* that have been ~~heat treated and are hermetically sealed~~subjected to a heat treatment sufficient to attain a core temperature of at least 40°C for at least one minute, or a time/temperature equivalent that inactivates *G. salaris*;

~~2) mechanically dried eviscerated fish;~~

~~3~~2) naturally dried eviscerated fish (i.e. sun-dried or wind-dried);

~~4~~3) frozen eviscerated fish that have been subjected to minus 18°C or lower temperatures;

~~5~~4) frozen fish fillets or steaks that have been subjected to minus 18°C or lower temperatures;

~~6~~5) chilled eviscerated fish that have been harvested from seawater with a salinity of at least 25 parts per thousand (ppt);

~~7~~6) chilled fish fillets or steaks derived from fish that have been harvested from seawater with a salinity of at least 25 ppt;

~~8~~7) chilled fish products from which the skin, fins and gills have been removed;

~~9~~8) non-viable fish roe;

~~10~~9) fish oil;

~~11~~10) fish *meal*;

~~12~~11) fish skin leather.

[…]

CHAPTER 10.4.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

[…]

Article 10.4.3.

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

In this article, all statements referring to ISAV include HPR deleted ISAV and HPR0 ISAV.

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 ISAV, regardless of the ISAV 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 ISAV;

~~2) mechanically dried eviscerated fish 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 ISAV;~~

~~3~~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 ISAV;

~~4~~3) fish oil;

~~5~~4) fish skin leather.

[…]

CHAPTER 10.5.

INFECTION WITH SALMONID ALPHAVIRUS

[…]

Article 10.5.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with SAV 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 SAV, regardless of the SAV 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 60°C for at least 60 minutes, or a time/temperature equivalent that inactivates SAV;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least 60 minutes, or a time/temperature equivalent that inactivates SAV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least 60 minutes or a time/temperature equivalent that inactivates SAV;

~~4~~3) fish oil;

~~5~~4) fish skin leather.

[…]

CHAPTER 10.6.

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

[…]

Article 10.6.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with IHNV 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 IHNV, regardless of the IHNV 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 90°C for at least 30 seconds, or a time/temperature equivalent that inactivates IHNV;

~~2)~~ ~~mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 90°C for at least 30 seconds, or a time/temperature equivalent that inactivates IHNV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 90°C for at least 30 seconds, or a time/temperature equivalent that inactivates IHNV;

~~4~~3) fish oil;

~~5~~4) fish skin leather.

[…]

CHAPTER 10.7.

INFECTION WITH KOI HERPESVIRUS

[…]

Article 10.7.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with KHV 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 KHV, regardless of the KHV 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 50°C for at least ~~three~~one minute~~s~~, or a time/temperature equivalent that inactivates KHV;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 50°C for at least three minutes, or a time/temperature equivalent that inactivates KHV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least 50°C for at least ~~three~~one minute~~s~~, or a time/temperature equivalent that inactivates KHV;

~~4~~3) fish oil.

[…]

CHAPTER 10.8.

INFECTION WITH RED SEA BREAM VIRUS

[…]

Article 10.8.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with RSIV 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 RSIV, regardless of the RSIV 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 30 minutes, or a time/temperature equivalent that inactivates RSIV;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 56°C for at least 30 minutes, or a time/temperature equivalent that inactivates RSIV;~~

~~3~~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 30 minutes, or a time/temperature equivalent that inactivates RSIV;

~~4~~3) fish oil;

~~5~~4) fish skin leather.

[…]

CHAPTER 10.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[…]

Article 10.9.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with SVCV 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 SVCV, regardless of the SVCV 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 ~~90~~60°C for at least 60 ~~seconds~~minutes, or a time/temperature equivalent inactivates SVCV;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 90°C for at least 60 seconds, or a time/temperature equivalent that inactivates SVCV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~90~~60°C for at least 60 ~~seconds~~minutes, or a time/temperature equivalent that inactivates SVCV;

~~4~~3) fish oil.

[…]

CHAPTER 10.10.

INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

[…]

Article 10.10.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with VHSV 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 VHSV, regardless of the VHSV 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 ~~90~~60°C for at least 60 ~~seconds~~minutes, or a time/temperature equivalent that inactivates VHSV;

~~2) mechanically dried eviscerated fish that have been subjected to a heat treatment sufficient to attain a core temperature of at least 90°C for at least 60 seconds, or any a time/temperature equivalent that inactivates VHSV;~~

~~3~~2) fish *meal* that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~90~~60°C for at least 60 ~~seconds~~minutes, or a time/temperature equivalent that inactivates VHSV;

~~4~~3) naturally dried, eviscerated fish (i.e. sun-dried or wind-dried);

~~5~~4) fish oil;

~~6~~5) fish skin leather.

[…]

Annex 20. Item 8.4.4. – Articles 11.X.3. for Mollusc disease-specific chapters

(TRACK CHANGES VERSION)

CHAPTER 11.1.

INFECTION WITH ABALONE HERPESVIRUS

[…]

Article 11.1.3.

Measures for the ~~i~~importation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with abalone herpesvirus status of the exporting country, zone or compartment

~~1)~~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*~~conditions~~ related to infection with abalone herpesvirus regardless of the infection with abalone herpesvirus status of the *exporting country*, *zone* or *compartment*~~:, when authorising the importation or transit of the following *aquatic animal products* from the species referred to in Article 11.1.2. which are intended for any purpose and which comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~121~~50°C for at least ~~3~~five minutes ~~and 36 seconds~~, or a time/temperature equivalent that inactivates AbHV~~;~~.

   ~~a) heat sterilised hermetically sealed abalone products (i.e. a heat treatment at 121˚C for at least 3.6 minutes or any time/temperature equivalent);~~

   ~~b~~2) ~~mechanically dried abalone products (i.e. that have been subjected to a heat treatment sufficient to attain a core temperature of at least 100121˚C for at least 3 minutes and 36 seconds, 30 minutes or anya time/temperature equivalent which has been demonstrated to that inactivates AbHV).~~

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.1.2., other than those referred to in point 1 of Article 11.1.3., *Competent Authorities* should require the conditions prescribed in Articles 11.1.7. to 11.1.11. relevant to the infection with abalone herpesvirus status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.1.2. but which could reasonably be expected to pose a *risk* of spread of infection with abalone herpesvirus, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.1.

INFECTION WITH ABALONE HERPESVIRUS

[…]

Article 11.1.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with abalone herpesvirus 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 infection with abalone herpesvirus regardless of the infection with abalone herpesvirus 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 50°C for at least five minutes, or a time/temperature equivalent that inactivates AbHV.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.2.

INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.3.

Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *B. exitiosa* status of the exporting country, zone or compartment

~~1)~~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*~~conditions~~ related to infection with *B. exitiosa*, regardless of the infection with *B. exitiosa* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 11.2.2. which are intended for any purpose and which comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 100°C for at least 15 minutes, or a time/temperature equivalent that inactivates *B. exitiosa*;

~~a)~~~~1~~2) frozen oyster meat; ~~and~~

~~b)~~~~2~~3) frozen half-shell oysters.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.2.2., other than those referred to in point 1 of Article 11.2.3., *Competent Authorities* should require the conditions prescribed in Articles 11.2.7. to 11.2.11. relevant to the infection with *B. exitiosa* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.2.2. but which could reasonably be expected to pose a *risk* of spread of infection with *B. exitiosa*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.2.

INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. exitiosa* 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 infection with *B. exitiosa*, regardless of the infection with *B. exitiosa* 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 100°C for at least 15 minutes, or a time/temperature equivalent that inactivates *B. exitiosa*;

2) frozen oyster meat;

3) frozen half-shell oysters.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.3.

INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.3.

Measures for the ~~i~~importation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *B. ostreae* status of the exporting country, zone or compartment

~~1)~~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*~~conditions~~ related to infection with *B. ostreae*, regardless of the infection with *B. ostreae* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 11.3.2. which are intended for any purpose and which comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 100°C for at least 15 minutes, or a time/temperature equivalent that inactivates *B. ostreae;*

~~a)~~~~1~~2) frozen oyster meat; ~~and~~

~~b)~~~~2~~3) frozen half-shell oysters.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.2.2., other than those referred to in point 1 of Article 11.2.3., *Competent Authorities* should require the conditions prescribed in Articles 11.2.7. to 11.2.11. relevant to the infection with *B. ostreae* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.2.2. but which could reasonably be expected to pose a *risk* of spread of infection with *B. ostreae*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.3.

INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. ostreae* 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 infection with *B. ostreae*, regardless of the infection with *B. ostreae* 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 100°C for at least 15 minutes, or a time/temperature equivalent that inactivates *B. ostreae*;

2) frozen oyster meat;

3) frozen half-shell oysters.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.4.

INFECTION WITH *MARTEILIA REFRINGENS*

[…]

Article 11.4.3.

Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *M. refringens* status of the exporting country, zone or compartment

~~1)~~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*~~conditions~~ related to infection with *M. refringens*, regardless of the infection with *M. refringens* status of the *exporting country*, *zone* or *compartment:* ~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.4.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three~~3~~ minutes and 36 seconds, or a time/temperature equivalent that inactivates *M. refringens*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.4.2., other than those referred to in point 1 of Article 11.4.3., *Competent Authorities* should require the conditions prescribed in Articles 11.4.7. to 11.4.11. relevant to the infection with *M. refringens* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.4.2. but which could reasonably be expected to pose a *risk* of spread of infection with *M. refringens*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.4.

INFECTION WITH *MARTEILIA REFRINGENS*

[…]

Article 11.4.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *M. refringens* 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 infection with *M. refringens*, regardless of the infection with *M. refringens* 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 121°C for at least three minutes and 36 seconds, or a time/temperature equivalent that inactivates *M. refringens*.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.5.

INFECTION WITH *PERKINSUS MARINUS*

[…]

Article 11.5.3.

Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *P. marinus* status of the exporting country, zone or compartment

~~1)~~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*~~conditions~~ related to infection with *P. marinus*, regardless of the infection with *P. marinus* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.5.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~121~~60°C for at least ~~three~~60 minutes ~~and 36 seconds~~, or a time/temperature equivalent that inactivates *P. marinus*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.5.2., other than those referred to in point 1 of Article 11.5.3., *Competent Authorities* should require the conditions prescribed in Articles 11.5.7. to 11.5.11. relevant to the infection with *P. marinus* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.5.2. but which could reasonably be expected to pose a *risk* of spread of infection with *P. marinus*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.5.

INFECTION WITH *PERKINSUS MARINUS*

[…]

Article 11.5.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *P. marinus* 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 infection with *P. marinus*, regardless of the infection with *P. marinus* 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 60°C for at least 60 minutes, or a time/temperature equivalent that inactivates *P. marinus*.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.6.

INFECTION WITH *PERKINSUS OLSENI*

[…]

Article 11.6.3.

Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *P. olseni* status of the exporting country, zone or compartment

~~1)~~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* ~~conditions~~ related to infection with *P. olseni*, regardless of the infection with *P. olseni* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.6.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~121~~60°C for at least ~~three~~60 minutes ~~and 36 seconds minutes~~, or a time/temperature equivalent that inactivates *P.olseni*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.6.2., other than those referred to in point 1 of Article 11.6.3., *Competent Authorities* should require the conditions prescribed in Articles 11.6.7. to 11.6.11. relevant to the infection with *P. olseni* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.6.2. but which could reasonably be expected to pose a *risk* of spread of infection with *P. olseni*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.6.

INFECTION WITH *PERKINSUS OLSENI*

[…]

Article 11.6.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *P. olseni* 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 infection with *P. olseni*, regardless of the infection with *P. olseni* 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 60°C for at least 60 minutes, or a time/temperature equivalent that inactivates *P.olseni*.

[…]

(TRACK CHANGES VERSION)

CHAPTER 11.7.

INFECTION WITH *XENOHALIOTIS CALIFORNIENSIS*

[…]

Article 11.7.3.

Measures for the ~~i~~importation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *X. californiensis* status of the exporting country, zone or compartment

~~1)~~The ~~following~~ *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 ~~the~~ these *aquatic animal products* ~~listed below~~, *Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *X. californiensis*, regardless of the infection with *X. californiensis* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of heat sterilised hermetically sealed abalone products (i.e. a heat treatment at 121˚C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.7.2. which are intended for any purpose and which comply with Article 5.4.1.~~

~~a~~1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~121~~95°C for at least ~~3~~five minutes ~~and 36 seconds~~, ~~(~~or a time/temperature equivalent that inactivates *X. californiensis*.~~;~~

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.7.2., other than those referred to in point 1 of Article 11.7.3., *Competent Authorities* should require the conditions prescribed in Articles 11.7.7. to 11.7.11. relevant to the infection with *X. californiensis* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.7.2. but which could reasonably be expected to pose a *risk* of spread of infection with *X. californiensis*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

(CLEAN VERSION)

CHAPTER 11.7.

INFECTION WITH *XENOHALIOTIS CALIFORNIENSIS*

[…]

Article 11.7.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *X. californiensis* 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 infection with *X. californiensis*, regardless of the infection with *X. californiensis* 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 95°C for at least five minutes, or a time/temperature equivalent that inactivates *X. californiensis*.

[…]

Annex 21. Item 8.5. – Article 11.5.1. and 11.5.2. of Chapter 11.5. Infection with *Perkinsus marinus*

CHAPTER 11.5.

INFECTION WITH *PERKINSUS MARINUS*

Article 11.5.1.

For the purposes of the *Aquatic Code*, infection with *Perkinsus marinus* means *infection* with the *pathogenic agent* *P. marinus* of the Family Perkinsidae.

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

Article 11.5.2.

Scope

The recommendations in this chapter apply to: ~~Eastern oyster~~American cupped oyster *(Crassostrea virginica)*, ~~Pacific oyster *(Crassostrea gigas)*~~, ~~Suminoe oyster *(Crassostrea ariakensis)*~~, ~~soft shell clam *(Mya arenaria)*~~, ~~Baltic clam *(Macoma balthica)*~~ Ariake cupped oyster (*Magallana* [Syn. *Crassostrea*] *ariakensis*), Cortez oyster (*Crassostrea corteziensis*) and palmate oyster (*Saccostrea palmula*)~~.hard shell clam *(Mercenaria mercenaria)*~~. ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

[…]

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 of disease and mortality can start as early as 10 days post-stocking. ~~Clinical~~ Signs ~~include:~~ of disease in moribund prawns ~~sink to bottom,~~ may include 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).~~ Behavioural changes such as frequent sinking to the bottom of tanks may also be noted.

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 WOAH 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.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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 a DNA preservative ~~DNAzol~~ for PCR testing. If material cannot be fixed it may be frozen, but repeated freezing and thawing of samples should be avoided.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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~~ Isolation					±	±	±	NA				
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 WOAH 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

Histological examination of AHPND infected shrimp reveals that pathological changes are limited to the hepatopancreas. 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. No B-, F- and R-cells are seen in the hepatopancreatic tubule and some nuclei of tubule epithelial cells are enlarged (karyomegaly). No significant bacterial involvement appears during this phase ~~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~~ 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013; 2014).

iii) In *Penaeus vannamei* AHPND tolerant lines, a chronic phase can be observed. 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~~ isolation

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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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 Accession No.: 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 13 (AP3): Sirikharin *et al.*, 2015; GenBank Accession No.: JALL01000066.1; amplicon size: 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 24 (TUMSAT-Vp3): Tinwongger *et al.*, 2014; GenBank Accession No.: AB972427; amplicon size: 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 35 (VpPirA-284): Han *et al.*, 2015a; GenBank Accession No.: KM067908; amplicon size: 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 46 (VpPirB-392): Han *et al.*, 2015a; GenBank Accession No.: KM067908; amplicon size: 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 57 (AP4): Dangtip *et al.*, 2015; GenBank Accession No.: JPKS01000000; amplicon size: 1269 bp			
PirA and PirB toxin genes	Primary Fwd AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA Nested Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 µM each	Primary 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 Nested 94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C
Method 8 (AP4): Dangtip *et al.*, 2015; GenBank : JPKS01000000; amplicon size: 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. Isothermal loop-mediated amplification protocol (LAMP)

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method: Koiwai *et al.*, 2017; GenBank Accession No.: AB972427.1			
Toxin PirAB-like	F3: TGA-TAA-TGC-ATT-CTA-TCA-TCA-GC B3: ATT-TGA-AAG-ACC-AAA-TGA-AAC-C FIP-F1c: GTG-AGC-ACC-TTC-TTA-GTG-GTA-ATA FIP-F2: GTT-GTA-ATT-AAC-AAT-GGC-GCT-AG	F3: 5.0 pmol B3: 5.0 pmol	65°C/60 min and 80°C/5 min

	BIP-B1c: TGA-CGG-AAT-TTA-ACC-CTA-ACA-ATG-C BIP-B2: GCT-TTG-AAA-GCA-TAG-TTA-GGA-TC	FIP: 40 pmol BIP: 40 pmol	

4.4.~~3~~4. 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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

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 agent isolation

[1] For example transboundary commodities.

iii) A positive result by real-time PCR

iv) A positive result by conventional PCR

v) A positive result by bioassay

vi) A positive result by LAMP

vii) 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 (no data are currently available). 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 purpose	Source populations	Tissue or sample types	Species	DSe	DSp	Reference test	Citation

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

7. References

ARANGUREN CARO L.F, MAI H.N., KANRAR S., CRUZ-FLORES R. & DHAR A.K. (2020a). A mutant of *Vibrio parahaemolyticus pir*AB_{VP} (+) that carries binary toxin genes but does not cause acute hepatopancreatic necrosis disease. *Microorganisms*, **8**, 1549.

Aranguren Caro L.F., Mai H.N., Noble B. & Dhar A.K. (2020b). Acute hepatopancreatic necrosis disease (VP_{AHPND}), a chronic disease in shrimp (*Penaeus vannamei*) population raised in latin America. J. Invertebr. Pathol., 174, 107424. doi: 10.1016/j.jip.2020.107424. Epub 2020 Jun 11.PMID: 32535000

CRUZ-FLORES R., MAI H.N & DHAR A.K. (2019). Multiplex SYBR Green and duplex TaqMan real-time PCR assays for the detection of *Photorhabdus* Insect-Related (Pir) toxin genes *pirA* and *pirB*. *Mol. Cell. Probes*, **43**, 20–28.

DABU I.M., LIM J.J., ARABIT P.M.T., ORENSE S.J.A.B., TABARDILLO J.A., CORRE V.L. & MANINGAS M.B.B. (2017). The first record of acute hepatopancreatic necrosis disease in the Philippines. *Aquacult. Res.*, **48**, 792–799.

DANGTIP S., SIRIKHARIN R, SANGUANRUT P., THITAMADEE S, SRITUNYALUCKSANA K., TAENGCHAIYAPHUM S., MAVICHAK R., PROESPRAIWONG P. & FLEGEL T.W. (2015). AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. *Aquaculture Rep.*, **2**, 158–162.

DE LA PENA L.D., CABILLON N.A.R., CATEDRAL D.D., AMAR E.C., USERO R.C., MONOTILLA W.D., CALPE A.T., FERNANDEZ D.D. & SALOMA C.P. (2015). Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. *Dis. Aquat. Org.*, **116**, 251–254.

~~FLEGEL T.W. & LO C.F. (2014). Free release of primers for specific detection of bacterial isolates that cause acute hepatopancreatic necrosis disease (AHPND). Published by the Network of Aquaculture Centres in Asia Pacific, Bangkok, Thailand.~~ ~~https://enaca.org/enclosure/?id=88~~

GOMEZ-GIL B., SOTO-RODRÍGUEZ S., LOZANO R. & BETANCOURT-LOZANO M. (2014). Draft genome sequence of *Vibrio parahaemolyticus* strain M0605, which causes severe mortalities of shrimps in Mexico. *Genome Announc.*, **2**, e00055-14.

GOMEZ-JIMENEZ S., NORIEGA-OROZCO L., SOTELO-MUNDO R.R., CANTU-ROBLES V.A., COBIAN-GUEMES A.G., COTA-VERDUGO R.G., GAMEZ-ALEJO L.A., DEL POZO-YAUNER L., GUEVARA-HERNANDEZ E., GARCIA-OROZCO K.D., LOPEZ-ZAVALA A.A. & OCHOA-LEYVA A. (2014). High-quality draft genomes of two *Vibrio parahaemolyticus* strains aid in understanding acute hepatopancreatic necrosis disease of cultured shrimps in Mexico. *Genome Announc.*, **2**, e00800-14.

HAN J.E., TANG K.F.J., TRAN L.H. & LIGHTNER D.V. (2015a). *Photorhabdus* insect related (*Pir*) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Org.*, **113**, 33–40.

HAN J.E., TANG K.F.J., PANTOJA C.R., WHITE B.L. & LIGHTNER D.V. (2015b). qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. *Aquaculture*, **442**, 12–15.

HONG X.P., XU D., ZHUO Y., LIU H.Q. & LU L.Q. (2016). Identification and pathogenicity of *Vibrio parahaemolyticus* isolates and immune responses of *Penaeus* (*Litopenaues*) *vannamei* (Boone). *J. Fish Dis.,* **39**, 1085–1097.

JOSHI J., SRISALA J., SAKAEW W., PRACHUMWAT A., SRITUNYALUCKSANA K., FLEGEL T.W. & THITAMADEE S. (2014a). Identification of bacterial agent(s) for acute hepatopancreatic necrosis syndrome, a new emerging shrimp disease. *Suranaree J. Sci. Technol*. Available from: http://ird.sut.ac.th/e-journal/ Journal/pdf/140283.pdf.

JOSHI J., SRISALA J., TRUONG V.H., CHEN I.T., NUANGSAENG B., SUTHIENKUL O., LO C.F., FLEGEL T.W., SRITUNYALUCKSANA K. & THITAMADEE S. (2014b). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, **428–429**, 297–302.

KARUNASAGAR I., KARUNASAGAR I., VENUGOPAL M.N. & NAGESHA C.N. (1987). Survival of *Vibrio parahaemolyticus* in estuarine and sea water and in association with clams. *Syst. Appl. Microbiol.*, **9**, 316–319.

KOIWAI K., TINWONGGER S., NOZAKI R., KONDO H. & HIRONO I. (2016). Detection of acute hepatopancreatic necrosis disease strain of *Vibrio parahaemolyticus* using loop-mediated isothermal amplification. *J. Fish Dis*., **39**, 603–606.

KONDO H., TINWONGGER S., PROESPRAIWONG P., MAVICHAK R., UNAJAK S., NOZAKI R. & HIRONO I. (2014). Draft genome sequences of six strains of *Vibrio parahaemolyticus* isolated from early mortality syndrome/acute hepatopancreatic necrosis disease shrimp in Thailand. *Genome Announc.*, **2**, e00221-14.

KONDO H., VAN P.T., DANG L.T. & HIRONO I. (2015). Draft genome sequences of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. *Genome Announc.*, **3**, e00978-15.

KUMAR V., BELS L.D., COUCK L., BARUAH K., BOSSIER P. & BROECK W.V.D. (2019). PirABVP Toxin Binds to Epithelial Cells of the Digestive Tract and Produce Pathognomonic AHPND Lesions in Germ-Free Brine Shrimp. *Toxins*, **11**, 717.

LEE C.T., CHEN I.T., YANG Y.T., KO T.P., HUANG Y.T., HUANG J.Y., HUANG M.F., LIN S.J., CHEN C.Y., LIN S.S., LIGHTNER D.V., WANG A.H., WANG H.C., HOR L.I. & LO C.F. (2015). The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proc. Natl Acad. Sci. USA*., **112**, 10798–10803.

LIGHTNER D.V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, LA, USA.

LO C.-F., LEU J.-H., HO C.-H., CHEN C.-H., PENG S.-E., CHEN Y.-T., CHOU C.-M., YEH P.-Y., HUANG C.-J., CHOU H.-Y., WANG C.-H. & KOU G.-H. (1996). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat. Org.,* **25**, 133–141.

MAI N.H., ARANGUREN L.F.C, CRUZ-FLORES R. & DHAR A.K. (2021). Development of a Recombinase Polymerase Amplification (RPA) assay for acute hepatopancreatic necrosis disease (AHPND) detection in Pacific white shrimp (*Penaeus vannamei*). *Mol. Cell. Probes*, **57**, 101710.

MAI H.N., CRUZ-FLORES R. & DHAR A.K. (2020). Development of an indirect Enzyme Linked Immunoassay (iELISA) using monoclonal antibodies against Photorhabdus insect related toxins, $PirA^{Vp}$ and $PirB^{Vp}$ released from *Vibrio* spp. *J. Microbiol. Methods*, **176**, 106002.

MUNTADA-GARRIGA J.M., RODRIGUEZ-JEREZ J.J., LOPEZ-SABATER E.I. & MORA-VENTURA M.T. (1995). Effect of chill and freezing temperatures on survival of *Vibrio parahaemolyticus* inoculated in homogenates of oyster meat. *Lett. Appl. Microbiol*., **20**, 225–227.

NACA (2014). Acute hepatopancreatic necrosis disease card (updated June 2014). Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.

NUNAN L., LIGHTNER D., PANTOJA C. & GOMEZ-JIMENEZ S. (2014). Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Dis. Aquat. Org.,* **111**, 81–86.

~~POWERS Q.M., ARANGUREN L.F., FITZSIMMONS K.M., MCLAIN J.E. & DHAR A.K. (2021). Crayfish (*Cherax quadricarinatus*) susceptibility to acute hepatopancreatic necrosis disease (AHPND). *J. Invertebr. Pathol*., **186**, 107554.~~

~~SCHOFIELD P.J., NOBLE B.L, ARANGUREN CARO L.F., MAI H.N., PADILLA T.J, MILLABAS J. & DHAR A.K. (2020). Pathogenicity of Acute Hepatopancreatic Necrosis Disease (AHPND) on the freshwater prawn, *Macrobrachium rosenbergii*, and Pacific White Shrimp, *Penaeus vannamei*, at various salinities. *Aquac. Res*., **52**, 1480–1489.~~

SIRIKHARIN R., TAENGCHAIYAPHUM S., SANGUANRUT P., CHI T.D., MAVICHAK R., PROESPRAIWONG P., NUANGSAENG B., THITAMADEE S., FLEGEL T.W. & SRITUNYALUCKSANA K. (2015). Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. *PLoS ONE*, **10**, e0126987. doi:10.1371/journal.pone.0126987.

SOTO-RODRIGUEZ S.A., GOMEZ-GIL B., LOZANO-OLVERA R., BETANCOURT-LOZANO M. & MORALES-COVARRUBIAS M.S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. *Appl. Environ. Microbiol.*, **81**, 1689–1699.

THOMSON W.K. & THACKER C.L. (1973). Effect of temperature on *Vibrio parahaemolyticus* in oysters at refrigerator and deep freeze temperatures. *Can. Inst. Food Sci. Tech. J*., **6**, 156–158.

TINWONGGER S., PROESPRAIWONG P., THAWONSUWAN J., SRIWANAYOS P., KONGKUMNERD J., CHAWEEPACK T., MAVICHAK R., UNAJAK S., NOZAKI R., KONDO H. & HIRONO I. (2014). Development of PCR diagnosis method for shrimp acute hepatopancreatic necrosis disease (AHPND) strain of *Vibrio parahaemolyticus*. *Fish Pathol.,* **49**, 159–164.

TRAN L.H., FITZSIMMONS K. & LIGHTNER D.V. (2014). AHPND/EMS: From the academic science perspective to the production point of view. *Aquaculture Asia Pacific*, **10**, 14–18.

TRAN L., NUNAN L., REDMAN R.M., MOHNEY L.L., PANTOJA C.R., FITZSIMMONS K. & LIGHTNER D.V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Org*., **105**, 45–55.

WEISBURG W.G., BARNS S.M., PELLETIER D.A. & LANE D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*., **173**, 697–703.

YANG Y.T., CHEN I.T., LEE C.T., CHEN C.Y., LIN S.S., HOR L.I., TSENG T.C., HUANG Y.T., SRITUNYALUCKSANA K., THITAMADEE S., WANG H.C. & LO C.F. (2014). Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. *Genome Announc.*, **2**, e00816-14.

*
* *

NB: There are WOAH Reference Laboratories for acute hepatopancreatic necrosis disease
(please consult the WOAH 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 WOAH Reference Laboratory for any further information on
acute hepatopancreatic necrosis disease

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

CHAPTER 2.2.3.

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, with juveniles always being the most severely affected. 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 WOAH 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 *H. penaei* ~~NHP~~ in apparently healthy shrimp has not been evaluated. ~~Faeces samples have not been validated to the same level as hepatopancreas samples.~~

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. 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 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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 screening populations for infection with ~~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*~~ in cell culture. 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 flagella hook ~~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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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			
H. penaei/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			
H. penaei/ Flagella hook gene-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

~~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~~ flagella hook gene separately.

Pathogen/ target gene	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1: Aranguren *et al.*, 2010; GenBank Accession No.: MH230908.1; amplicon size: 379 bp			
H. penaei/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; GenBank Accession No.: JQAJ01000001.1; amplicon size: 333 bp			
H. penaei/ Flagella hook gene ~~protein~~	Fwd FlgE 1143F: AGG-CAA-ACA-AAC-CCT-TG Rev FlgE 1475R: GCG-TTG-GGA-AAG-TT	~~0.2 µM~~ 200 nM	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 should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

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

[1] For example transboundary commodities.

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

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 (no data are currently available for either). 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.

7. References

AGUIRRE-GUZMAN G., SANCHEZ-MARTINEZ J.G., PÉREZ-CASTAÑEDA R. & ORTA-RODRIGUEZ R. (2010). Detection of necrotizing hepatopancreatitis (NHP) in wild shrimp from Laguna Madre, Mexico by a multiplex polymerase chain reaction. *Thai J. Vet. Med.*, **40**, 337–341.

ARANGUREN L.F., BRIÑEZ B., ARAGON L., PLATZ C., CARABALLO X., SUAREZ A. & SALAZAR M. (2006). Necrotizing hepatopancreatitis (NHP) infected *Penaeus vannamei* female broodstock: effect on reproductive parameters nauplii and larvae quality. *Aquaculture*, **258**, 337–343.

ARANGUREN L.F. & DHAR ARUN K. (2018). Detection and quantification of *Hepatobacter penaei* bacteria (NHPB) by new PCR and real-time quantitative PCR assays. *Dis. Aquat. Org*., **131**,: 49–57.

ARANGUREN L.F., TANG K.F.J. & LIGHTNER D.V. (2010). Quantification of the bacterial agent of necrotizing hepatopancreatitis (NHP-B) by real-time PCR and comparison of survival and NHP load of two shrimp populations. *Aquaculture*, **307**, 187–192.

AVILA-VILLA L.A., GOLLAS-GALVAN T., MARTINEZ-PORCHAS M., MENDOZA-CANO F. & HERNANDEZ-LOPEZ J. (2012). Experimental infection and detection of necrotizing hepatopancreatitis bacterium in the American lobster *Homarus americanus*. *Sci. World J.*, **2012**, 979381, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3356760/

BEKAVAC A., BECK A., DRAGIČEVIĆ P., DRAGUN Z., MAGUIRE I., IVANKOVIĆ D., FIKET Ž., GRAČAN R. , HUDINA S. (2022). Disturbance in invasion? Idiopathic necrotizing hepatopancreatitis in the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in Croatia. *J. Fish Dis*., **45**, 261–276.

BRADLEY-DUNLOP D.J., PANTOJA C. & LIGHTNER. D.V. (2004). Development of monoclonal antibodies for detection of necrotizing hepatopancreatitis in penaeid shrimp. *Dis. Aquat. Org.,* **60**, 233–240.

BRINEZ B., ARANGUREN F. & SALAZAR M. (2003). Fecal samples as DNA source for the diagnosis of necrotizing hepatopancreatitis (NHP) in *Penaeus vannamei* broodstock. *Dis. Aquat. Org.,* **55**, 69–72.

CRABTREE B.G., ERDMAN M.M., HARRIS D.L. & HARRIS I.T. (2006). Preservation of necrotizing hepatopancreatitis bacterium (NHPB) by freezing tissue collected from experimentally infected *Litopenaeus vannamei*. *Dis. Aquat. Org.,* **70**, 175–179.

DEL RÍO-RODRIGUEZ R.E., SOTO-RODRÍGUEZ S., LARA-FLORES M., CU-ESCAMILLA A.D. & GOMEZ-SOLANO M.I. (2006). A necrotizing hepatopancreatitis (NHP) outbreak in a shrimp farm in Campeche, Mexico: A first case report. *Aquaculture*, **255**, 606–609.

FRELIER P.F., LOY J.K. & KRUPPENBACH B. (1993). Transmission of necrotizing hepatopancreatitis in *Penaeus vannamei*. *J. Invertebr. Pathol,* **61**, 44–48.

FRELIER P.F., SIS R.F., BELL T.A. & LEWIS D.H. (1992). Microscopic and ultrastructural studies of necrotizing hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) cultured in Texas. *Vet. Pathol*., **29**, 269–277.

GRACIA-VALENZUELA M.H., LUZ ANGELICA ÁVILA-VILLA L.A., GLORIA YEPIZ-PLASCENCIA G., HERNÁNDEZ-LÓPEZ J., MENDOZA-CANO F., GARCÍA-SANCHEZ G. & GOLLAS-GALVÁN T. (2011). Assessing the viability of necrotizing hepatopancreatitis bacterium (NHPB) stored at –20°C for use in forced-feeding infection of *Penaeus* (*Litopenaeus*) *vannamei*. *Aquaculture*, **311**, 105–109.

IBARRA-GAMEZ J.C., GALAVÍZ-SILVA L. & MOLINA-GARZA Z.J. (2007). Distribution of necrotizing hepatopancreatitis bacterium (NHPB) in cultured white shrimp, *Litopenaeus vannamei*, from Mexico. *Cienc. Mar.,* **33,** 1–9.

KROL R.M., HAWKINS W.E. & OVERSTREET R.M. (1991). Rickettsial and mollicute infections in hepatopancreatic cells of cultured pacific white shrimp (*Penaeus vannamei*). *J. Invertebr. Pathol.*, **57**, 362–370.

LEYVA J.M., MARTINEZ-PORCHAS M., HERNANDEZ-LOPEZ J., VARGAS-ALBORES F.& T. GOLLAS-GALVAN (2018). Identifying the causal agent of necrotizing hepatopancreatitis in shrimp: Multilocus sequence analysis approach *Aquaculture Res*., 1–8.

LIGHTNER D.V (ed.) (1996). A handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, USA, 304 p.

LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquac. Soc*., **36**, 229–248.

LIGHTNER D.V. & REDMAN R.M. (1994). An epizootic of necrotizing hepatopancreatitis in cultured penaeid shrimp (Crustacea: Decapoda) in northwestern Peru. *Aquaculture*, **122**, 9–18.

LIGHTNER D.V., REDMAN R.M., PANTOJA C.R., TANG K.F.J., NOBLE B.L., SCHOFIELD P., MOHNEY L.L., NUNAN L.M. & NAVARRO S.A. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J. Invertebr. Pathol.*, **110**, 174–183.

LOY J.K., DEWHIRST F.E., WEBER W., FRELIER P.F., GARBAR T.L., TASCA S.I & TEMPLETON J.W. (1996). Molecular phylogeny and *in situ* detection of the etiologic agent of necrotizing hepatopancreatitis in shrimp. *Appl. Environ. Microbiol*., **62**, 3439–3445.

LOY J.K. & FRELIER P.F. (1996). Specific, nonradioactive detection of the NHP bacterium in *Penaeus vannamei* by *in situ* hybridization. *J. Vet. Diagn. Invest.,* **8**, 324–331.

LOY J.K., FRELIER P.F., VARNER P. & TEMPLETON J.W. (1996). Detection of the etiologic agent of necrotizing hepatopancreatitis in cultured *Penaeus vannamei* from Texas and Peru by polymerase chain reaction. *Dis. Aquat. Org*., **25**, 117–122.

MORALES-COVARRUBIAS M.S. (2010). Enfermedades del camarón. Detección mediante análisis en fresco e histopatología. Editorial Trillas, SA de CV., Av. Río Churubusco 385, Col. Pedro María Anaya, México, D.F. Segunda edición. ISBN: ISBN 978-607-17-0436-8. 1-180.

MORALES-COVARRUBIAS M.S., OSUNA-DUARTE A.G., GARCIA-GASCA A., LIGHTNER D.V. & MOTA-URBINA J.C. (2006). Prevalence of necrotizing hepatopancreatitis in female broodstock of *Penaeus vannamei* with unilateral eyestalk ablation and hormone injection. *J. Aquat. Anim. Health*, **18**, 19–25.

MORALES-COVARRUBIAS M.S., RUIZ-LUNA A., MOURA-LEMUS A.P., SOLÍS MONTIEL V.T. & CONROY G. (2011). Prevalencia de enfermedades de camarón blanco (*Litopenaeus vannamei*) cultivado en ocho regiones de latinoamérica. *Rev. Cient.* (*Maracaibo*), **XXI**, 434–446.

NUNAN L.M., PANTOJA C.R., GOMEZ-JIMENEZ S. & LIGHTNER D.V. (2013). "*Candidatus* Hepatobacter penaei," an intracellular pathogenic enteric bacterium in the hepatopancreas of the marine shrimp *Penaeus vannamei* (Crustacea: Decapoda). *Appl. Environ. Microbiol*., **79**, 1407–1409.

VINCENT A.G., BRELAND V.M. & LOTZ J.M. (2004). Experimental infection of Pacific white shrimp *Litopenaeus vannamei* with necrotizing hepatopancreatitis (NHP) bacterium by *per os* exposure. *Dis. Aquat. Org.,* **61**, 227–233

VINCENT A.G. & LOTZ J.M. (2005). Time course of necrotizing hepatopancreatitis (NHP) in experimentally infected *Litopenaeus vannamei* and quantification of NHP-bacterium using real-time PCR. *Dis. Aquat. Org.,* **67**, 163–169.

VINCENT A.G. & LOTZ J.M. (2007). Effect of salinity on transmission of necrotizing hepatopancreatitis bacterium (NHPB) to Kona stock *Litopenaeus vannamei. Dis. Aquat. Org.,* **75**, 265–268.

*
* *

NB: There is a WOAH Reference Laboratory for infection with *Hepatobacter penaei*
(necrotising hepatopancreatitis)
(please consult the WOAH 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 WOAH 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). IHHNV genotypes in Ecuador and Peru were found to be within a separate lineage of IHHNV type 2 genotypes circulating within these countries (Aranguen Caro *et al.,* 2022). 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~~ Portunidae	*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.

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).

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 WOAH 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

Specimens suitable for testing for infection with IHHNV include postlarvae (PL), juveniles and adults. While IHHNV may infect all life stages, virus load ~~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).

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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~~ bioassay

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~~ molecular techniques can be found in Section B.~~2.5~~ 5.5. 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~~ 5.3 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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					±	±	±	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 WOAH 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 is important and can ~~should~~ be checked using a suitable method as appropriate to the circumstances ~~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 Accession No.: ~~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 Accession No.: ~~Acc. No~~ AF218266; amplicon size 389 bp ~~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 Accession No.: ~~Acc. No~~ AF218266; amplicon size 356 bp ~~product~~			
IHHNV and IHHNV-related EVEs Between the non-structural and capsid protein-coding regions	Fwd 77012F: ~~TAC-TCC-GGA-CAC-CCA-ACC-A~~ ATC-GGT-GCA-CTA-CTC-GGA Rev 77353R: ~~GGC-TCT-GGC-AGC-AAA-GGT-AA~~-TCG-TAC-TGG-CTG-TTC-ATC	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 Accession No.: ~~Acc. No~~ AF218266; amplicon size 392 bp ~~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 Accession No.: ~~Acc. No~~ AF218266; amplicon size 309 bp ~~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~~
~~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~~

Primer	Product	Sequence (5′-3′)	G+C%/Temp.	GenBank & References	Specificity
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 should be ~~is~~ verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands must be sequenced and analysed ~~and compared with published~~ in comparison with reference 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 molecular 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

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 criterion ~~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

~~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:

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

vi) Positive result by bioassay

[1] For example transboundary commodities.

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~~ no data are currently available for either). 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.

7. References

ALY S.M., MANSOUR S.M., THABET R.Y. & MABROK M. (2021). Studies on infectious myonecrosis virus (IMNV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in cultured penaeid shrimp in Egypt. *Dis. Aquat. Org.*, **143**, 57–67.

ARANGUREN CARO L.F., GOMEZ-SANCHEZ M.M., PIEDRAHITA Y., MAI H.N., CRUZ-FLORES R., ALENTON R.R.R.& DHAR A.K. (2022). Current status of infection with infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Peruvian and Ecuadorian shrimp industry. *PLoS One*, 17(8):e0272456. doi: 10.1371/journal.pone.0272456.

ARUNRUT N., PROMBUN P., SAKSMERPROME V., FLEGEL T. W. & KIATPATHOMCHAI W. (2011). Rapid and sensitive detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *J. Virol. Methods,* **171**, 21–25.

BELL T.A. & LIGHTNER D.V. (1984). IHHN virus: infectivity and pathogenicity studies in *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture*, **38**, 185–194.

BELL T.A. & LIGHTNER D.V. (1987). IHHN disease of *Penaeus stylirostris*: effects of shrimp size on disease expression. *J. Fish Dis*., **10**, 165–170.

BELL T.A. & LIGHTNER D.V. (1988). A Handbook of Normal Shrimp Histology. Special Publication No. 1, World Aquaculture Society, Baton Rouge, Louisiana, USA, 114 pp.

BONAMI J.R., TRUMPER B., MARI J., BREHELIN M. & LIGHTNER D.V. (1990). Purification and characterization of IHHN virus of penaeid shrimps. *J. Gen. Virol*., **71**, 2657–2664.

CHAYABURAKUL K., LIGHTNER D.V., SRIURAIRATTANA S., NELSON K.T. & WITHYACHUMNARNKUL B. (2005). Different responses to infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* and *P. vannamei*. *Dis. Aquat. Org.*, **67**, 191–200.

CHAYABURAKUL K., NASH G., PRATANPIPAT P., SRIURARAIRATANA S. & WITHYACHUMNARNKUL B. (2004). Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. *Dis. Aquat. Org*., **60**, 89–96.

CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon. In:* Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.

COWLEY J.A., RAO M., COMAN G.J. & COWLEY J. (2018). Real-time PCR tests to specifically detect Infectious hypodermal and haemopoietic necrosis virus (IHHNV) lineages and an IHHNV endogenous viral element (EVE) integrated in the genome of Black Tiger shrimp (*Penaeus monodon*). *Dis. Aquat. Org*., **129**, 145–158.

DHAR A.K., ROUX M.M. & KLIMPEL K.R. (2001). Detection and quantification of Infectious hypodermal and hematopoeitic necrosis virus and White spot virus in shrimp using real-time quantitative PCR and SYBR green chemistry. *J. Clin. Microbiol*., **39**, 2835–2845.

ENCINAS-GARCIA T., MENDOZA-CANO F., ENRÍQUEZ-ESPINOZA T., LUKEN-VEGA L., VICHIDO-CHÁVEZ R. & SÁNCHEZ-PAZ A. (2015). An improved validated SYBR green-based real-time quantitative PCR assay for the detection of the *Penaeus stylirostris* densovirus in penaeid shrimp. *J. Virol. Methods*, **212**, 53–58.

FERNANDO M.C., ENRIQUEZ-ESPINOZA T., VALENZUELA-CASTILLO A., ENCINAS-GARCIA T. & SANCHEZ-PAZ A. (2016). High Occurrence of the Decapod Penstyldensovirus (PstDV1) Detected in Postlarvae of *Penaeus vannamei* Produced in Commercial Hatcheries of Mexico. *EcoHealth.* **13**, 591–596.

~~JAGADEESAN V., EZHIL PRAVEENA P., OTTA S.K. & JITHENDRAN K.P. (2019). Classical runt deformity syndrome cases in farmed *Penaeus vannamei* along the east coast of India. *In:* BRAQCON 2019: World Brackishwater Aquaculture Conference, Jithendran K.P., Saraswathy R., Balasubramanian C.P., Kumaraguru Vasagam K.P., Jayasankar V., Raghavan R., Alavandi S.V. & Vijayan K.K., eds. Journal of Coastal Research, Special Issue No. 86, pp. 107–111. Coconut Creek (Florida), ISSN 0749-0208.~~

KALAGAYAN G., GODIN D., KANNA R., HAGINO G., SWEENEY J., WYBAN J. & BROCK J. (1991). IHHN virus as an etiological factor in runt-deformity syndrome of juvenile *Penaeus vannamei* cultured in Hawaii. *J. World Aquacult. Soc*., **22**, 235–243.

KRABSETSVE K., CULLEN B.R. & OWENS L. (2004). Rediscovery of the Australian strain of infectious hypodermal and haematopoietic necrosis virus. *Dis. Aquat. Org*., **61**, 153–158.

LIGHTNER D.V. (ED.) (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA, 304 pp.

LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquaculture Soc.* **36**, 229–248.

LIGHTNER D.V., MOHNEY L.L., WILLIAMS R.R. & REDMAN R.M. (1987). Glycerol tolerance of IHHN virus of penaeid shrimp. *J. World Aquac. Soc*., **18**, 196–197.

LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free (SPF) shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. In: Shellfish Safety and Quality, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK, 384–424.

LIGHTNER D.V. & REDMAN R.M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture*, **164**, 201–220.

LIGHTNER D.V., REDMAN R.M. & BELL T.A. (1983). Infectious hypodermal and hematopoietic necrosis a newly recognized virus disease of penaeid shrimp. *J. Invertebr. Pathol*., **42**, 62–70.

MARI J., BONAMI J.R. & LIGHTNER D.V. (1993). Partial cloning of the genome of infectious hypodermal and hematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. *J. Gen. Virol.,* **74**, 2637–2643.

MONTGOMERY-BROCK D., TACON A.G.J., POULOS B., & LIGHTNER D.V. (2007). Reduced replication of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Litopenaeus vannamei* held in warm water. *Aquaculture*, **265**, 41–48.

MORALES-COVARRUBIAS M.S., NUNAN L.M., LIGHTNER D.V., MOTA-URBINA J.C., GARZA-AGUIRRE M.C. & CHAVEZ-SANCHEZ M.C. (1999). Prevalence of IHHNV in wild broodstock of *Penaeus stylirostris* from the upper Gulf of California, Mexico*. J. Aquat. Anim. Health*, **11**, 296–301.

MOTTE, E., YUGCHA E., LUZARDO J., CASTRO F., LECLERCQ G., RODRÍGUEZ J., MIRANDA P., BORJA O., SERRANO J., TERREROS M., MONTALVO K., NARVÁEZ A., TENORIO N., CEDEÑO V., MIALHE E. & BOULO V. (2003). Prevention of IHHNV vertical transmission in the white shrimp *Litopenaeus vannamei. Aquaculture,* **219**, 57–70.

NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (2000). Use of polymerase chain reaction (PCR) for the detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in penaeid shrimp. *Mar. Biotechnol*., **2**, 319–328.

NUNAN L.M., ARCE S.M., STAHA R.J. & LIGHTNER D.V. (2001). Prevalence of Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and White spot syndrome virus (WSSV) in *Litopenaeus vannamei* in the Pacific Ocean off the coast of Panama. *J. World Aquacult. Soc*., **32**, 330–334.

PANTOJA C.R., LIGHTNER D.V. & HOLTSCHMIT K.H. (1999). Prevalence and geographic distribution of IHHN parvovirus in wild penaeid shrimp (Crustacea: Decapoda) from the Gulf of California, Mexico*. J. Aquat. Anim. Health*, **11**, 23–34.

PENZES J.J., SODERLUND-VENERMO M., CANUTI M., EIS-HUBINGER A.M., HUGHES J., COTMORE S.F. & HARRACH B. (2020). Reorganizing the family *Parvoviridae*: a revised taxonomy independent of the canonical approach based on host association. *Arch. Virol*., **165**, 2133–2146. https://doi.org/10.1007/s00705-020-04632-4

PRIMAVERA, J.H. & QUINITIO E.T. (2000). Runt-deformity syndrome in cultured giant tiger prawn *Penaeus monodon. J. Crust. Biol*., **20**, 796–802.

SAKSMERPROME V., JITRAKORN S., CHAYABURAKUL K., LAIPHROM S., BOONSUA K. & FLEGEL T.W. (2011). Additional random, single to multiple genome fragments of *Penaeus stylirostris* densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis. *Virus Res*., **160**, 180–190.

SELLARS M.J., COWLEY J.A., MUSSON D., RAO M., MENZIES M.L, COMAN G. J. & MURPHY B.S. (2019). Reduced growth performance of Black Tiger shrimp (*Penaeus monodon*) infected with infectious hypodermal and hematopoietic necrosis virus. *Aquaculture*, **499**, 160–166.

SHIKE H., DHAR A.K., BURNS J.C., SHIMIZU C., JOUSSET F.X., KLIMPEL K.R. & BERGOIN M. (2000). Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito Brevidensoviruses. *Virology*, **277**, 167–177.

SUN Z.F., HU C. Q., REN C. H. & SHEN Q. (2006). Sensitive and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimps by loop-mediated isothermal amplification. *J. Virol. Methods,* **131**, 41–46.

TAENGCHAIYAPHUM S., BUATHONGKAM P., SUKTHAWORN S., WONGKHALUANG P., SRITUNYALUCKSANA K. & FLEGEL T.W. (2021). Shrimp Parvovirus Circular DNA Fragments Arise From Both Endogenous Viral Elements and the Infecting Virus. *Front. Immunol*., **12**, 729528. doi: 10.3389/fimmu.2021.729528.

TAENGCHAIYAPHUM S., WONGKHALUANG P., SITTIKANKAEW K., KAROONUTHAISIRI N., FLEGEL T.W. & SRITUNYALUCKSANA K. (2022). Shrimp genome sequence contains independent clusters of ancient and current Endogenous Viral Elements (EVE) of the parvovirus IHHNV. *BMC Genomics,* **23**, 565. doi: 10.1186/s12864-022-08802-3.

TANG K.F.J., DURAND S.V., WHITE B.L., REDMAN R.M., PANTOJA C.R. & LIGHTNER D.V. (2000). Postlarvae and juveniles of a selected line of *Penaeus stylirostris* are resistant to infectious hypodermal and hematopoietic necrosis virus infection. *Aquaculture*, **190**, 203–210.

TANG K.F.J. & LIGHTNER D.V. (2001). Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. *Dis. Aquat. Org.,* **44**, 79–85.

TANG K.F.J. & LIGHTNER D.V. (2006). Infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Res.,* **118**, 185–191.

TANG K.F.J., NAVARRO S.A. & LIGHTNER D.V. (2007). A PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the virus-related sequences in the genome of *Penaeus monodon*. *Dis. Aquat. Org.,* **74**, 165–170.

TANG K.F.J., POULOS B.T., WANG J., REDMAN R.M., SHIH H.H. & LIGHTNER D.V. (2003). Geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. *Dis. Aquat. Org*., **53**, 91–99.

~~WEI Y.W., FAN D.D. & CHEN J. (2017). The mussel *Mytilus edulis* L. as an important reservoir of infectious hypodermal and hematopoietic necrosis virus (IHHNV). *Aquaculture Res.,* **48**, 1346–1350.~~

XIA X.X., YU Y.X., HU L.H., MANFRED W. & PAN Y.J. (2015). Rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) by real-time, isothermal recombinase polymerase amplification assay. *Arch. Virol.,* **160**, 987–994.

*
* *

NB: There are WOAH Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus (please consult the WOAH web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH 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*,~~ tentatively assigned to the family *Totiviridae* ~~a member of the family *Totiviridae*~~ (~~Fauquet *et al.*, 2005~~; Lightner, 2011; Nibert, 2007; Poulos *et al.,* 2006; Wickner *et al.,* 2011).

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. A sudden onset of clinical signs may ~~have a sudden onset~~ occur 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.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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.
[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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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 Accession No.: KJ636783.2; amplicon size: 328/139 bp			
IMNV Capsid protein gene (nested-PCR)	~~Outer~~ Primary Fwd 4587F: CGA-CGC-TGC-TAA-CCA-TAC-AA Rev 4914R: ACT-CGG-CTG-TTC-GAT-CAA-GT ~~Inner~~ Nested 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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratories designated for IMN.

6.1. Apparently healthy animals or animals of unknown health status [1]

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~~

i) Positive result by real-time RT-PCR

ii) 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 criterion ~~criteria~~ is met:

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

[1] 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~~

~~ii) 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

iv) 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 (no data are currently available). 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 (*n*=30)	100 (*n*=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.

7. References

ALY S.M., MANSOUR S.M., THABET R.Y. & MABROK M. (2021). Studies on infectious myonecrosis virus (IMNV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in cultured penaeid shrimp in Egypt. *Dis. Aquat. Org.,* **143**, 57–67. doi: 10.3354/dao03556. PMID: 33570040.

ANDRADE T.P.D., SRISUVAN T., TANG K.F.J. & LIGHTNER D.V. (2007). Real-time reverse transcription polymerase chain reaction assay using TaqMan probe for detection and quantification of infectious myonecrosis virus (IMNV). *Aquaculture*, **264**, 9–15.

BELL T.A. & LIGHTNER D.V. (1988). A Handbook of Normal Penaeid Shrimp Histology. World Aquaculture Society, Baton Rouge, LA, USA, 114 p.

CHAIVISUTHANGKURA P., SENAPIN S., WANGMAN P., LONGYANT S. & SITHIGORNUL P. (2013). Simple and rapid detection of infectious myonecrosis virus using an immunochromatographic strip test. *Arch Virol*., **158**, 1925–1930.

CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon. In:* Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.

DANTAS M.D., CHAVANTE S.F., TEIXEIRA D.I., LIMA J.P. & LANZA D.C. (2015). Analysis of new isolates reveals new genome organization and a hypervariable region in infectious myonecrosis virus (IMNV). *Virus Res*., **20**, 66–71. doi: 10.1016/j.virusres.2015.03.015. Epub 2015 Apr 4. PMID: 25849112.

DA SILVA S.M.B.C., LAVANDER H.D., DE SANATANA LUNA M.M., DA SILVA A.O.M.E., GALVEZ A.O. & COIMBRA M.R.M. (2015). *Artemia franciscana* as a vector for infectious myonecrosis virus (IMNV) to *Litopenaeus vannanmei* juvenile. *J. Invertebr. Pathol.*, **126,** 1–5.

~~FAUQUET C.M., MAYO M.A., MANILOFF J., DESSELBERGER U. & BALL L.A., EDITORS (2005). Totiviridae. *In:* Virus Taxonomy: Classification and Nomenclature of Viruses. Eighth Report of the International Committee on the Taxonomy of Viruses, Elsevier, San Francisco, USA, pp. 571–580.~~

KOIWAI K., KODERA T., THAWONSUWAN J., RIANI S., KAWASE M., KONDO H. & HIRONO I. (2018). Rapid diagnosis of three shrimp RNA viruses using RT-PCR-DNA chromatography. *J. Fish Dis.,* 2018 May 28. doi: 10.1111/jfd.12821. Epub ahead of print. PMID: 29806113.

KUNANOPPARAT A., CHAIVISUTHANGKURA P., SENAPIN S., LONGYANY S., RUKPRATANPORN S., FLEGEL T.W. & SITHIGORNGUL P. (2011). Detection of infectious myonecrosis virus using monoclonal antibody specific to N and C fragments of the capsid protein expressed heterologously. *J. Virol. Methods*, **171**, 141–148.

LEE C.S. & O'BRYEN P.J., EDITORS (2003). Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. World Aquaculture Society, Baton Rouge, LA, USA, 293 p.

LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquac. Soc.*, **36**, 229–248.

LIGHTNER D.V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. *J. Invertebr. Pathol.*, **106,** 110–130.

LIGHTNER D.V., PANTOJA C.R., POULOS B.T., TANG K.F.J., REDMAN R.M., PASOS DE ANDRADE T. & BONAMI J.R. (2004). Infectious myonecrosis: new disease in Pacific white shrimp. *Global Aquaculture Advocate,* **7**, 85.

LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. *In:* Shellfish Safety and Quality, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK, pp. 384–424.

LOY D.S., LIU S., MOGLER M.A., LOY D.J., BLITVICH B.J. & BARTHOLOMAY L.C. (2015). Characterization of newly revealed sequences in the infectious myonecrosis virus genome in *Litopenaeus vannamei*. *J. Gen. Virol.*, 96 (Pt **7**), 1821–1819.

MAI H.N., HANGGONO B., CARO L.F.A., KOMARUDDIN U., NUR'AINI Y.L.& DHAR A.K. (2019). Novel infectious myonecrosis virus (IMNV) genotypes associated with disease outbreaks on Penaeus vannamei shrimp farms in Indonesia. *Arch. Virol.,* **164**, 3051–3057. doi: 10.1007/s00705-019-04408-5. Epub 2019 Sep 17. PMID: 31531743.

MOSS S.M. & MOSS D.R. (2009). Chapter 17: Selective breeding of penaeid shrimp. *In:* Shellfish Safety and Quality, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK. pp. 425–452.

NAIM S., BROWN J.K. & NIBERT M.L. (2014). Genetic diversification of penaeid shrimp infectious myonecrosis virus between Indonesia and Brazil. *Virus Res*., **189**, 99–105.

NAIM S., TANG K.F.J., YANG M., LIGHTNER D.V. & NIBERT M.L. (2015). Extended genome sequences of penaeid shrimp infectious myonecrosis virus strains from Brazil and Indonesia. *Arch. Virol*., **160**, 1579–1583.

NIBERT M.L. (2007). '2A-like' and 'shifty heptamer' motifs in penaeid shrimp infectious myonecrosis virus, a monosegmented double-stranded RNA virus. *J. Gen. Virol.*, **88**, 1315–1318.

NUNES A.J.P., CUNHA-MARTINS P. & VASCONSELOS-GESTEIRA T.C. (2004). Carcinicultura ameaçada. *Rev. Panoram. Aquic.*, **83**, 37–51 (in Portuguese).

POULOS B.T. & LIGHTNER D.V. (2006). Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). *Dis. Aquat. Org*., **73**, 69–72.

POULOS B.T., TANG K.F.J., PANTOJA C.R., BONAMI J.R. & LIGHTNER D.V. (2006). Purification and characterization of infectious myonecrosis virus of penaeid shrimp. *J. Gen. Virol.*, **87**, 987–996.

SAHUL HAMEED A.S., ABDUL MAJEED S., VIMAL S., MADAN N., RAJKUMAR T., SANTHOSHKUMAR S. & SIVAKUMAR S. (2017). Studies on the occurrence of infectious myonecrosis virus in pond-reared *Litopenaeus vannamei* (Boone, 1931) in India. *J. Fish Dis.,* **40**, 1823–1830. doi: 10.1111/jfd.12655. Epub 2017 Jun 20. PMID: 28631825

SANTHOSH KUMAR S., SIVAKUMAR S., ABDUL MAJEED S., VIMAL S., TAJU G. & SAHUL HAMEED A.S. (2021). *In vitro* propagation of infectious myonecrosis virus in C6/36 mosquito cell line. *J. Fish Dis.,* **44**, 987–992. doi: 10.1111/jfd.13359. Epub 2021 Feb 25. PMID: 33631045.

SENAPIN S., PHEWSAIYA K., BRIGGS M. & FLEGEL T.W. (2007). Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture*, **266**, 32–38.

TANG K.F.J., PANTOJA C.R., POULOS B.T., REDMAN R.M. & LIGHTNER D.V. (2005). *In situ* hybridization demonstrates that *Litopenaeus vannamei, L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Dis. Aquat. Org*., **63**, 261–265.

TANG K.F.J., PANTOJA C.R., REDMAN R.M. & LIGHTNER D.V. (2007). Development of *in situ* hybridization and RT-PCR assay for the detection of a nodavirus (*Pv*NV) that causes muscle necrosis in *Penaeus vannamei*. *Dis. Aquat. Org*., **75**, 183–190.

VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.

VIEIRA-GIRÃO P.R.N., FALCÃO C.B., ROCHA I.R.C.B., LUCENA H.M.R., COSTA F.H.F. & RÁDIS-BAPTISTA G. (2017). Antiviral Activity of Ctn[15–34], A Cathelicidin-Derived Eicosapeptide, Against Infectious Myonecrosis Virus in *Litopenaeus vannamei* Primary Hemocyte Cultures. *Food Environ. Virol.*, **9**, 277–286. doi: 10.1007/s12560-017-9285-5. Epub 2017 Feb 16. PMID: 28210987.

WHITE-NOBLE B.L., LIGHTNER D.V., TANG K.F.J. & REDMAN R. (2010). Lab challenge for selection of IMNV-resistant white shrimp. *Global Aquaculture Advocate*, July/August, 71–73.

Wickner R.B., Ghabrial S.A., Nibert M.L., Patterson J.L. & Wang C.C. (2011). Totiviridae. *In:* Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on the Taxonomy of Viruses, Elsevier, San Diego, USA.

*
* *

NB: At the time of publication (2022) there was no WOAH Reference Laboratory for infection with infectious myonecrosis virus (please consult the WOAH 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 killing the animals and used as non-lethal sampling of genetically valuable broodstock.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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~~ bioassay

The success of ~~pathogen isolation~~ bioassay depend~~s~~ 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.~~

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. 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 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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, i.e. by testing two aliquots.

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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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			
TSV/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~~ amplicon size: 231 bp			
TSV/ORF 2	Fwd: 9992: AAG-TAG-ACA-GCC-GCG-CTT	Primers/620 nM each	Reverse transcription 60°C/30 min

	Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC		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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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) [1].

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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

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. In addition, apparently ~~Alternatively,~~ healthy populations are sampled, when ~~in~~ surveys are carried out to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

[1] Reference to specific commercial products as examples does not imply their endorsement by WOAH. This applies to all commercial products referred to in this *Aquatic Manual*.

2 For example transboundary commodities.

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~~

i) A positive result by real-time RT-PCR

ii) 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 criterion ~~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

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 Tables 6.3.1 and 6.3.2. (~~none~~ no data are currently available for either). 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.

7. References

AUDELO DEL VALLE J., CLEMENT-MELLADO O., MAGANA-HERNANDEZ A., FLISSER A., MONTIEL-AGUIRRE F. & BRISENO-GARCIA B. (2003). Infection of cultured human and monkey cell lines with extract of penaeid shrimp infected with Taura syndrome virus. *Emerg. Infect. Dis.,* **9**, 265–266.

BONAMI J.R., HASSON K.W., MARI J., POULOS B.T. & LIGHTNER D.V. (1997). Taura syndrome of marine penaeid shrimp: characterization of the viral agent. *J. Gen. Virol.,* **78**, 313–319.

BROCK J.A. (1997). Special topic review: Taura syndrome, a disease important to shrimp farms in the Americas. *World J. Microbiol Biotechnol.,* **13**, 415–418.

BROCK J.A., GOSE R., LIGHTNER D.V. & HASSON K.W. (1995). An overview on Taura syndrome, an important disease of farmed *Penaeus vannamei*. *In:* Swimming through Troubled Water, Proceedings of the Special Session on Shrimp Farming, Aquaculture '95, Browdy C.L. & Hopkins J.S., eds. San Diego, California, 1–4 February 1995. World Aquaculture Society, Baton Rouge, Louisiana, USA, 84–94.

CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon. In:* Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.

CLIFFORD H.C. (1998). Management of ponds stocked with blue shrimp *Litopenaeus stylirostris*. *In:* Proceedings of the First Latin American Shrimp Farming Congress, D.E. Jory, ed. Panama City, Panama, 1–11.

DHAR A.K., COWLEY J.A., HASSON K.W. & WALKER P.J. (2004). Taura syndrome virus and yellow head virus of penaeid shrimp. *Adv. Virus Res.,* 64, 353–421.

DIXON H. & DORADO J. (1997). Managing Taura syndrome in Belize: a case study. *Aquaculture Magazine*, May/June, 30–42.

FEGAN D.F. & CLIFFORD H.C. III. (2001). Health management for viral diseases in shrimp farms. *In:* The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture. Aquaculture 2001, Browdy C.L. & Jory D.E., eds. The World Aquaculture Society, Baton Rouge, Louisiana, USA, 168–198.

GARZA J.R., HASSON K.W., POULOS B.T., REDMAN R.M., WHITE B.L. & LIGHTNER D.V. (1997). Demonstration of infectious taura syndrome virus in the feces of sea gulls collected during an epizootic in Texas. *J. Aquat. Anim. Health,* **9**, 156–159.

HASSON K.W., HASSON J., AUBERT H., REDMAN R.M. & LIGHTNER D.V. (1997). A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological detection using cDNA genomic probes. *J. Virol. Methods,* **66**, 227–236.

HASSON K.W., LIGHTNER D.V., MARI J. & BONAMI J.R., POULOS B.T., MOHNEY L.L., REDMAN R.M. & BROCK J.R. (1999a). The geographic distribution of Taura Syndrome Virus (TSV) in the Americas: determination by histology and *in situ* hybridization using TSV-specific cDNA probes. *Aquaculture*, **171**, 13–26.

HASSON K.W., LIGHTNER D.V., MOHNEY L.L., REDMAN R.M., POULOS B.T. & WHITE B.M. (1999b). Taura syndrome virus (TSV) lesion development and the disease cycle in the Pacific white shrimp *Penaeus vannamei*. *Dis. Aquat. Org.,* **36**, 81–93.

HASSON K.W., LIGHTNER D.V., POULOS B.T., REDMAN R.M., WHITE B.L., BROCK J.A. & BONAMI J.R. (1995). Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. *Dis. Aquat. Org.,* **23**, 115–126.

INTRIAGO P., JIMENEZ R., MACHUCA M., BARNIOL R., KRAUSS E. & SALVADOR X. (1997). Experiments on toxicosis as the cause of Taura Syndrome in *Penaeus vannamei* (Crustacea: Decapoda) in Ecuador. *In:* Diseases in Asian Aquaculture III, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, the Philippines, 365–379.

JIMENEZ R. (1992). Sindrome de Taura (Resumen). *In:* Acuacultura del Ecuador. Camara Nacional de Acuacultura, Guayaquil, Ecuador, 1–16.

JIMENEZ R., BARNIOL R., DE BARNIOL L. & MACHUCA M. (2000). Periodic occurrence of epithelial viral necrosis outbreaks in *Penaeus vannamei* in Ecuador. *Dis. Aquat. Org.,* **42**, 91–99.

LIGHTNER D.V. (ED.) (1996a). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA, 304 pp.

LIGHTNER D.V. (1996b). Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. *Rev. sci. tech. Office int. Epiz*., **15**, 579–601.

LIGHTNER D.V. (1999). The penaeid shrimp viruses TSV, IHHNV, WSSV, and YHV: current status in the Americas, available diagnostic methods and management strategies. *J. Appl. Aquac.*, **9**, 27–52.

LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance*. J. World Aquac. Soc.,* **36**, 229–248.

LIGHTNER D.V. & REDMAN R.M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture*, **164**, 201–220.

LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. *In:* Shellfish Safety and Quality, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK. pp. 384-424.

LIGHTNER D.V., REDMAN R.M., HASSON K.W. & PANTOJA C.R. (1995). Taura syndrome in *Penaeus vannamei* (Crustacea: Decapoda): gross signs, histopathology and ultrastructure. *Dis. Aquat. Org.,* **21**, 53–59.

LIGHTNER D.V., REDMAN R.M., PANTOJA C.R., TANG K.F.J., NOBLE B.L., SCHOFIELD P., MOHNEY L.L., NUNAN L.M. & NAVARRO S.A. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J. Invertebr. Pathol.,* **110**, 174–183.

LOTZ J.M. (1997). Effect of host size on virulence of Taura virus to the marine shrimp *Penaeus vannamei* (Crustacea: Penaeidae). *Dis. Aquat. Org.,* **30**, 45–51.

LOTZ J.M., ANTON, L.S. & SOTO M.A. (2005). Effect of chronic Taura syndrome virus infection on salinity tolerance of *Litopenaeus vannamei*. *Dis. Aquat. Org.,* **65**, 75–78.

LUO P., HU C.Q., REN C.H. & SUN Z.F. (2004). Taura syndrome virus and mammalian cell lines. *Emerg. Infect. Dis.,* **10**, 2260–2261.

MARI J., BONAMI J.R. & LIGHTNER D.V. (1998). Taura syndrome of penaeid shrimp: cloning of viral genome fragments and development of specific gene probes. *Dis. Aquat. Org.,* **33**, 11–17.

NAVARRO S.A., TANG K.F.J & LIGHTNER D.V. (2009). An improved Taura syndrome virus (TSV) RT-PCR using newly designed primers. *Aquaculture*, **293**, 290–292.

NIELSEN L., SANG-OUM W., CHEEVADHANARAK S. & FLEGEL T.W. (2005). Taura syndrome virus (TSV) in Thailand and its relationship to TSV in China and the Americas. *Dis Aquat. Org,* **63**, 101–106.

NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (1998). Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura Syndrome Virus (TSV) in experimentally infected shrimp. *Dis. Aquat. Org.,* **34**, 87–91.

NUNAN L.M., TANG-NELSON K. & LIGHTNER D.V. (2004). Real-time RT-PCR determination of viral copy number in *Penaeus vannamei* experimentally infected with Taura syndrome virus. *Aquaculture*, **229**, 1–10.

OVERSTREET R.M., LIGHTNER D.V., HASSON K.W., MCILWAIN S. & LOTZ J. (1997). Susceptibility to TSV of some penaeid shrimp native to the Gulf of Mexico and southeast Atlantic Ocean. *J. Invertebr. Pathol.,* **69**, 165–176.

PANTOJA C.R., NAVARRO S.A., NARANJO J., LIGHTNER D.V. & GERBA C.P. (2004). Nonsusceptibility of primate cells to Taura syndrome virus. *Emerg. Infect. Dis.,* **10**, 2106–2112.

POULOS B.T., KIBLER R., BRADLEY-DUNLOP D., MOHNEY L.L. & LIGHTNER D.V. (1999). Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. *Dis. Aquat. Org.,* **37**, 99–106.

ROBLES-SIKISAKA R., HASSON K.W., GARCIA D.K., BROVONT K., CLEVELAND K., KLIMPEL K.R. & DHAR A.K. (2002). Genetic variation and immunohistochemical differences among geographical isolates of Taura syndrome virus of penaeid shrimp. *J. Gen. Virol.,* **83**, 3123–3130.

ROBLES-SIKISAKA R., GARCIA D.K., KLIMPEL K.R. & DHAR A.K. (2001). Nucleotide sequence of 3'-end of the genome of Taura syndrome virus of shrimp suggests that it is related to insect picornaviruses. *Arch. Virol.,* **146**, 941–952.

SRISUVAN T., TANG K.F.J. & LIGHTNER D.V. (2005). Experimental infection of *Penaeus monodon* with Taura syndrome virus (TSV). *Dis. Aquat. Org*., **67**, 1–8.

TANG K.F.J. & LIGHTNER D.V. (2005). Phylogenetic analysis of Taura syndrome virus isolates collected between 1993 and 2004 and virulence comparison between two isolates representing different genetic variants. *Virus Res.*, **112**, 69–76.

TANG K.F.J., WANG J. & LIGHTNER D.V. (2004). Quantitation of Taura syndrome virus by real-time RT-PCR with a TaqMan assay. *J. Virol. Methods,* **115**, 109–114.

TU C., HUANG H.T., CHUANG S.H., HSU J.P., KUO S.T., LI N.J., HUS T.L., LI M.C. & LIN S.Y. (1999). Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. *Dis. Aquat. Org.,* **38**, 159–161.

VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.

VERGEL J.C.V., CABAWATAN L.D.P., MADRONA V.A.C., ROSARIO A.F.T., STA. ANA J.B.M., TARE M.V.R. & MANINGAS M.B.B. (2019). Detection of Taura Syndrome Virus (TSV) in *Litopenaeus vannamei* in the Philippines. *Philipp. J. Fish*., **26**, 8–14.

WERTHEIM J.O., TANG K.F.J., NAVARRO S.A. & LIGHTNER D.V. (2009). A quick fuse and the emergence of Taura syndrome virus. *Virology,* **390**, 324–329.

WHITE B.L., SCHOFIELD P.J., POULOS B.T. & LIGHTNER D.V. (2002). A laboratory challenge method for estimating Taura Syndrome virus resistance in selected lines of Pacific White Shrimp *Penaeus vannamei*. *J. World Aquac. Soc*., **33**, 341–348.

WYBAN J.A. (1992). Selective breeding of specific pathogen-free (SPF) shrimp for high health and increased growth. *In:* Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K.L., eds. The Oceanic Institute, Honolulu, Hawaii, USA, 257–268.

YU C.I. & SONG Y.L. (2000). Outbreaks of Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. *Fish Pathol.,* **5**, 21–24.

ZARAIN-HERZBERG M. & ASCENCIO F. (2001). Taura syndrome in Mexico: follow-up study in shrimp farms of Sinaloa. *Aquaculture,* **193**, 1–9.

*
* *

NB: There is a WOAH Reference Laboratory for infection with Taura syndrome virus
(please consult the WOAH 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 WOAH 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).

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with WSSV in accordance with Chapter 1.5. has not yet been completed]

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 stoc king 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.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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 Section B.5.5. 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 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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.
[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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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.: NC_003225			
WSSV/ ~~ORF X~~Capsid protein	Fwd WSS1011F: TGG-TCC-CGT-CCT-CAT-CTC-AG Rev WSS1079R: GCT-GCC-TTG-CCG-GAA-ATT-A Probe: 6FAM-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-TAMRA	900 nM 900 nM 250 nM	~~45 cycles of: 95°C/15 sec and 60°C/1 min~~ 50°C/2 min, 95°C/10 min, then 45 cycles of: 94°C/15 sec and 60°C/1 min
Method 2: Sritunyalucksana, 2006[1]; GenBank Accession No.: AF440570			
WSSV/ ~~ORF X~~Capsid protein	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-AGC-CG-TAMRA	900 nM 900 nM 250 nM	~~45 cycles of: 95°C/15 sec and 60°C/1 min~~ 50°C/2 min, 95°C/10 min, then 45 cycles of: 94°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.: AF440570; amplicon size: 1447/941 bp			
WSSV ~~(nested PCR)~~	~~Outer~~ Primary Fwd 146F1: ACT-ACT-AAC-TTC-AGC-CTA-TCTAG Rev 146R1: TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min
	~~Inner~~ Nested Fwd 146F2: GTA-ACT-GCC-CCT-TCC-ATC-TCC-A Rev 146R2: TAC-GGC-AGC-TGC-TGC-ACC-TTG	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min

Commercial PCR kits are available. Please consult the WOAH Register for kits that have been certified by WOAH (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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

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

vi) Positive result by *in-situ* hybridisation

[1] For example transboundary commodities.

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% (*n*=71)	100% (*n*=71)	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% (*n*=71)	100% (*n*=71)	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% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022
Real-time PCR (Sritunyalucksana et al., 2006)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	82.9% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022

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% (*n*=1591)	99.4% (*n*=1591)	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.

7. References

BALASUBRAMANIAN G., SUDHAKARAN R., SYED MUSTHAQ S., SARATHI M. & SAHUL HAMEED A.S. (2006). Studies on the inactivation of white spot syndrome virus of shrimp by physical and chemical treatments, and seaweed extracts tested in marine and freshwater animal models. *J. Fish Dis*., **29**, 569–572.

CHANG C.-F., SU M.-S., CHEN H.-Y. & LIAO I.C. (2003). Dietary β-1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. *Fish Shellfish Immunol.*, **15**, 297–310.

CHANG P.S., CHEN H.C. & WANG Y.C. (1998). Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by *in situ* hybridization**.** *Aquaculture*, **164**, 233–242.

CHANG P.S., LO C.F., WANG Y.C. & KOU G.H. (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Dis. Aquat. Org.,* **27**, 131–139.

CHANG Y., CHEN T., LIU W., HWANG J.& LO C. (2011). Assessment of the roles of copepod *Apocyclops royi* and bivalve mollusk *Meretrix lusoria* in white spot syndrome virus transmission. *Mar. Biotechnol*., **13**, 909–917.

CHEN I.T, AOKI T., HUANG Y.T., HIRONO I., CHEN T.C., HUANG J.Y., CHANG G.D., LO C.F., WANG H.C. (2011). White spot syndrome virus induces metabolic changes resembling the Warburg effect in shrimp hemocytes in the early stage of infection. *J. Virol.,* **85**, 12919–12928.

CHEN W.Y., ZHANG H., GU L., LI F. & YANG F. (2012). Effects of high salinity, high temperature and pH on capsid structure of white spot syndrome virus. *Dis. Aquat. Org.,***101**, 167–171.

CHOTIGEAT W., TONGSUPA S., SUPAMATAYA K. & PHONGDARA A. (2004). Effect of fucoidan on disease resistance of black tiger shrimp. *Aquaculture*, **233**, 23–30.

CLAYDON K., CULLEN B. & OWENS L. (2004). OIE white spot syndrome virus PCR gives false-positive results in *Cherax quadricarinatus. Dis. Aquat. Org.,* **62**, 265–268.

CORBEL V., ZUPRIZAL Z., SHI C., HUANG, SUMARTONO, ARCIER J.-M. & BONAMI J.-R. (2001). Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J. Fish Dis*., **24**, 377–382.

CUELLAR-ANJEL J., WHITE-NOBLE B., SCHOFIELD P., CHAMORRO R. & LIGHTNER D.V. (2012). Report of significant WSSV-resistance in the Pacific white shrimp, *Litopenaeus vannamei*, from a Panamanian breeding program. *Aquaculture*, **368–369**, 36–39.

DESRINA, PRAYITNO S.B, VERDEGEM M.C.J, VERRETH J.A.J. & VLAK J.M. (2022). White spot syndrome virus host range and impact on transmission. *Rev. Aquacult*., 1–18.

DESRINA, VERRETH J.A.J., PRAYITNO S.B., ROMBOUT J.H.W.M., VLAK J.M. & VERDEGEM M.C.J. (2013). Replication of white spot syndrome virus (WSSV) in the polychaete *Dendronereis* spp. *J. Invertebr. Pathol*., **114**, 7–10.

DURAND S.V. & LIGHTNER D.V. (2002). Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *J. Fish Dis.*, **25**, 381–389.

DURAND S.V., TANG K.F.J. & LIGHTNER D.V. (2000). Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus. *J. Aquat. Anim. Health,* **12**, 128–135.

EAST I.J. (2008). Addressing the problems of using the polymerase chain reaction technique as a stand-alone test for detecting pathogens in aquatic animals. *Sci. Tech. Rev*., **27**, 829–837.

ESCOBEDO-BONILLA C. M., ALDAY-SANZ V., WILLE M., SORGELOOS P., PENSAERT M.B. & NAUWYNCK H.J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J. Fish Dis.*, **31**, 1–18.

FLEGEL T.W. (1997). Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotechnol.,* **13**, 433–442.

HASSON K.W., FAN Y., REISINGER T., VENUTI J. & VARNER P.W. (2006). White-spot syndrome virus (WSSV) introduction into the Gulf of Mexico and Texas fresh water systems through imported, frozen bait-shrimp. *Dis. Aquat. Org*., **71**, 91–100.

HARYADI D., VERRETH J.A.J., VERDEGEM M.C.J. & VLAK J.M. (2015). Transmission of white spot syndrome virus (WSSV) from *Dendronereis* spp. (Peters) (Nereididae) to penaeid shrimp. *J. Fish Dis*., **38**, 419-428.

HE J. & ZHOU H. (1996). Infection route and host species of white spot syndrome baculovirus. *Acta Sci. Natur. Univ. Sunyatseni*, **38**, 65–69.

HEIDARIEH M., SOLTANI M., MOTAMEDI SEDEH F. & SHEIKHZADEH N. (2013). Low water temperature retards white spot syndrome virus replication in *Astacus leptodactylus* Crayfish. *Acta Sci. Vet.*, **41,** 1–6.

HUANG J. & YU J. (1995). A new staining method for on-site observation of viral inclusion bodies of penaeid shrimp. (*Chinese J*.). *Mar. Fish. Res.*, **16**, 31–39.

HUANG J., YU J., WANG X.-H., SONG X.-L., MA C.-S., ZHAO F.-Z. & YANG C.-H. (1995). Survey on the pathogen and route of transmission of baculoviral hypodermal and hematopoietic necrosis in shrimp by ELISA of monoclone antibody. (*Chinese J*.). *Mar. Fish. Res.*, **16**, 40–50.

HUANG Y., YIN Z., WENG S., HE J. & LI S. (2012). Selective breeding and preliminary commercial performance of *Penaeus vannamei* for resistance to white spot syndrome virus (WSSV). *Aquaculture*, **364–365**, 111–117.

KONO T., SAVAN R., SAKAI M., & ITAMI T. (2004). Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. *J. Virol. Methods*, **115**, 59–65.

LEI Z.-W., HUANG J., SHI C.-Y., ZHANG L.-J. & YU K.-K. (2002). Investigation into the hosts of white spot syndrome virus (WSSV). *Oceanol. Limnol. Sin.*, **33**, 250–258.

LI Q., ZHANG J.H., CHEN Y.J. & YANG F. (2004). White spot syndrome virus (WSSV) infectivity for *Artemia* at different developmental stages. *Dis. Aquat. Org*., **57**, 261–264.

LIGHTNER D.V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. Baton Rouge, Louisiana, USA: World Aquaculture Society, 1996.

LIU B., YU Z.M., SONG X.X. & GUAN Y.Q. (2007). Studies on the transmission of WSSV (white spot syndrome virus) in juvenile *Marsupenaeus japonicus* via marine microalgae. *J. Invertebr. Pathol*., **95**, 87–92.

LO C.F., AOKI T., BONAMI J.R., FLEGEL T.W., LEU J.H., LIGHTNER D.V., STENTIFORD G., SÖDERHÄLL K., WALKER P.W. WANG H.C.., XUN X., YANG F. & VLAK J.M. (2012). *Nimaviridae*. *In:* Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses, King A.M.Q., Adams M.J., Carstens E.B., & Lefkowitz E.J., eds. Elsevier Academic Press, San Diego, CA. USA, pp 229–234.

LO C.F., HO C.H., CHEN C.H., LIU K.F., CHIU Y.L., YEH P.Y., PENG S.E., HSU H.C., LIU H.C., CHANG C.F., SU M.S., WANG C.H. & KOU G.H. (1997). Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis. Aquat. Org.,* **30**, 53–72.

LO C.F., HO C.H., PENG S.E., CHEN C.H., HSU H.C., CHIU Y.L., CHANG C.F., LIU K.F., SU M.S., WANG C.H. & KOU G.H. (1996b). White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis. Aquat. Org.*, **27**, 215–225.

LO C.F. & KOU G.H. (1998). Virus-associated white spot syndrome of shrimp in Taiwan: a review. *Fish Pathol.,* **33**, 365–371.

LO C.F., LEU J.H., HO C.H., CHEN C.H., PENG S.E., CHEN Y.T., CHOU C.M., YEH P.Y., HUANG C.J., CHOU H.Y., WANG C.H. & KOU G.H. (1996a). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat .Org.*, **25**, 133–141.

MAEDA M., ITAMI T., MIZUKI E., TANAKA R., YOSHIZU Y., DOI K., YASUNAGA-AOKI C., TAKAHASHI Y. & KAWARABATA T. (2000). Red swamp crawfish (*Procambarus clarkii*): an alternative experimental host in the study of white spot syndrome virus. *Acta Virol.,* **44**, 371–374.

MCCOLL K.A., SLATER J., JEYASEKARAN G., HYATT A.D. & CRANE M.ST.J. (2004). Detection of White Spot Syndrome virus and Yellow head virus in prawns imported into Australia. *Australian Vet. J.*, **82**, 69–74.

MOMOYAMA K., HIRAOKA M., INOUYE K., KIMURA T. & NAKANO H. (1995). Diagnostic techniques of the rod-shaped nuclear virus infection in the kuruma shrimp, *Penaeus japonicus. Fish Pathol.,* **30**, 263–269.

MOMOYAMA K., HIRAOKA M., NAKANO H., KOUBE H., INOUYE K. & OSEKO N. (1994). Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Histopathological study. *Fish Pathol.,* **29**, 141–148.

MOMOYAMA K., HIRAOKA M., NAKANO H. & SAMESHIMA M. (1998). Cryopreservation of penaeid rod-shaped DNA virus (PRDV) and its survival in sea water at different temperatures. *Fish Pathol.,* **33**, 95–96.

MOODY N.J.G., MOHR P.G., WILLIAMS L.M., CUMMINS D.M., HOAD J., SLATER J., VALDETER S.T., COLLING A., SINGANALLUR N.B., GARDNER I.A., GUDKOVS N. & CRANE M.ST.J. (2022). Performance characteristics of two real-time, TaqMan polymerase chain reaction assays for the detection of WSSV in clinically diseased and apparently health prawns. *Dis. Aquat. Org*., https://www.int-res.com/prepress/d03687.html.

NAKANO H., HIRAOKA M., SAMESHIMA M., KIMURA T. & MOMOYAMA K. (1998). Inactivation of penaeid rod-shaped DNA virus (PRDV), the causative agent of penaeid acute viraemia (PAV), by chemical and physical treatments. *Fish Pathol.,* **33**, 65–71.

NUNAN L.M. & LIGHTNER D.V. (1997). Development of a non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). *J. Virol. Methods,* **63**, 193–201.

NUNAN L.M. & LIGHTNER D.V. (2011). Optimized PCR assay for detection of white spot syndrome virus (WSSV). *J. Virol. Methods,* **171**, 318–321.

NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (1998). The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. *Aquaculture,* **160**, 19–30.

POULOS B.T., PANTOJA C.R., BRADLEY-DUNLOP D., AGUILAR J. & LIGHTNER D.V. (2001). Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Dis. Aquat. Org.,* **47**, 13–23.

SAHUL HAMEED A.S., ANILKUMAR M., STEPHEN RAJ M.L. & JAYARAMAN K. (1998). Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture*, **160**, 31–45.

SAHUL HAMEED A.S., YOGANANDHAN K., SATHISH S., RASHEED M., MURUGAN V. & JAYARAMAN K. (2001). White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*). *Aquaculture*, **201**, 179–186.

SANCHEZ-PAZ A. (2010). White spot syndrome virus: an overview on an emergent concern. *Vet. Res.*, **41**, 43.

SATHEESH KUMAR S., ANANDA BHARATHI R., RAJAN J.J.S., ALAVANDI S.V., POORNIMA M., BALASUBRAMANIAN C.P. & PONNIAH A.G. (2013). Viability of white spot syndrome virus (WSSV) in sediment during sun-drying (drainable pond) and under non-drainable pond conditions indicated by infectivity to shrimp. *Aquaculture*, **402–403**, 119–126.

SITHIGORNGUL W., RUKPRATANPORN S., PECHARABURANIN N., LONGYANT S., CHAIVISUTHANGKURA P. & SITHIGORNGUL P. (2006). A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. *Dis. Aquat. Org.,* **72**, 101–106.

SONG X., HUANG J., WANG C., YU J., CHEN B. & YANG C. (1996). Artificial infection of brood shrimp of *Penaeus chinensis* with hypodermal and hematopoietic necrosis baculovirus. *J. Fish. China,* **20**, 374–378.

SRITUNYALUCKSANA K., SRISALA J., MCCOLL K., NIELSEN L. & FLEGEL T.W. (2006). Comparison of PCR methods for white spot syndrome virus (WSSV) infections in penaeid shrimp. *Aquaculture,* 255, 95−104.

STENTIFORD G.D., BONAMI J.R. & ALDAY-SANZ V. (2009). A critical review of susceptibility of crustaceans to Taura Syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. *Aquaculture,* **291**, 1–17.

STENTIFORD G.D. & LIGHTNER D.V. (2011). Cases of white spot disease (WSD) in European shrimp farms. *Aquaculture,* **319**, 302–306.

TSAI M.F., KOU G.H., LIU H.C., LIU K.F., CHANG C.F., PENG S.E., HSU H.C., WANG C.H. & LO C.F. (1999). Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. *Dis. Aquat. Org.,* **38**, 107–114.

VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Sea birds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.

VENEGAS C.A., NONAKA L., MUSHIAKE K., SHIMIZU K., NISHIZAWA T. & MUROGA K. (1999). Pathogenicity of penaeid rod-shaped DNA virus (PRDV) to kuruma prawn in different developmental stages. *Fish Pathol.,* **34**, 19–23.

VIDAL O.M., GRANJA C.B., ARANGUREN F., BROCK J.A. & SALAZAR M. (2001). A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. *J. World Aquac. Soc.,* **32**, 364–372.

VIJAYAN K.K., STALIN RAJ V., BALASUBRAMANIAN C.P., ALAVANDI S.V., THILLAI SEKHAR V. & SANTIAGO T.C. (2005). Polychaete worms – a vector for white spot syndrome virus (WSSV). *Dis. Aquat. Org.,* **63**, 107–111.

WANG C.H., YANG H.N., TANG C.Y., LU C.H., KOU G.H. & LO C.F. (2000). Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. *Dis. Aquat. Org.*, **41**, 91–104.

WANG H.C., HIRONO I, MANINGAS M.B.B., SOMBOONWIWA K., STENTIFORD G. & ICTV REPORT CONSORTIUM. (2019). ICTV Virus Taxonomy Profile: *Nimaviridae*. *In:* Virus Taxonomy: The ICTV 10th Report on Virus Classification and Taxon Nomenclature. The ICTV website (www.ictv.global/report/nimaviridae).

WANG M., CHEN Y., ZHAO Z., WENG S., YANG J., LIU S., LIU C., YUAN F., AI B., ZHANG H., ZHANG M., LU L., YUAN K., YU Z., MO B., LIU X., GAI C., LI Y., LU R., ZHONG Z., ZHENG L., FENG G., LI S.C. & HE J. (2021). A convenient polyculture system that controls a shrimp viral disease with a high transmission rate. *Commun Biol*., **4**, 1276.

WITHYACHUMNARNKUL B. (1999). Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). *Dis. Aquat. Org.,* **39**, 21–27.

WONGTEERASUPAYA C., VICKERS J.E., SRIURAIRATANA S., NASH G.L., AKARAJAMORN A., BOONSAENG V., PANYIM S., TASSANAKAJON A., WITHYACHUMNARNKUL B. & FLEGEL T.W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis. Aquat. Org.,* **21**, 69–77.

WU J.L., SUZUKI K., ARIMOTO M., NISHIZAWA T. & MUROGA K. (2002). Preparation of an Inoculum of White Spot Syndrome Virus for Challenge Tests in Penaeus japonicus. *Fish Pathol.,* **37**, 65–69.

WU W., WU B., YE T., HUANG H., DAI C., YUAN J. & WANG W. (2013). TCTP is a critical factor in shrimp immune response to virus infection. *PloS One*, *8*, e74460.

YAN D.C., DONG S.L., HUANG J., YU X.M., FENG M.Y. & LIU X.Y. (2004). White spot syndrome virus (WSSV) detected by PCR in rotifers and rotifer resting eggs from shrimp pond sediments. *Dis. Aquat. Org.,* **59**, 69–73.

YAN D.C., DONG S.L., HUANG J.& ZHANG J.S. (2007). White spot syndrome virus (WSSV) transmission from rotifer inoculum to crayfish. *J. Invertebr. Pathol*., **94**, 144–148.

YOGANANDHAN K., SYED MUSTHAQ S., NARAYANAN R.B. & SAHUL HAMEED A.S. (2004). Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans. *J. Fish Dis.,* **27**, 517–522.

ZENG Y. (2021). Molecular epidemiology of white spot syndrome virus in the world. *Aquaculture*, **537**, 736509. https://doi.org/10.1016/j.aquaculture.2021.736509.

ZHAN W.B., WANG Y.H., FRYER J.L., YU K.K., FUKUDA H. & MENG Q.X. (1998). White Spot Syndrome Virus Infection of Cultured Shrimp in China. *J. Aquat. Anim. Health*, **10**, 405–410.

ZHANG J.S., DONG S.L. DONG Y.W., TIAN X.L., CAO Y.C. & LI Z.J., YAN D.C. (2010). Assessment of the role of brine shrimp *Artemia* in white spot syndrome virus (WSSV) transmission. *Vet. Res. Commun*., **34**, 25–32.

ZHANG J.S., DONG S.L., DONG Y.W., TIAN X.L. & HOU C.Q. (2008). Bioassay evidence for the transmission of WSSV by the harpacticoid copepod *Nitocra* sp. *J. Invertebrate Pathol*., **97**, 33–39.

ZHANG J.S., DONG S.L., TIAN X.L., DONG Y.W., LIU X.Y. & YAN D.C. (2006). Studies on the rotifer (*Brachionus urceus* Linnaeus, 1758) as a vector in white spot syndrome virus (WSSV) transmission. *Aquaculture*, **261**, 1181–1185.

*
* *

NB: There are WOAH Reference Laboratories for infection with white spot syndrome virus
(please consult the WOAH web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH 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

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with *A. invadans* in accordance with Chapter 1.5. has not yet been completed]

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

Family	Scientific name	Common name
	Puntius sophore	pool barb
	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

Family	Scientific name	Common name
	Leiopotherapon unicolor	spangled perch
	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~~ There are no published protocols for *A. invadans* 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.

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).

3.3. 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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~~ Observation for clinical signs

Using observational data of clinical signs (see Section 2.3.2 *Clinical signs, including behavioural changes*) for targeted surveillance, a 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*. 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.

4.2. 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~~3. 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~~3.1. 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~~3.2 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~~4. Cell culture for isolation

4.~~3~~4.1. 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).

4.34.2. 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*.

4.34.3. 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 4.4.3.1), 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 4.4.3.1. *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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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*. Oidtmann *et al.* (2008) demonstrated cross reactivity of the Phadee *et al*. (2004b) assay with *A. frigidophilus* when more than 10 ng of template DNA of *A. frigidophilus* was used in the PCR.

Pathogen/ target gene	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1: Vandersea *et al.*, 2006); GenBank Accession No.: AF396684; ~~Product~~ amplicon 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~~ AF396684; ~~Product~~ amplicon 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~~ amplicon size: 564			
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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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~~ were 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* (as described in Section 4.1 *Observation for clinical signs*). ~~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. If a Competent Authority does not have the capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratories designated for EUS.

6.1. Apparently healthy animals or animals of unknown health status [1]

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* [2]

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

[1] For example transboundary commodities.

[2] 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 *Test*[*s*] *recommended for surveillance to demonstrate freedom in apparently healthy populations*).

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. and 6.3.2. (no data are currently available for either). 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.

7. References

ADIL B., SHANKAR K.M., NAVEEN KUMAR B.T., PATIL R., BALLYAYA A., RAMESH K.S., POOJARY S.R., BYADGI O.V. & SIRIYAPPAGOUDER P. (2013). Development and standardization of a monoclonal antibody-based rapid flow-through immunoassay for the detection of *Aphanomyces invadans* in the field. *J. Vet. Sci.*, **14**, 413–419.

AFZALI S.F., HASSAN M.D., ABDUL-RAHIM A.M., SHARIFPOUR I. & SABRI J. (2013). Isolation and identification of *Aphanomyces* species from natural water bodies and fish farms in Selangor, Malaysia. *Malaysian Appl. Biol.*, **42**, 21–31.

ANDREW T., HUCHZERMEYER K., MBEHA B. & NENGU S. (2008). Epizootic ulcerative syndrome affecting fish in the Zambezi river system in Southern Africa. *Vet. Rec.,* **163**, 629–632.

BALDOCK F.C., BLAZER V., CALLINAN R., HATAI K., KARUNASAGAR I., MOHAN C.V. & BONDAD-REANTASO M.G. (2005). Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): Re-examination of causal factors, case definition and nomenclature. *In:* Diseases in Asian Aquaculture V, Walker P., Lester R. & Bondad-Reantaso M.G., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 555–585.

BALASURIYA L.K.S.W. (1994). Epizootic ulcerative syndrome in fish in Sri Lanka, country status report. *In:* Proceeding of the ODA Regional Seminar on Epizootic Ulcerative, Robert R.J., Campbell B. & MacRae I.H., eds. Aquatic Animal Health Research Institute, Bangkok, Thailand, pp 39–47.

BLAZER V.S., VOGELBEIN W.K., DENSMORE C.L., MAY E.B., LILLEY J.H. & ZWERNER D.E. (1999). Aphanomyces as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. *J. Aquat. Anim. Health,* **11**, 340–349.

BONDAD-REANTASO M.G., LUMANLAN S.C., NATIVIDAD J.M. & PHILLIPS M.J. (1992). Environmental monitoring of the epizootic ulcerative syndrome (EUS) in fish from Munoz, Nueva Ecija in the Philippines. *In:* Diseases in Asian Aquaculture 1, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 475–490.

CATAP E.S. & MUNDAY B.L. (1998). Effects of variations of water temperature and dietary lipids on the expression of experimental epizootic ulcerative syndrome (EUS) in sand whiting, Sillago ciliata. *Fish Pathol.,* **33**, 327–335.

CAVALIER-SMITH T. & CHAO E.E.Y. (2006). Phylogeny and Megasystematics of Phagotrophic Heterokonts (Kingdom Chromista). *J. Mol. Evol.,* **62**, 388–420.

CHINABUT S. & ROBERTS R.J. (1999). Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS). Aquatic Animal Health Research Institute, Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33 pp. ISBN 974-7604-55-8.

CHINABUT S., ROBERTS R.J., WILLOUGHBY G.R. & PEARSON M.D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.*, **18**, 41–47.

CRUZ-LACIERDA E.R. & SHARIFF M. (1995). Experimental transmission of epizootic ulcerative syndrome (EUS) in snakehead, *Ophicephalus striatus. Dis. Asian Aquac*., **II**., 327–336.DIEGUEZ-URIBEONDO J., GARCIA M.A., CERENIUS L., KOZUBÍKOVÁ E., BALLESTEROS I., WINDELS C., WEILAND J., KATOR H., SÖDERHÄLL K. & MARTÍN M.P. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genetics and Biology*, **46**, 365–376.

EGUSA S. & MASUDA N. (1971). A new fungal disease of Plecoglossus altivelis. *Fish Pathol.,* **6**, 41–46.

EUROPEAN FOOD SAFETY AUTHORITY EFSA (2011a). Scientific Opinion on Epizootic Ulcerative Syndrome. *EFSA J*., **9**, 2387.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2011b). Report of the technical hearing meeting on Epizootic Ulcerative Syndrome (EUS). *EFSA Support. Publ*., **8**, 1–16.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (1986). Report of the expert consutation on ulcerative fish diseases in the Asia-Pacific region (TCP/RAS/4508). Bangkok, August 1986. FAO, Regional Office for Asia and the Pacific, Bangkok, Thailand.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (2009). Report of the international emergency disease investigation task force on a serious fish disease in Southern Africa, 18–26 May 2007, FAO, Rome, Italy, 70 pp.

FRASER G.C., CALLINAN R.B. & CALDER L.M. (1992). *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.*, **15**, 173–181.

GOMO C., HANYIRE T., MAKAYA P. & SIBANDA S. (2016). Outbreak of epizootic ulcerative syndrome (EUS) in Seranochromis robustus fish spesies in Darwendale dam, Zimbabwe. *African J. Fish. Sci*., **4**, 204–205.

HANJAVANIT C. (1997). Mycotic granulomatosis found in two species of ornamental fishes imported from Singapore. *Mycoscience*, 38, 433–436.

HATAI K. & EGUSA S. (1979). Studies on pathogenic fungus of mycotic granulomatosis III. Development of the medium for MG-fungus. *Fish Pathol.,* **13**, 147–152.

HATAI K., EGUSA S., TAKAHASHI S. & OOE K. (1977). Study on the pathogenic fungus of mycotic granulomatosis – I. Isolation and pathogenicity of the fungus from cultured-ayu infected with the disease. *Fish Pathol.,* **12**, 129–133.

HATAI K., NAKAMURA K., AN RHA S., YUASA K. & WADA S. (1994). *Aphanomyces* infection in dwarf gourami (*Colisa lalia*). *Fish Pathol.,* **29**, 95–99.

HAWKE J.P., GROOTERS A.M. & CAMUS A.C. (2003). Ulcerative Mycosis Caused by *Aphanomyces invadans* in Channel Catfish, Black Bullhead, and Bluegill from Southeastern Louisiana. *J. Aquat. Anim. Health*., **15**, 120–127.

HERBERT B., JONES J.B.B., MOHAN C.V. V. & PERERA R.P.P. (2019). Impacts of epizootic ulcerative syndrome on subsistence fisheries and wildlife. *Rev. Sci. Tech.,* **38**, 459–475.

HUCHZERMEYER C.F., HUCHZERMEYER K.D.A., CHRISTISON K.W., MACEY B.M., COLLY P.A., HANG'OMBE B.M. & SONGE M.M. (2018). First record of epizootic ulcerative syndrome from the Upper Congo catchment: An outbreak in the Bangweulu swamps, Zambia. *J. Fish Dis*., **41**, 87–94.

HUCHZERMEYER K.D.A. & VAN DER WAAL B.C.W. (2012). Epizootic ulcerative syndrome: Exotic fish disease threatens Africa's aquatic ecosystems. *J. S. Afr. Vet. Assoc*., **83**, 1–6.

IBERAHIM N.A., TRUSCH F. & VAN WEST P. (2018). *Aphanomyces invadans*, the causal agent of Epizootic Ulcerative Syndrome, is a global threat to wild and farmed fish. *Fungal Biol. Rev.,* **44**, 1–13.

KHAN M.H., MARSHALL L., THOMPSON K.D., CAMPBELL R.E. & LILLEY J.H. (1998). Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the *Oomycete* fish pathogen, *Aphanomyces invadans*. *Bull. Eur. Assoc. Fish Pathol.,* **18**, 192–197.

KIRYU Y., SHIELDS J.D., VOGELBEIN W.K., KATOR H. & BLAZER V.S. (2003). Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus. Dis. Aquat. Org*., **54**, 135–146.

KUMAR P., SARKAR P., STEFI RAJU V., MANIKANDAN V., GURU A., ARSHAD A., ELUMALAI P. & AROCKIARAJ J. (2020). Pathogenicity and Pathobiology of Epizootic Ulcerative Syndrome (EUS) Causing Fungus *Aphanomyces invadans* and Its Immunological Response in Fish. *Rev. Fish. Sci. Aquac*., **28**, 358–375.

LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J.~~, FALLIS A., LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J.~~ (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Bangkok: The Aquatic Animal Health Research Institute.

LILLEY J.H., HART D., PANYAWACHIRA V., KANCHANAKHAN S., CHINABUT S., SÖDERHÄLL K. & CERENIUS L. (2003). Molecular characterization of the fish-pathogenic fungus Aphanomyces invadans. *J. Fish Dis.,* **26**, 263–275.

LILLEY J.H., HART D., RICHARDS R.H., ROBERTS R.J., CERENIUS L. & SODERHALL K. (1997a). Pan-Asian spread of single fungal clone results in large scale fish kills. *Vet. Rec.*, **140**, 653–654.

LILLEY J.H., PETCHINDA T. & PANYAWACHIRA V. (2001). *Aphanomyces invadans* zoospore physiology: 4. *In vitro* viability of cysts. *The AAHRI Newsletter*, **10**, 1–4.

LILLEY J.H. & ROBERTS R.J. (1997). Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. *J. Fish Dis.*, **20**, 135–144.

LILLEY J.H., THOMPSON K.D. & ADAMS A. (1997b). Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis. *Dis. Aquat. Org*., **30**, 187–197.

LUMANLAN-MAYO S.C., CALLINAN R.B., PACLIBARE J.O., CATAP E.S. & FRASER, G.C. (1997). Epizootic ulcerative syndrome (EUS) in rice-fish culture systems: an overview of field experiments 1993-1995. *In:* Diseases in Asian Aquaculture III, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 129–138.

MCHUGH K.J., CHRISTISON K.W., WEYL O.L.F. & SMIT N.J. (2014). Histological Confirmation of Epizootic Ulcerative Syndrome in Two Cyprinid Species from Lake Liambezi, Zambezi Region, Namibia. *African Zool*., **49**, 311–316.

MCKENZIE R.A. & HALL W.T.K. (1976). Dermal ulceration of mullet (*Mugil cephalus*). *Aust. Vet. J.,* **52**, 230–231.

MILES D.J.V., POLCHANA J., LILLEY J.H., KANCHANAKHAN S., THOMPSON K.D. & ADAMS A. (2001). Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture*, **195**, 1–15.

MILES D.J.C., THOMPSON K.D., LILLEY J.H. & ADAMS A. (2003). Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody. *Dis. Aquat. Org*., **55**, 77–84.

NOGA E.J. & DYKSTRA M.J. (1986). Oomycete fungi assocaited with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). *J. Fish Dis*., **9**, 47–53.

OIDTMANN B. (2012). Review of biological factors relevant to import risk assessments for epizootic ulcerative syndrome (*Aphanomyces invadans*). *Transbound. Emerg. Dis*., **59**, 26–39.

OIDTMANN B., STEINBAUER GEIGER S. & HOFFMANN R.W. (2008). Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. *Dis. Aquat. Org*., **82**, 185–207.

PAGRUT N.K., GANGULY S., JAISWAL V. & SINGH C. (2017). An overview on epizootic ulcerative syndrome of fishes in India: A comprehensive report. *J. Entomol. Zool. Stud*., **5**, 1941–1943.

PHADEE P., KURATA O. & HATAI K. (2004a). A PCR method for the detection of *Aphanomyces piscicida*. *Fish Pathol.,* **39**, 25–31.

PHADEE, P., KURATA, O., HATAI K., HIRONO I. & AOKI T. (2004b). Detection and identification of fish-pathogenic *Aphanomyces piscicida* using polymerase chain reaction (PCR) with species-specific primers. *J. Aquat. Anim. Health*, **16**, 220–230.

PRADHAN P.K., MOHAN C.V., SHANKAR K.M., KUMAR B.M. & DEVARAJA G. (2007). Yearlings of Indian major carps resist infection against the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*. *Current Science*, **92**, 1430–1434.

TONGUTHAI K. (1985). A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, Thailand, 39 pp.

TSUI C.K.M., MARSHALL W., YOKOYAMA R., HONDA D., LIPPMEIER J.C., CRAVEN K.D., PETERSON P.D. & BERBEE M.L. (2009). Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. *Mol. Phylogenet. Evol*., **50**, 129–140.

VANDERSEA M.W., LITAKER R.W., YONNISH B., SOSA E., LANDSBERG J.H., PULLINGER C., MOON-BUTZIN P., GREEN J., MORRIS J.A., KATOR H., NOGA E.J. & TESTER P.A. (2006). Molecular assays for detecting *Aphanomyces invadans* in ulcerative mycotic fish lesions. *Appl. Environ. Microbiol.,* **72**, 1551–1557.

VISHWANATH T., MOHAN C. & SHANKAR K. (1998). Epizootic Ulcerative Syndrome (EUS), associated with a fungal pathogen, in Indian fishes: histopathology – 'a cause for invasiveness'. *Aquaculture*, **165**, 1–9.

WADA S., AN RHA S., KONDOH T., SUDA H., HATAI K. & ISHII H. (1996). Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida. Fish Pathol.,* **31**, 71–80.

WILLOUGHBY L.G. & ROBERTS R.J. (1994). Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. *J. Fish Dis.,* **17**, 541–543.

*
* *

NB: There is currently (2022) no WOAH Reference Laboratories for infection with *Aphanomyces invadans*
(please consult the WOAH 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

None known

~~*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). European perch from distinct geographical areas with and without a history of EHNV have been tested under experimental conditions and have demonstrated susceptibility to EHN (Becker *et al.,* 2016). 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 (Becker *et al.,* 2019; 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 and sporadic outbreaks involving small numbers of European perch (Becker *et al,* 2019; Whittington *et al*., 2010).

See WOAH 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 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 i.e. European perch where these are available, otherwise rainbow trout or the other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample epidemiological units ~~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~~ apparently healthy fish collected in such a way that all parts of the farm or affected waterbody 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

~~Non-lethal samples (blood, fin, gill, integument or mucous) are unsuitable for testing EHNV~~ Not applicable.

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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.

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~~ lines for virus isolation~~/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 WOAH 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 [F~~C~~BS] 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% F~~C~~BS 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.

The identity of viruses in cell culture is determined by PCR and amplicon sequencing.

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 diagnostic sample should be tested in duplicate, i.e. by testing two aliquots.

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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

4.4.1. Real-time PCR

The ranavirus real-time screening protocol in use at the WOAH 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~~	~~5' CTC ATC GTT CTG GCC ATC A 3'~~	~~Pallister *et al.,* 2007~~
~~RANA CON R~~	~~5' TCC CAT CGA GCC GTT CA 3'~~	
~~**Probe**~~		
~~RANA CON Pr~~	~~5' 6**FAM** CAC AAC ATT ATC CGC ATC **MGB** 3'~~	
~~**Primer**~~		
~~C1096~~	~~GAC TGA CCA ACG CCA GCC TTA ACG~~	~~Jaramillo *et al.,* 2012~~
~~C1097~~	~~GCG GTG GTG TAC CCA GAG TTG TCG~~	
~~**Primer**~~		
~~RanaF1~~	~~CCA GCC TGG TGT ACG AAA ACA~~	~~Stilwell *et al.,* 2018~~
~~RanaR1~~	~~ACT GGG ATG GAG GTG GCA TA~~	
~~**Probe**~~		
~~RanaP1~~	~~6FAM TGG GAG TCG AGT ACT AC MGB~~	

~~***Primer and probe sequences***~~

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Pallister *et al.*, 2007); GenBank Accession No.: DQ457105			
Ranavirus/MCP	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

Method 2 (Jaramillo *et al.,* 2012); GenBank Accession No.:			
Ranavirus/MCP	C1096 GAC-TGA-CCA-ACG-CCA-GCC-TTA-ACG C1097 GCG-GTG-GTG-TAC-CCA-GAG-TTG-TCG	12.5 pM for each primer	40 cycles of 95°C/30 sec; 58°C/30 sec
Method 3 (Stilwell *et al.,* 2018); GenBank Accession No.:			
Ranavirus/MCP	Fwd: RanaF1: CCA-GCC-TGG-TGT-ACG-AAA-ACA Rev: RanaR1 ACT-GGG-ATG-GAG-GTG-GCA-TA Probe: RanaP1 FAM-TGG-GAG-TCG-AGT-ACT-AC-MGB	900 nM for each primer, 250 nM for probe	40 cycles of 95°C/30 sec; 60°C/45 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. Both MCP1 and MCP2 target a region within the capsid protein gene (Marsh *et al.,* 2002).

~~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 (Marsh *et al.*, 2002): ~~Product~~ amplicon size MCP-1 is 321 bp and ~~product~~ amplicon 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~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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 [1]. The primary affinity purified ~~rabbit~~ anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the WOAH 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 µg 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 antibody 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.

1 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.

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).

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 WOAH 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~~ reported (Whittington & Steiner, 1993). 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 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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

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 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 followed by 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 followed by 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.~~

[2] For example transboundary commodities.

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. (no data are currently available). 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.

7. References

AHNE W., BEARZOTTI M., BREMONT M. & ESSBAUER S. (1998). Comparison of European systemic piscine and amphibian iridoviruses with epizootic haematopoietic necrosis virus and frog virus 3. *J. Vet. Med.* [*B*], **45**, 373–383.

AHNE W., OGAWA M. & SCHLOTFELDT H.J. (1990). Fish viruses: transmission and pathogenicity of an icosahedral cytoplasmic deoxyribovirus isolated from sheatfish *Silurus glanis*. *J. Vet. Med*. [*B*], **37**, 187–190.

AHNE W., SCHLOTFELDT H.J. & THOMSEN I. (1989). Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). *J. Vet. Med.* [*B*], **36**, 333–336.

ARIEL E. & BANG JENSEN B. (2009). Challenge studies of European stocks of redfin perch, *Perca fluviatilis* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), with epizootic haematopoietic necrosis virus. *J. Fish Dis.,* **32**, 1017–1025.

Ariel E, Holopainen R, Olenen NJ & Tapiovaara H (2010). Comparative study of ranavirus isolates from cod (*Gadua morhua)* and turbot (*Psetta maxima*) wuth reference to other ranaviruses. Archives of Virology **155**, 1261-1271

ARIEL E., NICOLAJSEN N., CHRISTOPHERSEN M.-B., HOLOPAINEN R., TAPIOVAARA H. & BANG JENSEN B. (2009). Propagation and isolation of ranaviruses in cell culture. *Aquaculture,* **294**, 159–164.

BECKER J.A., GILLIGAN D., ASMUS M., TWEEDIE A. & WHITTINGTON R.J. (2019). Geographic distribution of Epizootic haematopoietic necrosis virus (EHNV) in freshwater fish in south eastern Australia: lost opportunity for a notifiable pathogen to expand its geographic range. *Viruses*, **11**, 315 doi:10.3390/v11040315

BECKER J.A., TWEEDIE A., GILLIGAN D., ASMUS M. & WHITTINGTON R. J. (2016). Susceptibility of Australian Redfin Perch *Perca fluviatilis* Experimentally Challenged with Epizootic Hematopoietic Necrosis Virus (EHNV). *J. Aquat. Anim. Health*, **28**, 122–130.

BLOCH B. & LARSEN J.L. (1993). An iridovirus-like agent associated with systemic infection in cultured turbot *Scophthalmus maximus* fry in Denmark. *Dis. Aquat. Org*., **15**, 235–240.

BRYAN L.K., BALDWIN C.A., GRAY M.J. & MILLER D.L. (2009). Efficacy of select disinfectants at inactivating Ranavirus. *Dis. Aquat. Org.,* **84**, 89–94.

CHINCHAR V.G. (2002). Ranaviruses (family Iridoviridae): emerging cold-blooded killers – brief review. *Arch. Virol.*, **147**, 447–470.

CHINCHAR G., ESSBAUER S., HE J.G., HYATT A., MIYAZAKI T., SELIGY V. & WILLIAMS T. (2005). Family Iridoviridae. *In:* Virus Taxonomy. Classification and Nomeclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses, Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds. Academic Press, San Diego, California, USA, 145–161.

CINKOVA K., RESCHOVA S., KULICH P. & VESELY T. (2010). Evaluation of a polyclonal antibody for the detection and identification of ranaviruses from freshwater fish and amphibians. *Dis. Aquat. Org.,* **89**, 191–198.

CRANE M.S.J., YOUNG J. & WILLIAMS L. (2005). Epizootic haematopoietic necrosis virus (EHNV): growth in fish cell lines at different temperatures. *Bull. Eur. Assoc. Fish Pathol*., **25**, 228–231.

DRURY S.E.N., GOUGH R.E. & CALVERT I. (2002). Detection and isolation of an iridovirus from chameleons (*Chamaeleo quadricornis* and *Chamaeleo hoehnelli*) in the United Kingdom. *Vet. Rec.,* **150**, 451–452.

FIJAN N., MATASIN Z., PETRINEC Z., VALPOTIC I. & ZWILLENBERG L.O. (1991). Isolation of an iridovirus-like agent from the green frog (*Rana esculenta* L.). *Veterinarski Arhiv,* **61**, 151–158.

HEDRICK R.P., MCDOWELL T.S., AHNE W., TORHY C. & DE KINKELIN P. (1992). Properties of three iridovirus-like agents associated with systemic infections of fish. *Dis. Aquat. Org.,* **13**, 203–209.

~~HOLOPAINEN R., HONKANEN J., JENSEN B.B., ARIEL E. & TAPIOVAARA H. (2011). Quantitation of ranaviruses in cell culture and tissue samples. *J. Virol. Methods,* **171**, 225–233.~~

HOLOPAINEN R., OHLEMEYER S., SCHÜTZE H., BERGMANN S.M. & TAPIOVAARA H. (2009). Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Dis. Aquat. Org.,* **85**, 81–91.

HYATT A.D., GOULD A.R., ZUPANOVIC Z., CUNNINGHAM A.A., HENGSTBERGER S., WHITTINGTON R.J., KATTENBELT J. & COUPAR B.E.H. (2000). Comparative studies of piscine and amphibian iridoviruses. *Arch. Virol.,* **145**, 301–331.

HYATT A.D., WILLIAMSON M., COUPAR B.E.H., MIDDLETON D., HENGSTBERGER S.G., GOULD A.R., SELLECK P., WISE T.G., KATTENBELT J., CUNNINGHAM A.A.& LEE J. (2002). First identification of a ranavirus from green pythons (*Chondropython viridis*). *J. Wildl. Dis.,* **38**, 239–252.

JARAMILLO D., TWEEDIE A., BECKER J.A., HYATT A., CRAMERI S. & WHITTINGTON R.J. (2012). A validated quantitative polymerase chain reaction assay for the detection of ranaviruses (Family Iridoviridae) in fish tissue and cell cultures, using EHNV as a model. *Aquaculture*, **356–357**, 186–192.

LANGDON J.S. (1989). Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. *J. Fish Dis.*, **12**, 295–310.

LANGDON J.S. & HUMPHREY J.D. (1987). Epizootic Hematopoietic Necrosis a New Viral Disease in Redfin Perch *Perca fluviatilis* L. in Australia. *J. Fish Dis.*, **10**, 289–298.

LANGDON J.S., HUMPHREY J.D. & WILLIAMS L.M. (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *J. Fish Dis.*, **11**, 93–96.

LANGDON J.S., HUMPHREY J.D., WILLIAMS L.M., HYATT A.D. & WESTBURY H.A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. *J. Fish Dis.*, **9**, 263–268.

MAO J., THAM T.N., GENTRY G.A., AUBERTIN A. & CHINCHAR V.G. (1996). Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. *Virology*, **216**, 431–436.

MAO J.H., HEDRICK R.P. & CHINCHAR V.G. (1997). Molecular characterisation, sequence analysis and taxonomic position of newly isolated fish iridoviruses. *Virology*, **229**, 212–220.

MARSH I.B., WHITTINGTON R.J., O'ROURKE B., HYATT A.D. & CHISHOLM O. (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Molec. Cell. Probes*, **16**, 137–151.

PALLISTER J., GOULD A., HARRISON D., HYATT A., JANCOVICH J. & HEINE H. (2007). Development of real-time PCR assays for the detection and differentiation of Australian and European ranaviruses. *J. Fish Dis.,* **30**, 427–438.

POZET F., MORAND M., MOUSSA A., TORHY C. & DE KINKELIN P. (1992). Isolation and preliminary characterization of a pathogenic icosahedral deoxyribovirus from the catfish (*Ictalurus melas*). *Dis. Aquat. Org.,* **14**, 35–42.

REDDACLIFF L.A. & WHITTINGTON R.J. (1996). Pathology of epizootic haematopoeitic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss* Walbaum) and redfin perch (*Perca fluviatilis* L.). *J. Comp. Pathol.,* **115**, 103–115.

RIMMER A.E., BECKER J.A., TWEEDIE A. & WHITTINGTON R.J. (2012). Validation of high throughput methods for tissue disruption and nucleic acid extraction for ranaviruses (family Iridoviridae). *Aquaculture*, **338–341**, 23–28.

SPEARE R. & SMITH J.R. (1992). An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. *Dis. Aquat. Org.*, **14**, 51–57.

STILWELL N.K., WHITTINGTON R.J., HICK P.M., BECKER J.A., ARIEL E., VAN BEURDEN S., VENDRAMIN N., OLESEN N.J. & WALTZEKT.B. (2018). Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses. *Dis. Aquat. Org*., **128**, 105–116.

WHITTINGTON R.J., BECKER J.A. & DENNIS M.M. (2010). Iridovirus infections in finfish – critical review with emphasis on ranaviruses. *J. Fish Dis.,* **33**, 95–122.

WHITTINGTON R.J., KEARNS C., HYATT A.D., HENGSTBERGER S. & RUTZOU T. (1996). Spread of epizootic haematopoietic necrosis virus (EHNV) in redfin perch (*Perca fluviatilis*) in southern Australia. *Aust. Vet. J.*, **73**, 112–114.

WHITTINGTON R.J., PHILBEY A., REDDACLIFF G.L. & MACGOWN A.R. (1994). Epidemiology of epizootic haematopoietic necrosis virus (EHNV) infection in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum): findings based on virus isolation, antigen capture ELISA and serology. *J. Fish Dis.*, **17**, 205–218.

WHITTINGTON R.J. & REDDACLIFF G.L. (1995). Influence of environmental temperature on experimental infection of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) with epizootic haematopoietic necrosis virus, an Australian iridovirus. *Aust. Vet. J.,* **72**, 421–424.

WHITTINGTON R.J., REDDACLIFF L.A., MARSH I., KEARNS C., ZUPANOVIC Z. & CALLINAN R.B. (1999). Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout *Oncorhynchus mykiss* in southeastern Australia and a recommended sampling strategy for surveillance. *Dis. Aquat. Org.,* **35**, 125–130.

WHITTINGTON R.J. & STEINER K.A. (1993). Epizootic haematopoietic necrosis virus (EHNV): improved ELISA for detection in fish tissues and cell cultures and an efficient method for release of antigen from tissues. *J. Virol. Methods,* **43**, 205–220.

WOLF K., BULLOCK G.L., DUNBAR C.E. & QUIMBY M.C. (1968). Tadpole edema virus: a viscerotrophic pathogen for anuran amphibians. *J. Infect. Dis.,* **118**, 253–262.

ZUPANOVIC Z., MUSSO C., LOPEZ G., LOURIERO C.L., HYATT A.D., HENGSTBERGER S. & ROBINSON A.J. (1998). Isolation and characterisation of iridoviruses from the giant toad *Bufo marinus* in Venezuela. *Dis. Aquat. Org.,* **33**, 1–9.

*
* *

NB: There is a WOAH Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV) (please consult the WOAH 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 WOAH Reference Laboratories for any further information on infection with EHNV. The WOAH 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.

Annex 30. Item 10.2.3. – Section 2.2.1. of Chapter 2.3.9. Infection with SVCV

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*	~~B~~bream
	Aristichthys nobilis	~~B~~bighead carp
	Carassius auratus	~~G~~goldfish
	Ctenopharyngodon idella	~~G~~grass carp
	Cyprinus carpio	~~C~~common carp (all varieties and subspecies)
	Danio rerio	~~Z~~zebrafish
	Notemigonus crysoleucas	~~G~~golden shiner
	Pimephales promelas	~~F~~fathead minnow
	Percocypris pingi	Jinsha ~~bass~~barbel carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	~~R~~roach
Siluridae	*Silurus glanis*	Wels catfish

[…]

Annex 31. Item 10.3.1. – Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *B. exitiosa*

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*), Ariake cupped oyster (*Magallana* (syn. *Crassostrea*) *ariakensis*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*), and Olympia oyster (*Ostrea lurida*) ~~and Suminoe oyster (*Magallana* (syn. *Crassostrea*) *ariakensis*)~~.

2.2.~~1~~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. 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*).

[…]

Annex 32. Item 10.3.1. – Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *B. ostreae*

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: Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), European flat oyster (*Ostrea edulis*)~~,~~ and 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 33. Item 10.3.2. – Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *M. 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 clam (*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*).

[…]

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. surveillance of apparently healthy animals, presumptive diagnosis of clinically affected animals or confirmatory diagnosis of a suspect result from surveillance or presumptive 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 WOAH *Aquatic Code* Chapter 1.4 . *Aquatic animal disease surveillance.*

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 epidemiological units 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.~~ If weak, abnormally behaving discoloured or freshly dead (not decomposed) animals are present, such animals should be selected. If such animals are not present, animals should be selected in such a way that all epidemiological units of the farm or waterbody are proportionately represented in the sample.

~~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 provided they are not decomposed. 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 WOAH-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 WOAH-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

Virological examination of crustaceans is not routinely used for listed diseases. *Macrobrachium rosenbergii* has been isolated in insect cell lines, but it is not a recommended method.

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*.~~ Not applicable.

2.2.2. Virus isolation

~~For processing of tissues see Section 3 of disease-specific chapters in this *Aquatic Manual*.~~ Not applicable.

2.2.3. Treatment to neutralise enzootic viruses

Not applicable.

2.3. Bacteriological examination

Bacteriological examination of crustaceans is not routinely used for listed diseases, but it may be used for the strains of *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$) that cause acute hepatopancreatic necrosis disease (AHPND) and for ~~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 for experimental purposes

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~~ with the pathogenic 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 WOAH-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. *Infection with* Aphanomyces astaci (*crayfish plague*)

4.2. Storage of cultures

See Chapter 2.2.2.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the WOAH-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)
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 WOAH.

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~~ extracted from crustacean tissue. ~~The~~ Molecular techniques 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.

Each diagnostic sample~~s~~ should be tested in duplicate, i.e. by testing two aliquots. ~~and~~ Both aliquots 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~~ barrier pipette tips should be used for all sample preparation and PCR ~~preparation~~ steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where ~~the~~ nucleic acid extraction, amplification~~s~~ 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, pens/marker pens and paper (e.g. workbooks). Also, ensure all work-tops and ~~air-flow~~ cabinets/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~~ separate from the molecular biology laboratory and reagents

5.5.1. Sample preparation and types

Samples should be prepared to preserve the nucleic acid of the pathogen and should be handled and packaged with the greatest care to minimise the potential for cross-contamination among the samples or target degradation before the assay can be performed. ~~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%~~ 80% analytical grade 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 or freezer bricks 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*. In situations where it is not possible to get the specimens to the laboratory alive, they may be quick ~~freeze~~ frozen in the field using crushed dry-ice or ~~freeze~~ frozen 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%~~ 80% analytical grade 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%~~ 80% analytical grade 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).

vi) *Fixed tissues for* in-situ *hybridisation:* For this purpose, classic methods for preservation of the tissues are adequate. Neutral-buffered formalin is usually a good choice. Fixation for over 24–48 hours should be avoided; samples should be transferred to ethanol following formalin treatment.

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 analytical grade ethanol ~~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%~~ 80% analytical grade 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.

4. KEY REFERENCES FOR FURTHER READING

BELL T.A. & LIGHTNER D.V. (1988). A Handbook of Normal Shrimp Histology. Special Publication No. 1, World Aquaculture Society, Baton Rouge, Louisiana, USA.

BONDAD-REANTASO M.G., MCGLADDERY S.E., EAST I. & SUBASINGHE R.P. (2001). Asian Diagnostic Guide to Aquatic Animal Diseases. *FAO Fisheries Technical Paper*, No. 402, supplement 2. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 240 pp.

JOHNSON P.T. (1980). Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Prager, New York, USA, 440 pp.

LIGHTNER D.V. (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA. 304 pp.

LIGHTNER D.V., REDMAN R.M., PANTOJA C.R., TANG K.F.J, NOBLE B.L., SCHOFIELD P., MOHNEY L.L., NUNAN L.M. & NAVARRO S.A. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J. Invert. Pathol.,* **110**, 174–183.

LOTZ J.M. (1997). Special topic review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. *World J. Microbiol. Biotechnol*., **13**, 405–413.

MOODY N.J.G. & CRANE M.ST.J. (2016). Validation of diagnostic tests in the OIE manual for aquatic animals. In: Proc. 3rd OIE Global Conference on Aquatic Animal Health – "Riding the Wave of the Future", Ho Chi Minh City, Vietnam, 20–22 January 2015, pp.119–126.

QADIRI S.S.N., SOO-JIN KIM S.-J., KRISHNAN R., KIM J.-O., KOLE S., KIM W.-S. & OH M.-J. (2019). Localization and tissue tropism of viral haemorrhagic septicemia virus (VHSV) in experimentally infected juvenile olive flounder, *Paralichthys olivaceus*: An *in situ* hybridization and immunohistochemical study. *Aquaculture*, **505**, 242–252.

THITAMADEE S, PRACHUMWAT A., SRISALA J., JAROENLAK P., SALACHAN P.V., SRITUNYALUCKSANA K, FLEGEL T.W. & ITSATHITPHAISARN O. (2016). Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture*, **452**, 69–87.

VALVERDE E.J., BORREGO J.J., SARASQUETE M.C., ORTIZ-DELGADO J.B. & CASTRO D. (2017). Target organs for lymphocystis disease virus replication in gilthead seabream (*Sparus aurata*). *Vet. Res*., **48**, 21. doi 10.1186/s13567- 017-0428-3.

WALKER P.J. & MOHAN C.V. (2009). Viral disease emergence in shrimp aquaculture: origins, impact and the effectiveness of health management strategies. *Rev. Aquaculture*, **1**, 125–154.

*
* *

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

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. North American host species spiny-cheek crayfish (*Orconectes limosus*) has been shown to be a carrier of Group E (Kozubíková *et al.,* 2011).

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

The recommendations in this chapter apply to all species of crayfish in all three crayfish families (Cambaridae, Astacidae and Parastacidae).

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with *A. astaci* in accordance with Chapter 1.5. has not yet been completed]

~~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

[Under study]

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~~ that develop~~ment of~~ clinical disease and mortalities, and those that are infected ~~without associated~~ but do not display any significant clinical disease or mortalities. All life stages are considered susceptible to infection with *A. astaci*.

Species that develop clinical disease and mortalities include 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 (e.g. Holdich *et al*., 2009). Australian species of freshwater crayfish are also considered vulnerable to clinical disease and mortalities.

Species that can be infected but do not normally develop clinical disease include North American crayfish species such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Faxonius* spp. All North American crayfish species that have been investigated have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (reviewed by Svoboda *et al.* 2017).

~~*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.

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

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, which are prone to development of clinical disease, 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 reservoirs ~~carriers~~ of the infection without showing clinical signs. However, some strains of *A. astaci,* especially from group A, show lowered virulence, thus enabling ~~normally highly susceptible~~ European crayfish to act as reservoirs ~~carriers~~ as well (see review by Svoboda *et al*., 2017).

Colonisation of habitats~~, initially~~ by North American crayfish species carrying *A. astaci* ~~occupied by highly susceptible~~ is likely to result in an epizootic if crayfish species that are prone to expression of clinical disease are present ~~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).~~ None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity, and prevalence

When the infection first reaches a naïve population of ~~highly susceptible~~ crayfish species that are prone to clinical disease, high levels of mortality are usually observed within a short space of time, ~~so that in~~ and 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~~ a lower rate of 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~~ any noticeable mortalities (Viljamaa-Dirks *et al*., 2013).

On rare occasions, single specimens of ~~the highly susceptible~~ species that are prone to clinical disease 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* *prone to clinical disease*

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 do not usually show signs of disease ~~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).

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, or 3) through colonisation of non-native 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 occurred 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).

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).

2.3.5. 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 WOAH 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~~ crayfish species that are prone to clinical disease (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~~ crayfish species that are prone to clinical disease 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 prone to expression of clinical disease 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 species).

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 that are prone to clinical disease, sampled 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 that are prone to clinical disease, 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 melanised 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.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 WOAH 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 [1].

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 is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~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.

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling conditions

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

Method 1*: Vralstad *et al.,* 2009, Strand, 2013; GenBank Accession No.: AM947024			
Aphanomyces ~~astacus~~ astaci & *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 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~~ astaci/*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 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~~ amplicon size: 569 bp			
Aphanomyces ~~astacus~~ astaci & *A. fennicus*/ ITS	Fwd: GCT-TGT-GCT-GAG-GAT-GTT-CT Rev: CTA-TCC-GAC-TCC-GCA-TTC-TG-	500 nM 500 n	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 (Casabella-Herrero *et al.,* 2021).

4.5. Amplicon sequencing

~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference 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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

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.

[2] For example transboundary commodities.

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* in wet mounts

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~~ no data are currently available for either). 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.

7. References

ALDERMAN D.J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Rev sci. tech. Off. int. Epiz.,* **15**, 603–632.

ALDERMAN D.J. & POLGLASE J.L. (1985). Disinfection for crayfish plague. *Aquacult. Fish. Manage.,* **16**, 203–205.

ALDERMAN D.J. & POLGLASE J.L. (1986). *Aphanomyces astaci:* isolation and culture. *J. Fish Dis*., **9**, 367–379.

ALDERMAN D.J., POLGLASE J.L. & FRAYLING M. (1987). *Aphanomyces astaci* pathogenicity under laboratory and field conditions. *J. Fish Dis*., **10**, 385–393.

CASABELLA-HERRERO G., MARTÍNEZ-RÍOS M., VILJAMAA-DIRKS S., MARTÍN-TORRIJOS L. & DIÉGUEZ-URIBEONDO J. (2021). *Aphanomyces astaci* mtDNA: insights into the pathogen's differentiation and its genetic diversity from other closely related oomycetes. *Fungal Biol.,* **125**, 316–325. doi: 10.1016/j.funbio.2020.11.010. Epub 2020 Dec 2.

CERENIUS L. & SÖDERHÄLL K. (1984). Chemotaxis in *Aphanomyces astaci*, an arthropodparasitic fungus. *J. Invertabr. Pathol.,* **43**, 278–281.

CERENIUS L., SÖDERHÄLL K. & FULLER M.S. (1987). *Aphanomyces astaci* and *Aphanomyces* spp. *In:* Zoosporic fungi in teaching and research, Fuller M.S. & Jaworski A., eds. South-Eastern Publishing Corp., Athens, Georgia, USA. pp 64–65.

CERENIUS L., SÖDERHÄLL K., PERSSON M. & AJAXON R. (1988). The crayfish plague fungus, *Aphanomyces astaci* – diagnosis, isolation and pathobiology. *Freshwater Crayfish*, **7**, 131–144.

DI DOMENICO M., CURINI V., CAPRIOLI R., GIANSANTE C., MRUGAŁA A., MOJŽIŠOVÁ M., CAMMA C. & PETRUSEK A. (2021). Real-Time PCR assays for rapid Identification of common *Aphanomyces astaci* genotypes. *Front. Ecol. Evol.*, **9**, art 597585 doi:10.3389/fevo.2021.597585.

DIEGUEZ-URIBEONDO J., HUANG T.-S., CERENIUS L. & SÖDERHÄLL K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii. Mycol. Res*., **99**, 574–578.

GRANDJEAN F., VRÅLSTAD T., DIÉGUEZ-URIBEONDO J., JELIĆ M., MANGOMBI J., DELAUNAY C., FILIPOVÁ L., REZINCIUC S., KOZUBÍKOVÁ-BALCAROVA E., GYONNET D., VILJAMAA-DIRKS S. & PETRUSEK A. (2014). Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. *Vet. Microbiol*., **170**, 317–324.

HOLDICH D.M., REYNOLDS J.D., SOUTY-GROSSET C. & SIBLEY P.J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ec.*, **394–395**, 1–46.

HUANG T.S., CERENIUS L. & SÖDERHÄLL K. (1994). Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture*, **126**, 1–10.

KOZUBÍKOVÁ E., VILJAMAA-DIRKS S., HEINIKAINEN S. & PETRUSEK A. (2011). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague agent *Aphanomyces astaci. J. Invertebr. Pathol.,* **108**, 214–216.

MAKKONEN J., JUSSILA J., PANTELEIT J., KELLER N.S., SCHRIMPF A., THEISSINGER K., KORTET R., MARTÍN-TORRIJOS L., SANDOVAL-SIERRA J.V., DIÉGUEZ-URIBEONDO J. & KOKKO H. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology,* **145**, 1210–1218. https://doi.org/10.1017/S0031182018000227.

MARTIN-TORRIJOS L., CAMPOS LACH M., POU ROVIRA Q. & DIÉGUEZ-URIBEONDO J. (2017). Resistance to the crayfish plague, *Aphanomyces astaci* (Oomycota) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS ONE*, **12** (7), e0181226. https://doi.org/10.1371/journal. pone.0181226

MINARDI D., STUDHOLME D.J., OIDTMANN B., PRETTO T. & VAN DER GIEZEN M. (2019). Improved method for genotyping the causative agent of crayfish plague (*Aphanomyces astaci*) based on mitochondrial DNA. *Parasitology*, 146, 1022–1029, doi:10.1017/S0031182019000283

MINARDI D., STUDHOLME D.J., VAN DER GIEZEN M., PRETTO T. & OIDTMANN B. (2018). New genotyping method for the causative agent of crayfish plague (*Aphanomyces astaci*) based on whole genome data. *J. Invertebr. Pathol.*, **156**, 6–13.

OIDTMANN B., GEIGER S., STEINBAUER P., CULAS A. & HOFFMANN R.W. (2006). Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Dis. Aquat. Org.,* **72**, 53–64.

OIDTMANN B., HEITZ E., ROGERS D. & HOFFMANN R.W. (2002). Transmission of crayfish plague. *Dis. Aquat. Org.,* **52**, 159–167.

PAVIC D., ČANKOVIĆ M., PETRIĆ I., MAKKONEN J., HUDINA S., MAGUIRE I., VLADUŠIĆA T., ŠVER L., HRAŠĆANA R., ORLIĆ K., DRAGIČEVIĆ P. & BIELEN A. (2020) Non-destructive method for detecting *Aphanomyces astaci*, the causative agent of crayfish plague, on the individual level. *J. Invertebr. Pathol.*, **169**, 107274. https://doi.org/10.1016/j.jip.2019.107274

RENNERFELT E. (1936). Untersuchungen uber die Entwicklung und Biologie des Krebspestpilzes Aphanomyces astaci Schikora. Report of the Institute of Freshwater Research (Drottningholm, Sweden), **10**, 1–21.

SOUTY-GROSSET C., HOLDICH D.M., NOEL P.Y., REYNOLDS J.D. & HAFFNER P. (eds) (2006). Atlas of Crayfish in Europe. Muséum National d'Histoire Naturelle, Paris (Patrimoines naturels, 64), 188 p.

STRAND D.A., HOLST-JENSEN A., VILJUGREIN H., EDVARDSEN B., KLAVENESS D., JUSSILA J. & VRÅLSTAD T. (2011). Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Dis. Aquat. Org.*, **95**, 9–17.

STRAND D.A., JUSSILA J., VILJAMAA-DIRKS S., KOKKO H., MAKKONEN J., HOLST-JENSEN A., VILJUGREIN H. & VRÅLSTAD T. (2012). Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Vet. Microbiol.,* **160**, 99–107.

STRAND D.A. (2013) Environmental DNA monitoring of the alien crayfish plague pathogen *Aphanomyces astaci* in freshwater systems – Sporulation dynamics, alternative hosts and improved management tools. *Dissertation book, University of Oslo Faculty of Mathematics and Natural Sciences Department of Biosciences, Oslo*, ISSN 1501-7710, 73 p.

SVOBODA J., MRUGAŁA A., KOZUBÍKOVÁ-BALCAROVÁ E. & PETRUSEK A. (2017). Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J. Fish Dis.*, **40,**127–140. https://doi.org/10.1111/jfd.12472

SODERHALL K. & CERENIUS L. (1999) The crayfish plague fungus: history and recent advances. *Freshwater Crayfish*, **12**, 11-34.

THOMAS J.R., ROBINSON C.V., MRUGAŁA A., ELLISON A.R., MATTHEWS E., GRIFFITHS S.W., CONSUEGRA S. & CABLE J. (2020). Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish. *Parasitology*, **1–9**, https://doi.org/10.1017/S0031182020000165

TUFFS S & OIDTMANN B (2011). A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci*. *Vet. Microbiol.,* **153**, 345–353.

UNESTAM T. (1966). Studies on the crayfish plague fungus *Aphanomyces astaci*. II. Factors affecting zoospores and zoospore production. *Physiol. Plant.*, **19**, 1110–1119.

~~UNESTAM T. & SODERHALL K. (1977). Specialisation in crayfish defence and fungal aggressiveness upon crayfish plague infection. *Freshwater Crayfish*, **3**, 321–331.~~

VILJAMAA-DIRKS S. (2006). Improved detection of crayfish plague with a modified isolation method. *Freshwater Crayfish*, **15**, 376–382.

VILJAMAA-DIRKS S. & HEINIKAINEN S. (2019) A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. *J. Fish Dis.*, **42**, 413–422. https://doi.org/10.1111/jfd.12955

VILJAMAA-DIRKS S., HEINIKAINEN S., NIEMINEN M., VENNERSTRÖM P. & PELKONEN S. (2011). Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report. *Bull. Eur. Assoc. Fish Pathol*., **31**, 182–188.

VILJAMAA-DIRKS S., HEINIKAINEN S., TORSSONEN H., PURSIAINEN M., MATTILA J. & PELKONEN S. (2013). Distribution and epidemiology of genotypes of the crayfish plague *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland. *Dis. Aquat. Org*., **103**, 199–208.

VRALSTAD T., JOHNSEN S.I., FRISTAD R., EDSMAN L. & STRAND D.A. (2011). Potent infection reservoir of crayfish plague now permanently established in Norway. *Dis. Aquat. Org.,* **97**, 75–83.

VRALSTAD T., KNUTSEN A.K., TENGS T. & HOLST-JENSEN A. (2009). A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.,* **137**, 146–155.

~~WHITE T.J., BRUNS T., LEE S. & TAYLOR J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In:* PCR Protocols: A Guide to Methods and Applications, Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds. Academic Press, San Diego, California, USA, pp. 315–322.~~

*
* *

NB: There is a WOAH Reference Laboratory for infection with *Aphanomyces astaci* (crayfish plague)
(please consult the WOAH 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 WOAH 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 2.2.6.

INFECTION WITH *MACROBRACHIUM ROSENBERGII* NODAVIRUS (WHITE TAIL DISEASE)

1. Scope

Infection with *Macrobrachium rosenbergii* nodavirus means infection with the pathogenic agent *Macrobrachium rosenbergii* nodavirus (*Mr*NV) in the Family *Nodaviridae*. The disease is commonly known as white tail disease (WTD).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Two viruses are associated with WTD, namely *Mr*NV (primary) and extra small virus (XSV) (associate) (Qian *et al.,* 2003; Romestand & Bonami, 2003). MrNV is a necessary cause of WTD in prawns, however, the role of XSV in pathogenicity remains unclear.

*Mr*NV belongs in the family *Nodaviridae* (Bonami *et al.,* 2005). While the physico-chemical properties of *Mr*NV are consistent with those of other members of the *Nodaviridae*, it differs structurally and genetically from other nodaviruses within the two recognised genera, *Alphanodavirus* and *Betanodavirus* (Ho *et al*., 2017, 2018; Naveenkumar *et al*., 2013). Consequently, a third genus, *Gammanodavirus*, has been proposed for nodaviruses that infect crustaceans, including *Mr*NV and *Penaeus vannamei* nodavirus (*Pv*NV) (Naveenkumar *et al*., 2013).

XSV is the first sequenced satellite virus in aquatic animals and it is also the first record of a satellite-nodavirus association (Bonami *et al.,* 2005). XSV has been classified by the ICTV as *Macrobrachium* satellite virus 1 of the family *Sarthroviridae.*

2.1.2. Survival and stability in processed or stored samples

Both viral pathogens (*Mr*NV and XSV) are stable in processed or stored samples stored at –20 or –80°C. Storing the samples at –80°C is recommended for long-time storage and maintenance of pathogen virulence (Sahul Hameed & Bonami, 2012). The infected samples should be processed at low temperature to maintain the stability of the viruses. Viral inoculum prepared from infected prawn stored at –20°C caused 100% mortality in postlarvae (PL) of *M. rosenbergii* by immersion challenge (Qian *et al*., 2003; Sahul Hameed *et al*., 2004a). Ravi & Sahul Hameed (2016) found that MrNV in tissue suspensions was inactivated after exposure to 50°C for at least 5 min.

2.1.3. Survival and stability outside the host

Survival outside the host is not known.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Mr*NV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: giant river prawn (*Macrobrachium rosenbergii*).

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 MrNV according to Chapter 1.5. of the *Aquatic Code* are: white leg shrimp (*Penaeus vannamei*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results (but not active infection) have been reported in the following species:

Family	*Scientific name*	*Common name*
Aeshnidae	*Aeshna* sp.	dragonfly
Artemiidae	*Artemia* sp.	brine shrimps
Belostomatidae	*Belostoma* sp.	giant water bug
Dytiscidae	*Cybister* sp.	beetle
Notonectidae	*Notonecta* sp.	backswimmer
Palaemonidae	*Macrobrachium rude*	hairy river prawn
	Macrobrachium malcolmsonii	monsoon river prawn
Parastacidae	*Cherax quadricarinatus*	red claw crayfish
Penaeidae	*Penaeus japonicus*	kuruma prawn
	Penaeus indicus	Indian white prawn
	Penaeus monodon	giant tiger prawn

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

Experimental pathogenicity studies revealed that larvae, PL and early juveniles of *M. rosenbergii* are susceptible to *Mr*NV/XSV, whereas adults are resistant (Qian *et al*., 2003; Sahul Hameed *et al*., 2004a).

No mortality was observed either in naturally or experimentally (*Mr*NV/XSV) infected subadult and adult prawns. Experimental studies confirmed vertical transmission from infected broodstock to PL (Sudhakaran *et al*., 2007a).

2.2.4. Distribution of the pathogen in the host

*Mr*NV and XSV have been demonstrated in gill tissue, head muscle, heart, abdominal muscle, ovaries, pleopods and tail muscle, but not the hepatopancreas or eyestalk (Sahul Hameed *et al*., 2004a; Sri Widada *et al*., 2003).

2.2.5. Aquatic animal reservoirs of infection

One study has indicated the possibility that marine shrimp act as a reservoir for *Mr*NV and XSV and that these viruses maintain virulence in the shrimp tissue system (Sudhakaran *et al*., 2006).

2.2.6. Vectors

Aquatic insects such as giant water bug (*Belostoma* sp.), dragonfly (*Aesohna* sp.), beetle (*Cybister* sp.) and backswimmer (*Notonecta* sp.) may act as mechanical carriers for *Mr*NV/XSV and are a potential transmission risk to cultivated *Macrobrachium rosenbergii* (Sudhakaran *et al.,* 2008). It is recommended to remove these insects from freshwater prawn culture systems, especially at larval-rearing centres. Sudhakaran *et al*. (2008) demonstrated RT-PCR positives from insects, and infected C6/36 insect cell line with tissue homogenates from the insects. Viral replication was confirmed through EM and RT-PCR, but transmission from insects to naïve shrimp was not demonstrated.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Larvae, PL and juveniles of *M. rosenbergii* are susceptible to infection with *Mr*NV, which often causes high mortalities in these life stages. Mortality may reach a maximum in about 5 or 6 days after the appearance of the first clinical signs. Very few PL with infection with *Mr*NV survive beyond 15 days in an outbreak, but PL that survive may grow to market size.

Adults are resistant to infection with *Mr*NV, but act as carriers (Qian *et al*., 2003; Sahul Hameed *et al*., 2004a). Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems (Arcier *et al*., 1999; Qian *et al*., 2003; Sahul Hameed *et al*., 2004a; 2004b).

2.3.2. Clinical signs, including behavioural changes

Infected PL become opaque and develop a whitish appearance, particularly in the abdominal region. The whitish discoloration appears first in the second or third abdominal segment and gradually diffuses both anteriorly and posteriorly. In severe cases, degeneration of telson and uropods may occur. Floating exuviae (moults) in the tanks appear abnormal and resemble 'mica flakes' (Arcier *et al*., 1999). The infected PL show progressive weakening of their feeding and swimming ability (Sahul Hameed *et al*., 2004a).

2.3.3. Gross pathology

Infection with *Mr*NV is indicated by the whitish coloration of abdominal muscle.

2.3.4. Modes of transmission and life cycle

Transmission is vertical by trans-ovum and horizontal by the waterborne route (Qian *et al*., 2003; Sahul Hameed *et al*., 2004a; Sudhakaran *et al*., 2007a).

2.3.5. Environmental factors

Not available.

2.3.6. Geographical distribution

The disease was first reported in the French West Indies (Arcier *et al*., 1999), later in Asia-Pacific (Murwantoko *et al*., 2016; Owens *et al*., 2009; Qian *et al*., 2003; Saedi *et al*., 2012; Sahul Hameed *et al*., 2004b; Wang *et al*., 2008; Yoganandhan *et al*., 2006).

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

2.4. Biosecurity and disease control strategies

Preventive measures, such as screening of broodstock and PL, and good management practices may help to prevent infection with *Mr*NV in culture systems. As the life cycle of *M. rosenbergii* is completed under controlled conditions, specific pathogen-free (SPF) broodstock and PL can be produced (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al.,* 2005).

2.4.1. Vaccination

Not available

2.4.2. Chemotherapy including blocking agents

No known chemotherapeutic agents reported to treat *Mr*NV-infected prawn.

2.4.3. Immunostimulation

The immunomodulatory effect of recombinant capsid protein and recombinant RNA-dependent RNA polymerase (RdRp) protein of *Mr*NV has been studied and the protection of viral challenged post-larvae from *Mr*NV infection has been demonstrated (Farook *et al*., 2014; NaveenKumar *et al*., 2021).

2.4.4. Breeding resistant strains

None reported

2.4.5. Inactivation methods

A viral suspension treated with heat at 65°C for 2 hours destroyed infectivity of *Mr*NV and XSV in challenge experiments (Qian *et al*., 2003). The viral inoculum exposed to UV irradiation for a period of 5 minutes and more was totally inactivated and failed to cause mortality in PL of prawn (Ravi & Sahul Hameed, 2016).

2.4.6. Disinfection of eggs and larvae

Routine disinfection procedures followed for crustacean viral disease control are suggested.

2.4.7. General husbandry

*Mr*NV is transmitted both horizontally and vertically in culture systems (Qian *et al*., 2003; Sahul Hameed *et al*., 2004a; Sudhakaran *et al*., 2007a). Good husbandry practices, such as proper disinfection of tanks and water may help to prevent infection. It is recommended to remove insects from freshwater prawn culture systems, especially at larval-rearing centres. Specific pathogen-free (SPF) broodstock and PL can be obtained from disease free populations or by RT-PCR screening and selection of negative broodstock (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al.,* 2005).

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

PLs are most suitable for detection of *Mr*NV. PL showing clinical signs of disease can be sampled preferentially. Adults and juveniles can be sampled for MrNV however prevalence in these lifestages may be lower (see Section 2.3.1).

3.2. Selection of organs or tissues

The tissues most affected in moribund PLs/early juveniles are striated muscles of the abdomen, cephalothorax and tail. The whole PL body is preferred for detection of *Mr*NV (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005). All organs of adult *M. rosenbergii* except eyestalks and the hepatopancreas, are best for screening the viruses by RT-PCR.

3.3. Samples or tissues not suitable for pathogen detection

Eyestalks and the hepatopancreas of adult prawns are not suitable (Sri Widada *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

3.4. Non-lethal sampling

Pleopods (swimming legs) are a convenient source of RNA for non-destructive screening of *Mr*NV in adult prawn (Sahul Hameed *et al.,* 2004a).

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

3.5. Preservation of samples for submission

Infected larvae or PL with prominent signs of whitish muscle in the abdominal region are collected from disease outbreak areas. Samples are washed in sterile saline, transferred to sterile tubes, and transported to the laboratory. For general guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0.

3.5.1. Samples for pathogen isolation

Moribund or frozen PL samples can be used for isolation of viral pathogens using cell lines (C6/36 mosquito cell line (Sudhakaran et al., 2007b).

3.5.2. Preservation of samples for molecular detection

Infected samples stored at –80°C or samples preserved in 80% (v/v) analytical/reagent-grade (undenatured) ethanol should be used for RT-PCR for detection of *Mr*NV (Sri Widada *et al.,* 2003; Sahul Hameed *et al.,* 2004b; Yoganandhan *et al*., 2005).

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

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

Tissue samples for histopathology, immunohistochemistry or *in-situ* hybridisation should be fixed immediately after collection in neutral-buffered formalin or modified Davidson's fixative (Sri Widada *et al.,* 2003). 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 5.3. of 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 animals 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 identifying infection 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 life stage.

Ratings against purposes of use. For each recommended assay a qualitative rating against 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, cost, timeliness, and sample throughput. For a specific purpose of use, assays are rated as:

+++ =	Most suitable methods – desirable performance and operational characteristics;
++ =	Suitable method(s) acceptable performance and operational characteristics under most circumstances;
+ =	Less suitable methods – performance or operational characteristics may significantly limit application;
Shaded boxes =	Not appropriate for this purpose.

Level of validation. 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.

WOAH 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). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

Method	D. Surveillance of apparently healthy animals				E. Presumptive diagnosis of clinically affected animals				F. 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						++	++					
Cell culture												
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	1	+++	+++	+++	1
Conventional RT-PCR	++	++	++	1	+++	+++	+++	2				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	2
In-situ hybridisation						++	++	1		++	++	1
Bioassay												
LAMP	++	++	++	1	++	++	++	1				
Ab-ELISA												
Ag-ELISA					++	++	++	1				
Lateral flow assay					++	++	++	2				
Other methods[3]												

LV = level of validation, refers to the stage of validation in the WOAH 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

None to date

4.2. Histopathology and cytopathology

The most affected tissue in infected PL is striated muscle of the cephalothorax, abdomen and tail. Histological features include the presence of acute Zenker's necrosis of striated muscles, characterised by severe hyaline degeneration, necrosis and muscular lysis. Moderate oedema and abnormal open spaces among the affected muscle cells are also observed, as is the presence of large oval or irregular basophilic cytoplasmic inclusion bodies in infected muscles (Arcier *et al.,* 1999; Hsieh *et al.,* 2006).

4.3. Cell culture for isolation

*Mr*NV has been isolated in insect cell lines, but is not a recommended method (Sudhakaran *et al.,* 2007b) (Hernandez-Herrera *et al.,* 2007).

4.4. Nucleic acid amplification

PCR methods for MrNV and XSV are included in this section for completeness. However, the case definitions in Section 6 are based on detection methods for MrNV only.

PCR assays should always be run with the controls specified in Section 5.5. *Use of molecular 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

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time RT-PCR

Real-time RT-PCR assay can be performed using the SYBR Green dye based on the method described by Hernandez-Herrera *et al*. (2007) or the TaqMan assay described by Zhang *et al*. (2006).

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Hernandez-Herrera *et al.* (2007); GenBank Accession No.: AY222839)			
MrNv/RNA1	Fwd: AGG-ATC-CAC-TAA-GAA-CGT-GG Rev: CAC-GGT-CAC-AAT-CCT-TGC-G	500 nM 500 nM	40 cycles of: 95°C/15 sec, 60°C/5 sec and 72°C/10 sec
Method 2: Zhang *et al.* (2006); GenBank Accession No.: AY231436)			
MrNv/RNA1	Fwd: CAA-CTC-GGT-ATG-GAA-CTC-AAG-GT Rev: AGG-AAA-TAC-ACG-AGC-AAG-AAA-AGT-C Probe: FAM-ACC-CTT-CGA-CCC-CAG-CAA-TGG-TG-TAMARA	1000 nM 1000 nM 400 nM	50 cycles of: 94°C/30 sec and 58°C/30 sec
Method 3: Zhang *et al.* (2006); GenBank Accession No.: DQ174318)			
XSV	**Fwd: AGC-CAC-ACT-CTC-GCA-TCT-GA Rev: CTC-CAG-CAA-AGT-GCG-ATA-CG Probe: FAM-CAT-GCC-CCA-TGA-TCC-TCG-CA-TAMARA**	**1000 nM 1000 nM 400 nM**	**50 cycles of: 94°C/30 sec and 58°C/30 sec**

4.4.2. Conventional RT-PCR

The protocol for the conventional RT-PCR for detection of *Mr*NV/XSV developed by Sri Widada *et al.* (2003), Sahul Hameed *et al.* (2004a; 2004b) and Sudhakaran *et al*. (2007a) is recommended. *Mr*NV and XSV can be detected by conventional RT-PCR separately using a specific set of primers or these two viruses can be detected simultaneously using a single-tube one-step multiplex RT-PCR (Yoganandhan *et al*., 2005). Conventional Real-time RT-PCR is recommended in situations where high sensitivity is required.

Pathogen / target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1: One step RT-PCR (Sri Widada *et al*., 2003; Sahul Hameed *et al*., 2004a, b; Sudhakaran *et al*., 2007a) GenBank Accession No.: AY222840 (MrNV) and AY247793 (XSV)			
*Mr*Nv	Fwd: GCG-TTA-TAG-ATG-GCA-CAA-GG Rev: AGC-TGT-GAA-ACT-TCC-ACT-GG	0.02 nM 0.02 nM	30 cycles of: 94°C/40 sec, 55°C/40sec and 68°C/60 sec
XSV	Fwd: CGC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-TAA Rev: CCG-GAA-TTC-CGT-TAC-TGT-TCG-GAG-TCC-CAA	0.02 nM 0.02 nM	30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec
Method 2: nested RT-PCR using above-mentioned primers as external primers (Sudhakaran *et al*., 2007a)			
*Mr*Nv	Internal primers: Fwd: GAT-GAC-CCC-AAC-GTT-ATC-CT Rev: GTG-TAG-TCA-CTT-GCA-AGA-GG	0.02 nM 0.02 nM	30 cycles of: 94°C/60 sec, 55°C/60 sec and 72°C/60 sec
XSV	Internal primers: Fwd: ACA-TTG-GCG-GTT-GGG-TCA-TA Rev: GTG-CCT-GTT-GCT-GAA-ATA-CC-3	0.02 nM 0.02 nM	30 cycles of: 94°C/60 sec, 55°C/60 sec and 72°C/60 sec
Method 3: Multiplex RT-PCR (Yoganandhan *et al*., 2005) GenBank Accession No.: AY222840 (MrNV) and AY247793 (XSV)			
MrNV	Fwd: GAT-ACA-GAT-CCA-CTA-GAT-GAC-C Rev: GAC-GAT-AGC-TCT-GAT-AAT-CC	0.02 nM 0.02 nM	30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec
XSV	**Fwd: GGA-GAA-CCA-TGA-GAT-CAC-G Rev: CTG-CTC-ATT-ACT-GTT-CGG-AGT-C**	**0.02 nM 0.02 nM**	**30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec**

4.4.3. Other nucleic acid amplification methods: Loop-mediated isothermal amplification (LAMP)

Haridas *et al*. (2010) have applied loop-mediated isothermal amplification (LAMP) for rapid detection of *Mr*NV and XSV in the freshwater prawn. A set of four primers, two outer primers and two inner primers, have been designed separately for detection of *Mr*NV and XSV.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

The presence of *Mr*NV in infected cells can be demonstrated in histological sections using a DIG-labelled DNA *in-situ* hybridisation probe specific for *Mr*NV (Sri Widada *et al.,* 2003).

4.7. Immunohistochemistry

None developed.

4.8. Bioassay

Not used for diagnostic purposes.

4.9. Antibody- or antigen-based detection methods

4.9.1. ELISA

Antibody-based diagnostic methods for *Mr*NV include the ELISA described by Romestand & Bonami (2003) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (Qian *et al.,* 2006).

4.9.2. Lateral flow assay (LFA)

An antibody-based lateral flow assay (LFA) has been developed for the early detection of *Mr*NV in the PL stage (Jamalpure *et al*., 2021).

4.10. Other methods

None

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

Real-time RT-PCR is recommended for targeted surveillance to declare freedom from infection with *Mr*NV.

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. If a Competent Authority does not have the capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status[1]

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 *Mr*NV shall be suspected if at least one of the following criteria is met:

i) Positive result by real-time RT-PCR

ii) Positive result by conventional RT-PCR

iii) Positive result by LAMP

[1] For example transboundary commodities.

6.1.2. Definition of confirmed case in apparently healthy animals

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

i) Positive result by real-time RT-PCR result and positive conventional RT-PCR and sequence analysis

6.2 Clinically affected animals

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *Mr*NV shall be suspected if at least one of the following criteria is met:

i) Clinical signs consistent with infection by *Mr*NV

ii) Histopathology consistent with infection by *Mr*NV

iii) Positive result by real-time RT-PCR

iv) Positive conventional RT-PCR

v) Positive result by *in situ* hybridisation

vi) Positive result by LAMP

vii) Positive result by Ag ELISA

viii) Positive result by lateral flow assay

6.2.2. Definition of confirmed case in clinically affected animals

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

i) Positive result for real time RT-PCR and positive result by conventional RT-PCR with sequence analysis

ii) Positive result by ISH followed by positive result by conventional RT-PCR with sequence analysis

iii) Positive result by ISH followed by positive result by real-time RT-PCR

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *Mr*NV are provided in Tables 6.3.1. and 6.3.2 (no data are currently available). This information can be used for the design of surveys for infection with *Mr*NV, 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
RT-PCR	Diagnosis	Clinically affected PL from hatchery and nursery	Whole post-larvae	*Macrobrachium rosenbergii*	100 (*n*=20)	100 (*n*=20)	Western blot or ELISA	Sri Widada *et al*. (2003); Sahul Hameed *et al*. (2011)
Lateral flow immun oassay	Surveillance	PL from prawn hatcheries	Whole post-larvae	*Macrobrachium rosenbergii*	100 (*n*=80)	90 (*n*=80)	RT-PCR	Jamalpure *et al*. (2021)

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
RT-PCR: = reverse transcription 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,
RT-PCR: = reverse transcription polymerase chain reaction.

7. References

ARCIER J.-M., HERMAN F., LIGHTNER D.V., REDMAN R.M., MARI. J. & BONAMI J.-R. (1999). A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*. *Dis. Aquat. Org.*, **38**, 177–181.

BONAMI J.R., SHI Z., QIAN D. & SRI WIDADA J. (2005). White tail disease of the giant freshwater prawn, *Macrobrachium rosenbergii*: separation of the associated virions and characterization of MrNV as a new type of nodavirus. *J. Fish Dis.*, **28**, 23–31.

FAROOK M.A., SUNDAR RAJ N., MADAN N., VIMAL S., ABDUL MAJEED S., TAJU G., RAJKUMAR T., SANTHOSHKUMAR S., SIVAKUMAR S. & SAHUL HAMEED A.S. (2014). Immunomodulatory effect of recombinant *Macrobrachium rosenbergii* nodavirus capsid protein (r-MCP) against white tail disease of giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). *Aquaculture,* **433**, 395–403.

HARIDAS D.V., PILLAI D., MANOJKUMAR B., NAIR C.M. & SHERIEF P.M. (2010). Optimisation of reverse transcriptase loop-mediated isothermal amplification assay for rapid detection of *Macrobrachium rosenbergii* nodavirus and extra small virus in *Macrobrachium rosenbergii. J. Virol. Methods*, **167**, 61–67.

HERNANDEZ-HERRERA R.I., CHAPPE-BONNICHON V., ROCH P., SRI WIDADA J. & BONAMI J.R. (2007). Partial susceptibility of the SSN-1 fish cell line to a crustacean virus: a defective replication study. *J. Fish Dis.*, **30**, 673–679.

HO K.L., GABRIELSEN M., BEH P.L., KUEH C.L., THONG Q.X. & STREETLEY J. (2018). Structure of the *Macrobrachium rosenbergii* nodavirus: a new genus within the nodaviridae? *PLOS Biology*, **16**, e3000038.

HO K.L., KUEH C.L., BEH P.L., TAN W.S. & BHELLA D. (2017). Cryo-Electron microscopy structure of the *Macrobrachium rosenbergii* nodavirus capsid at 7 angstroms resolution. *Scientific Reports*, **7**, 2083.

HSIEH C.-Y., WU Z.-B., TUNG M.-C., TU C., LO S.-P., CHANG T.-C., CHANG C.-D., CHEN S.-C., HSIEH Y.-C. & TSAI S.-S. (2006). *In situ* hybridization and RT-PCR detection of *Macrobrachium rosenbergii* nodavirus in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), in Taiwan. *J. Fish Dis.*, **29**, 665–671. https://doi.org/10.1111/j.1365-2761.2006.00762.x

JAMALPURE S., VIMAL S., NAFEEZ AHMED A., SAHUL HAMEED A.S., PAKNIKAR K.M. & JYTIKA M.R. (2021). On-site detection of nodavirus in post larval (PL) stage of the giant prawn, *Macrobrachium rosenbergii*: A test to nip the problem in the bud. *Aquaculture*, **534**, 736292; https://doi.org/10.1016/j.aquaculture.2020.736292

MURWANTOKO M., ARIF B., ROOSMANTO R & MASASHI K. (2016). *Macrobrachium rosenbergii* nodavirus infection in a giant freshwater prawn hatchery in Indonesia. *Springer Plus*, **5**, 1729.

NAVEENKUMAR S., SHEKAR M., KARUNASAGAR I. & KARUNAS I. (2013). Genetic analysis of RNA1 and RNA2 of *Macrobrachium rosenbergii* nodavirus (MrNV) isolated from India. *Virus Res.,* **173**, 377–385. https://doi.org/10.1016/j.virusres.2013.01.003https://doi.org/10.1016/j.virusres.2013.01.003

NAVEEN KUMAR S., PRAVEEN R., INDRANI K. & KARUNASAGAR I. (2021). Recombinant viral proteins delivered orally through inactivated bacterial cells induce protection in *Macrobrachium rosenbergii* (de Man) against White Tail Disease. *J. Fish Dis.*, **44**, 601–612.

OWENS L., LA FAUCE K., JUNTUNEN K., HAYAKIJKOSOL O. & ZENG C. (2009). *Macrobrachium rosenbergii* nodavirus disease (white tail disease) in Australia. *Dis. Aquat. Org.,* **85**, 175–180.

QIAN D., LIU W., JIANXIANG W. & YU L. (2006). Preparation of monoclonal antibody against *Macrobrachium rosenbergii* Nodavirus and application of TAS-ELISA for virus diagnosis in post-larvae hatcheries in east China during 2000–2004. *Aquaculture*, **261**, 1144–1150.

QIAN D., SHI Z., ZHANG S., CAO Z., LIU W. LI L., XIE Y., CAMBOURNAC I. & BONAMI J.R. (2003). Extra small virus-like particles (XSV) and nodavirus associated with whitish muscle disease in the giant freshwater prawn, *Macrobrachium rosenbergii. J. Fish Dis*., **26**, 521–527.

RAVI N. & SAHUL HAMEED A.S. (2016). Effect of chemical and physical treatments on the inactivation of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). *Aquaculture Res.,* **47**, 1231–1237.

ROMESTAND B. & BONAMI J.R. (2003). A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of *Mr*NV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *J. Fish Dis*., **26**, 71–75.

SAEDI T. A., HASSAN M., WEN S. T., KHATIJAH Y., HASSAN M.D., KUA B.C., SOON G.T. & SUBHA B. (2012). Detection and phylogenetic profiling of nodavirus associated with white tail disease in Malaysian *Macrobrachium rosenbergii* de Man. *Mol. Biol. Rep*., **39**, 5785–5790.

SAHUL HAMEED A.S. & BONAMI J.R. (2012). White Tail Disease of Freshwater Prawn, *Macrobrachium rosenbergii*. *Indian J. Virol.,* **23**, 134–140.

SAHUL HAMEED A.S., RAVI M., FAROOK M.A., TAJU G., HERNANDEZ-HERRERA R.I. & BONAMI J.R. (2011). Screening the post-larvae of *Macrobrachium rosenbergii* for early detection of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) by RT–PCR and immunological techniques. *Aquaculture*, **317**, 42–47. http://dx.doi.org/10.1016/j.aquaculture.2011.04.022

SAHUL HAMEED A.S., YOGANANDHAN K., SRI WIDADA J. & BONAMI J.R. (2004a). Experimental transmission and tissue tropism of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and its associated small virus (XSV). *Dis. Aquat. Org.,* **62**, 191–196.

SAHUL HAMEED A.S., YOGANANDHAN K., SRI WIDADA J. & BONAMI J.R. (2004b). Studies on the occurrence of *Macrobrachium rosenbergii* nodavirus and extra small virus-like particles associated with white tail disease of *M. rosenbergii* in India by RT-PCR detection. *Aquaculture*, **238**, 127–133.

SRI WIDADA J., DURAND S., CAMBOURNAC I., QIAN D., SHI Z., DEJONGHE E., RICHARD V. & BONAMI J.R. (2003). Genome-based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant freshwater prawn, *Macrobrachium rosenbergii*: dot-blot, *in situ* hybridization and RT-PCR. *J. Fish Dis*., **26**, 583–590.

SRI WIDADA J., RICHARD V., SHI Z., QIAN D. & BONAMI J.R. (2004). Dot-Blot hybridization and RT-PCR detection of extra small virus (XSV) associated with white tail disease of prawn *Macrobrachium rosenbergii. Dis. Aquat. Org.,* **58**, 83–87.

SUDHAKARAN R., HARIBABU P., KUMAR S.R., SARATHI M., AHMED V.P., BABU V.S., VENKATESAN C. & HAMEEDL A.S. (2008). Natural aquatic insect carriers of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV). *Dis. Aquat. Org.*, **79**, 141–145. doi: 10.3354/dao01886.

SUDHAKARAN R., ISHAQ AHMED V.P., HARIBABU P., MUKHERJEE S.C., SRI WIDADA J., BONAMI J.R. & SAHUL HAMEED A.S. (2007a). Experimental vertical transmission of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) from brooders to progeny in *Macrobrachium rosenbergii* and *Artemia. J. Fish Dis*., **30**, 27–35.

SUDHAKARAN R., PARAMESWARAN V. & SAHUL HAMEED A.S. (2007b). *In vitro* replication of *Macrobrachium rosenbergii*) nodavirus and extra small virus (XSV) in C6/36 mosquito cell line. *J. Virol. Methods*, **146**, 112–118.

SUDHAKARAN R., SYED MUSTHAQ S., HARIBABU P., MUKHERJEE S.C., GOPAL C. & SAHUL HAMEED A.S. (2006). Experimental transmission of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in three species of marine shrimp (*Penaeus indicus*, *Penaeus japonicus* and *Penaeus monodon*). *Aquaculture*, **257**, 136–141.

WANG C.S., CHANG J.S., WEN C.M., SHIH H.H., & CHEN S.N. (2008). *Macrobrachium rosenbergii nodavirus* infection in *M. rosenbergii* (de Man) with white tail disease cultured in Taiwan. *J. Fish Dis*., **31**, 415–422.

YOGANANDHAN K., LEARTVIBHAS M., SRIWONGPUK S. & LIMSUWAN C. (2006). White tail disease of the giant freshwater prawn *Macrobrachium rosenbergii* in Thailand. *Dis. Aquatic. Org.*, **69**, 255–258.

YOGANANDHAN K., SRI WIDADA J., BONAMI J.R. & SAHUL HAMEED A.S. (2005). Simultaneous detection of *Macrobrachium rosenbergii* nodavirus and extra small virus by a single tube, one-step multiplex RT-PCR assay. *J. Fish Dis*., **28**, 65-69.

ZHANG H., WANG J., YUAN J., LI L., ZHANG J., BONAMI J.-R. & SHI Z. (2006). Quantitative relationship of two viruses (*Mr*NV and XSV) in white tail disease of *Macrobrachium rosenbergii*. *Dis. Aquat. Org.*, **71**, 11–17.

*
* *

NB: There is a WOAH Reference Laboratory for infection with *Macrobrachium rosenbergii* nodavirus (white tail disease) (please consult the WOAH web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3) any further information on infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

NB: FIRST ADOPTED IN 2009. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.9

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

1. Scope

Infection with yellow head virus genotype 1 means infection with the pathogenic agent yellow head virus genotype 1 (YHV1) of the Genus *Okavirus* and Family *Roniviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Yellow head virus genotype 1 (YHV1; species *Yellow head virus*) is one of eight known genotypes in the yellow head complex of viruses and is the only known genotype that causes yellow head disease. YHV1 forms enveloped, rod-shaped particles 40–50 nm × 150–180 nm (Chantanachookin *et al.,* 1993; Wongteerasupaya *et al*., 1995). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids appear as rods (diameter 20–30 nm) and possess a helical symmetry with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p20 and envelope glycoproteins gp64 and gp116) and a ~26 kb positive-sense single-stranded RNA genome. The nucleotide sequence of the ORF1b region of the viral genome has been used to determine the phylogenetic relationships of YHV1 and other yellow head virus genotypes (Dong *et al*., 2017; Mohr *et al*., 2015; Wijegoonawardane *et al.,* 2008a).

YHV1, yellow head virus genotype 2 (YHV2; species *Gill-associated virus*) and yellow head virus genotype 8 (YHV8; species *Okavirus 1*) have been formally classified by the International Committee on Taxonomy of Viruses (Walker *et al*., 2021). Four other genotypes in the complex (YHV3–YHV6) occur commonly in healthy *Penaeus monodon* in East Africa, Asia and Australia and are rarely or never associated with disease (Walker *et al.,* 2001; Wijegoonawardane *et al.,* 2008a). Of the remaining two yellow head virus genotypes, YHV7 was detected in diseased *P. monodon* in Australia (Mohr *et al*., 2015) and YHV8 was detected in *P. chinensis* suspected of suffering from acute hepatopancreatic necrosis disease (Liu *et al*., 2014). There is evidence of genetic recombination between genotypes (Wijegoonawardane *et al.*, 2009).

2.1.2. Survival and stability in processed or stored samples

YHV1, identified by either transmission electron microscopy (TEM) (Nunan *et al.,* 1998), or molecular methods (Durand *et al.,* 2000; McColl *et al.,* 2004), has been detected in frozen commodity prawns with infectivity demonstrated by bioassay.

2.1.3. Survival and stability outside the host

YHV1 remains viable in aerated seawater for up to 72 hours (Flegel *et al.*, 1995b).

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 YHV1 according to Chapter 1.5 of the *Aquatic Animal Health Code* (*Aquatic Code*) are: blue shrimp (*Penaeus stylirostris*), dagger blade grass shrimp (*Palaemonetes pugio*), giant tiger prawn (*Penaeus monodon*), jinga shrimp (*Metapenaeus affinis*) 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 YHV1 according to Chapter 1.5 of the *Aquatic Code* are: banana prawn (*Penaeus merguiensis*), carpenter prawn (*Palaemon serrifer*), kuruma prawn (*Penaeus japonicus*), northern brown shrimp (*Penaeus aztecus*), northern pink shrimp (*Penaeus duorarum*), northern white shrimp (*Penaeus setiferus*), Pacific blue prawn (*Palaemon styliferus*), red claw crayfish (*Cherax quadricarinatus*), Sunda river prawn (*Macrobrachium sintangense*) and yellow shrimp (*Metapenaeus brevicornis*).

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: acorn barnacle (*Chelonibia patula*), blue crab (*Callinectes sapidus*), cyclopoid copepod (*Ergasilus manicatus*), gooseneck barnacle (*Octolasmis muelleri*), Gulf killifish (*Fundulus grandis*) and paste shrimp (*Acetes* sp.).

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

Penaeus monodon are susceptible to YHV1 infection beyond PL15 (Khongpradit *et al.*, 1995). Lightner *et al*. (1998) YHV1 challenge caused disease in juveniles of *Penaeus aztecus, P. duorarum, P. setiferus,* and *P. vannamei* but postlarvae appeared resistant (Lightner *et al*. 1998). YHV1 infections are usually detected only when disease is evident, however infections have been detected in healthy wild populations of *P. stylirostris* (Castro-Longoria *et al.,* 2008). Natural YHV1 infections have been detected in *P. japonicus*, *P. merguiensis*, *P. setiferus*, *M. ensis*, and *P. styliferus* (Cowley *et al.,* 2002; Flegel *et al.,* 1995a; 1995b).

2.2.4. Distribution of the pathogen in the host

YHV1 targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia (Chantanachookin *et al*., 1993; Lightner, 1996).

2.2.5. Aquatic animal reservoirs of infection

YHV1 was detected by reverse-transcription polymerase chain reaction (RT-PCR) in clinically normal wild *P. stylirostris* collected for surveillance purposes in the Gulf of California in 2003 (Castro-Longoria *et al*., 2008). The infectious nature of the YHV1 detected was confirmed by experimental infections. There is also evidence that YHV1 can persist in survivors of experimental infection (Longyant *et al.*, 2005; 2006).

2.2.6. Vectors

There are no known vectors of YHV1.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In farmed *P. monodon*, YHV disease can cause up to 100% mortality within 3–5 days of the first appearance of clinical signs (Chantanachookin *et al.,* 1993). Mortalities can be induced by experimental exposure of *P. monodon* to YHV1 (Oanh *et al.,* 2011).

2.3.2. Clinical signs, including behavioural changes

Shrimp from late postlarvae (PL) stages onwards can be infected experimentally with YHV1. In cultured shrimp, infection can result in mass mortality occurring, usually in early to late juvenile stages. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax. However, these disease features are not particularly distinctive, and gross signs are not reliable even for preliminary diagnosis of YHV1.

Exceptionally high feeding activity followed by an abrupt cessation of feeding may occur within 2–4 days of the appearance of gross clinical signs of disease and mortality. Moribund shrimp may congregate at pond edges near the surface (Chantanachookin *et al.,* 1993).

2.3.3 Gross pathology

The yellow hepatopancreas of diseased shrimp may be exceptionally soft when compared with the brown hepatopancreas of a healthy shrimp (Chantanachookin *et al.,* 1993).

2.3.4. Modes of transmission and life cycle

YHV1 can be transmitted horizontally by ingestion of infected tissue, immersion in membrane-filtered tissue extracts, or by cohabitation with infected shrimp (Walker & Sittidilokratna, 2008). YHV1 replicates in the cytoplasm of infected cells in which long filamentous pre-nucleocapsids are abundant and virions bud into cytoplasmic vesicles in densely packed paracrystalline arrays for egress at the cytoplasmic membrane (Chantanachookin *et al.,* 1993).

2.3.5. Environmental factors

Elevated virus infection levels accompanied by disease can be precipitated by physiological stress induced by sudden changes in pH or dissolved oxygen levels, or other environmental factors (Flegel *et al*., 1997).

2.3.6. Geographical distribution

YHV1 has been reported in South-East Asia (Walker *et al.*, 2001). YHV1 has also been detected in *P. stylirostris* and *P. vannamei* in the Americas (Castro-Longoria *et al.,* 2008; Sanchez-Barajas *et al.*, 2009).

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

None available.

2.4.2. Chemotherapy including blocking agents

No effective commercial anti-viral product is yet available.

2.4.3. Immunostimulation

A multi-target dsRNA for simultaneous inhibition of YHV1 and white spot syndrome virus demonstrated inhibition of the two viruses when administered to shrimp by injection (Chaimongkon *et al.,* 2020)

2.4.4. Breeding resistant strains

Not reported.

2.4.5. Inactivation methods

YHV1 can be inactivated by heating at 60°C for 15 minutes (Flegel *et al.*, 1995b). Little information is available on other inactivation methods but the virus appears to be susceptible to treatment with chlorine at 30 parts per million (0.03 mg ml^{-1}) (Flegel *et al.,* 1997).

2.4.6. Disinfection of eggs and larvae

Not reported.

2.4.7. General husbandry

The focus is to exclude YHV1 from entering production systems; for example, by using specific pathogen-free (SPF) stock, batch testing stock and biosecurity measures to reduce entry into culture systems.

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

For diagnosis during a disease outbreak, moribund shrimp collected from pond edges are the preferred source of material for examination. Apparently healthy shrimp should also be collected from the same ponds. For surveillance in populations of apparently healthy shrimp, life stages from PL stage 15 onwards can provide tissue sources useful for testing.

3.2. Selection of organs or tissues

In moribund shrimp suspected to be infected with YHV1, pleopods, gill and lymphoid organ are the most suitable sample tissues. For screening or surveillance of juvenile or adult shrimp that appear grossly normal, pleopods or gills are preferred.

3.3. Samples or tissues not suitable for pathogen detection

Not determined.

3.4. Non-lethal sampling

Haemolymph can be used for non-lethal sampling.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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 bioassay

The success of bioassay 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.

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 (absolute) 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 can be frozen at –20°C or below for 1 month or less; for long-term storage. –80°C is recommended.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. 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 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, however, larger specimens should be processed and tested individually. Small life stages 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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 RT-PCR												
Conventional RT-PCR	++	++	++	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									++	+++	+++	1
In-situ hybridisation						++	++	1				
Bioassay					+	+	+	1				
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 WOAH 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.

[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

Fix the cephalothorax tissues of moribund shrimp suspected to be affected by YHV1 in Davidson's fixative, prepare tissue sections and stain with Meyer's haematoxylin and eosin (H&E) using standard histological procedures (Lightner, 1996). Examine tissues of ectodermal and mesodermal origin by light microscopy for the presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 µm in diameter or smaller (Chantanachookin *et al.,* 1993). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly informative.

Lymphoid organ spheroids are commonly observed in healthy *P. monodon* chronically infected with YHV1 or GAV and lymphoid organ necrosis often accompanies disease (Spann *et al.*, 1997). However, spheroid formation and structural degeneration of lymphoid organ tissue also result from infection by other shrimp viruses (Lightner, 1996).

4.3. Cell culture for isolation

Although primary shrimp cell culture methods are available, they are not recommended to isolate and identify YHV1 as a routine diagnostic method. No continuous cell lines suitable for YHV1 culture are available.

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

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

Not available.

4.4.2. Conventional RT-PCR

Three RT-PCR protocols are described. For all conventional RT-PCR protocols, assignment to YHV genotype 1 can be achieved by nucleotide sequence analysis of the RT-PCR amplicon. Reference sequences for YHV1 include:

Protocol 1 is a 1-step RT-PCR that can be used to detect YHV1 in affected shrimp. The protocol is as described by Mohr *et al.* (2015) and adapted from Wongteerasupaya *et al.* (1997). This protocol will detect YHV1 but not GAV or any of the other genotypes currently recognised.

Protocol 2 is a more sensitive multiplex nested RT-PCR that can be used to differentiate YHV1 from GAV. The protocol is as described by Mohr *et al*. (2015) and adapted from Cowley *et al.* (2004). The first stage of the multiplex nested RT-PCR (primary RT-PCR) was designed to detect YHV1 and GAV but has been reported to also detect YHV7 (Mohr *et al*., 2015). Both the primary RT-PCR and the nested PCR detected the novel YHV genotype from China (People's Rep. of) (Liu *et al*., 2014). In the second PCR step, a 277 bp product indicates detection of YHV and a 406 bp product indicates detection of GAV. The presence of both 406 bp and 277 bp products indicates a dual infection with GAV and YHV1. The nested PCR can be run as two separate assays specific for YHV1 or GAV by omitting either the G6 or Y3 primer, respectively. **NOTE:** Due to reported problems with primer specificity for some emerging strains, all PCR products generated using protocol 2 should be sequenced to confirm the virus genotype.

Protocol 3 is a multiplex nested RT-PCR protocol that can be used for screening shrimp for any of the seven genotypes of the yellow head complex of viruses. The protocol is as described by Mohr *et al*. (2015) and adapted from Wijegoonawardane *et al.* (2008b). Two primers were designed to each site, one accommodating sequence variations

amongst YHV1 isolates and the other variations amongst isolates of the other genotypes (Wijegoonawardane *et al.*, 2008b). It is not known whether this assay will detect the YHV genotype recently detected in China (People's Rep. of) (Liu *et al*., 2014).

Primer sequences

Pathogen / target gene	Primer (5'–3')	Concentration	Cycling parameters
Protocol 1 (Wongteerasupaya *et al.,* 1997; GenBank Accession No.: ??; amplicon size: 135 bp)			
YHV1 / ORF1b	10F: CCG-CTA-ATT-TCA-AAA-ACT-ACG 144R: AAG-GTG-TTA-TGT-CGA-GGA-AGT	180 nM 180 nM	40 cycles of 94°C for 30sec, 58°C for 45 sec, 68°C for 45 sec,
Protocol 2 (Cowley *et al.,* 2004; GenBank Accession No.: ??)			
YHV1 and GAV / ORF1b	Primary (Amplicon size: 794 bp) GY1: 5GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG GY4: GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG Nested for detection of YHV1 (Amplicon size: 277 bp) GY2: CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA Y3: ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT Nested for detection of GAV (Amplicon size: 406 bp) GY2: CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA G6: GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT	180 nM 180 nM 360 nM 360 nM 360 nM 360 nM	35 cycles of 95°C for 30 sec, 66°C for 30 sec, and 68 C for 45 sec
Protocol 3 (Wijegoonawardane *et al.,* 2008b; GenBank Accession No.: ??)			
YHV1 to YHV7 / ORF1b	**Primary (amplicon size: 359 bp)** **YC-F1ab pool:** **ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC** **ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC** **YC-R1ab pool:** **TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC** **TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC** **Nested (amplicon size: 147 bp)** **YC-F2ab pool:** **CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA** **CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA** **YC-R2ab pool:** **RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT** **GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT** **Mixed base codes: R(AG), Y(CT), M(AC), K(GT), S(GC), W(AT), H(ACT), B(GCT), V(AGC), D(AGT), N(AGCT).**	**180 nM** **180 nM** **180 nM** **180 nM** **180 nM** **180 nM** **180 nM** **180 nM**	**35 cycles of 94°C for 45 sec, 60°C for 45 sec, 68°C for 45 sec,** **35 cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 45 sec;**

The primer contains a mismatch for GAV but is specific for YHV1. The mismatch can cause false-positives with GAV in the nested PCR of protocol 2 where GAV generates an amplicon of very similar size to the expected size of the YHV1 amplicon. For GAV, the 7th base from left (T) is substituted for C so that the primer sequence for GAV should be 5'-CAT-CTG-CCC-AGA-AGG-CGT-CTA-TGA-3', according to the sequence data of the GAV genome (database accession numbers: NC_010306.1 and AF227196.2).

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

Not available.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

The protocol of Tang *et al.* (2002) is suitable for detecting YHV1 or GAV (Tang & Lightner, 1999). To preserve viral RNA accessibility, fix tissues sampled from live shrimp in neutral-buffered, modified Davidson's fixative without acetic acid (RF-fixative) (Hasson *et al.*, 1997). To achieve good tissue preservation whilst also preserving RNA accessibility, normal Davidson's fixative can be used as long as the fixation time is limited to 24 hours (maximum of 48 hours). Detailed methods can be found in Tang *et al.* (2002) YHV-infected cells give a blue to purple-black colour against the brown counter stain. Positive controls of YHV-infected tissue and negative controls of uninfected shrimp tissue should be included. The digoxigenin-labelled DNA probe can be prepared by PCR labelling using the following primers:

YHV1051F: 5'-ACA-TCT-GTC-CAG-AAG-GCG-TC-3'

YHV1051R: 5'-GGG-GGT-GTA-GAG-GGA-GAG-AG-3'

4.7. Immunohistochemistry

Not applicable.

4.8. Bioassay

The bioassay procedure is based on that described by Spann *et al.* (1997), but similar procedures have been described by several other authors (e.g. Lu *et al.,* 1994). The bioassay should be conducted in susceptible shrimp that have been determined to be free from YHV complex viruses.

Suspect YHV1-infected samples should be maintained at 4°C or on ice. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at –80°C or in liquid nitrogen until required. Viral inoculum should be prepared as described by Spann *et al.* (1997).

Juvenile shrimp of a known susceptible species are injected with viral inoculum. Negative controls (buffer injected) and positive controls (known YHV1 positive material) treatment groups are required. Shrimp should be maintained separately to prevent cross-contamination between treatments. Observe the shrimp and record mortalities for at least 21 days or until the test and positive control groups reach 100% mortality.

4.9. Antibody- or antigen-based detection methods (ELISA, etc.)

None has been successfully developed.

4.10. Other methods

None at present.

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

Nested RT-PCR (Protocol 3) is recommended for demonstrating freedom from YHV1 in an apparently healthy populations. Sequencing of any amplified PCR products is required to determine the YHV genotype. Two-step PCR negative results are required for YHV1.

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. If a Competent Authority does not have the capability 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 confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status[1]

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 YHV1 shall be suspected if the following criterion is met:

i) Positive result by a recommended RT-PCR detection test

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with YHV1 is considered to be confirmed if the following criterion is met:

i) A positive result by conventional RT-PCR and identification of YHV1 by sequence analysis of the amplicon

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 YHV1 shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs consistent with YHV1 infection

ii) Histopathology consistent with YHV1 infection

iii) Positive result by conventional RT-PCR

iv) Positive result by ISH

v) Positive result by bioassay

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with YHV1 is considered to be confirmed if the following criterion is met:

i) A positive result from each of two RT-PCR methods targeting non-overlapping parts of the genome followed by sequence analysis of the amplicons to identify YHV1

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with YHV1 are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). This information can be used for the design of surveys for infection with YHV1, 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

[1] For example transboundary commodities.

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.

7. References

CASTRO-LONGORIA R., QUINTERO-ARREDONDO N., GRIJALVA-CHON J.M. & RAMOS-PAREDES J. (2008). Detection of the yellow-head virus (YHV) in wild blue shrimp, *Penaeus stylirostris*, from the Gulf of California and its experimental transmission to the Pacific white shrimp, *Penaeus vannamei*. *J. Fish Dis.,* **31**, 953–956.

CHAIMONGKON D., ASSAVALAPSAKUL W., PANYIM S. & ATTASART P. (2020). A multi-target dsRNA for simultaneous inhibition of yellow head virus and white spot syndrome virus in shrimp. *J. Biotechnol*., **321**, 48–56. doi: 10.1016/j.jbiotec.2020.06.022. Epub 2020 Jun 29. PMID: 32615142.

CHANTANACHOOKIN C., BOONYARATPALIN S., KASORNCHANDRA J., DIREKBUSARAKOM S., AEKPANITHANPONG U., SUPAMATTAYA K., SRIURAITANA S. & FLEGEL T.W. (1993). Histology and ultrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow-head disease. *Dis. Aquat. Org.*, **17**, 145–157.

COWLEY J.A., CADOGAN L.C., WONGTEERASUPAYA C., HODGSON R.A.J., BOONSAENG V. & WALKER P.J. (2004). Multiplex RT-nested PCR differentiation of gill-associated virus (Australia) from yellow head virus (Thailand) of Penaeus monodon. J. *Virol. Methods*, **117**, 49–59. doi: 10.1016/j.jviromet.2003.11.018.

COWLEY J.A., HALL M.R., CADOGAN L.C., SPANN K.M. & WALKER P.J. (2002). Vertical transmission of gill-associated virus (GAV) in the black tiger prawn *Penaeus monodon. Dis. Aquat. Org*., **50**, 95–104.

DONG X., LIU S., ZHU L., WAN X., LIU Q., QIU L., ZOU P., ZHANG Q. & HUANG J. (2017) Complete genome sequence of an isolate of a novel genotype of yellow head virus from *Fenneropenaeus chinensis* indigenous in China. *Arch Virol* **162**, 1149-1152.

DURAND S.V., TANG K.F.J. & LIGHTNER D.V. (2000). Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus*. J. Aquatic Anim. Health,* **12**, 128–135.

FLEGEL T.W., BOONYARATPALIN S. & WITHYACHUMNARNKUL B. (1997). Current status of research on yellow-head virus and white-spot virus in Thailand. *In:* Diseases in Asian Aquaculture III, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, the Philippines, 285–296.

FLEGEL T.W., FEGAN D.F. & SRIURAIRATANA S. (1995a). Environmental control of infectious shrimp diseases in Thailand. *In:* Diseases in Asian Aquaculture II, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Asian Fisheries Society, Manila, the Philippines, 65–79.

FLEGEL T.W., SRIURAIRATANA S., WONGTERRASUPAYA C., BOONSAENG V., PANYIM S. & WITHYACHUMNARNKUL B. (1995b). Progress in characterization and control of yellow-head virus of *Penaeus monodon*. *In:* Swimming Through Troubled Water, Proceedings of the Special Session on Shrimp Farming, Aquaculture ’95, Browdy C.L. & Hopkins J.S., eds. World Aquaculture Society, Baton Rouge, USA, 76–83.

HASSON K.W., HASSON J., AUBERT H., REDMAN R.M. & LIGHTNER D.V. (1997). A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological assay using cDNA probes. *J. Virol. Methods,* **66**, 227–236.

KHONGPRADIT R., KASORNCHANDRA J. & BOONYARATALIN S. (1995). Susceptibility of the postlarval stages of black tiger shrimp (*Penaeus monodon*) to yellow-head baculovirus (YBV). *In:* Diseases in Asian Aquaculture II, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Asian Fisheries Society, Manila, the Philippines, p. 6.

LIGHTNER D.V. (ED.) (1996). Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp. World Aquaculture Society, Baton Rouge, USA.

LIGHTNER D.V., HASSON K. W., WHITE B. L & REMAN R. M. (1998) Experimental infection of western hemisphere penaeid shrimp with Asian white spot syndrome virus and Asian yellow head virus *J. Aquatic Anim. Health*, **10,** 271-281

LIU Q., HUANG J., YANG H.-L., YANG B., WANG H.-L., WANG Q.-T., LIU F. & ZHANG Q.-L. (2014) Detection of a new genotype of yellow-head virus in farmed shrimp suspicious of EMS/AHPNS infection. *Oceanol. Limnol. Sin.*, **45**, 703–709.

LONGYANT S., SATTAMAN S., CHAIVISUTHANGKURA P., RUKPRATANPORN S., SITHIGORNGUL W. & SITHIGORNGUL P. (2006). Experimental infection of some penaeid shrimps and crabs by yellow head virus (YHV). *Aquaculture,* **257**, 83–91.

LONGYANT S., SITHIGORNGUL P., CHAIVISUTHANGKURA P., RUKPRATANPORN S., SITHIGORNGUL W. & MENASVETA P. (2005). Differences in the susceptibility of palaemonid shrimp species to yellow head virus (YHV) infection. *Dis. Aquat. Org*., **64**, 5–12.

LU Y., TAPAY L.M., BROCK J.A. & LOH P.C. (1994). Infection of the yellow head baculo-like virus (YBV) in two species of penaeid shrimp *Penaeus stylirostris* (Stimpson) and *Penaeus vannamei* (Boone). *J. Fish Dis.*, **17**, 649–656.

MCCOLL K.A., SLATER J., JEYASEKARAN G., HYATT A.D. & CRANE M.ST.J. (2004). Detection of white spot syndrome virus and yellowhead virus in prawns imported into Australia. *Aust. Vet. J.,* **82**, 69–74.

MOHR P.G., MOODY N.J.G, HOAD J., WILLIAMS L.M., BOWATER R.O., CUMMINS D.M., COWLEY J.A. & CRANE M.ST.J. (2015). New yellow head virus genotype (YHV7) in giant tiger shrimp *Penaeus monodon* indigenous to northern Australia. *Dis. Aquat. Org*., **115**, 263–268.

MOODY N. ET AL (IN PREPARATION). Development of a real-time and conventional PCR assays for the detection of yellow head virus genotype 1.

NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (1998). The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. *Aquaculture,* **160**, 19–30.

OANH D.T., VAN HULTEN M.C., COWLEY J.A. & WALKER P.J. (2011). Pathogenicity of gill-associated virus and Mourilyan virus during mixed infections of black tiger shrimp (*Penaeus monodon*). *J. Gen. Virol*., **92**, 893–901.

SANCHEZ-BARAJAS M., LINAN-CABELLO M.A. & MENA-HERRERA A. (2009). Detection of yellow-head disease in intensive freshwater production systems of *Litopenaeus vannamei*. *Aquacult. Int.,* **17,** 101–112.

SPANN K.M., COWLEY J.A., WALKER P.J. & LESTER R.J.G. (1997). A yellow-head-like virus from *Penaeus monodon* cultured in Australia. *Dis. Aquat. Org.*, **31**, 169–179.

TANG K.F.J. & LIGHTNER D.V. (1999). A yellow head virus gene probe: nucleotide sequence and application for *in situ* hybridization. *Dis. Aquat. Org*., **35**, 165–173.

TANG K.F.J., SPANN K.M., OWENS L. & LIGHTNER D.V. (2002). *In situ* detection of Australian gill-associated virus with a yellow head virus gene probe. *Aquaculture,* **205**, 1–5.

WALKER P.J., COWLEY J.A. SPANN K.M., HODGSON R.A.J. HALL M.R & WITHYACHUMNARNKUL B. (2001). Yellow head complex viruses: Transmission cycles and topographical distribution in the Asia-Pacific Region. *In:* The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001, Browdy C.L. & Jory D.E., eds. World Aquaculture Society, Baton Rouge, LA, USA, 292–302.

Walker P.J. & Sittidilokratna N. (2008). Yellow Head Virus. *In:* Encyclopedia of Virology, third edition. Academic Press, 476–483. https://doi.org/10.1016/B978-012374410-4.00779-2

WIJEGOONAWARDANE P.K.M., COWLEY J.A., SITTIDILOKRATNA, N., PHETCHAMPAI, N., COWLEY, J.A., GUDKOVS, N. & WALKER P.J. (2009). Homologous genetic recombination in the yellow head complex of nidoviruses infecting *Penaeus monodon shrimp*. *Virology* doi: 1016/j.virol.2009.04.015.

WIJEGOONAWARDANE P.K.M., COWLEY J.A., PHAN T., HODGSON R.A.J., NIELSEN L., KIATPATHOMCHAI W. & WALKER P.J. (2008a). Genetic diversity in the yellow head nidovirus complex. *Virology* **380**, 213–225.

WIJEGOONAWARDANE P.K.M., COWLEY J.A. & WALKER P.J. (2008b). Consensus RT-nested PCR to detect yellow head virus genotypes in penaeid shrimp. *J. Virol. Methods*, **153**, 168–175.

WONGTEERASUPAYA C., BOONSAENG V., PANYIM S., TASSANAKAJON A., WITHYACHUMNARNKUL B. & FLEGEL T.W. (1997). Detection of yellow-head virus (YHV) of *Penaeus monodon* by RT-PCR amplification. *Dis. Aquat. Org.,* **31**, 181–186.

WONGTEERASUPAYA C., SRIURAIRATANA S., VICKERS J.E., AKRAJAMORN A., BOONSAENG V., PANYIM S., TASSANAKAJON A., WITHYACHUMNARNKUL B. & FLEGEL T.W. (1995). Yellow-head virus of P*enaeus monodon* is an RNA virus. *Dis. Aquat. Org.,* **22**, 45–50.

*
* *

NB: There is a WOAH Reference Laboratory for infection with yellow head virus genotype 1 (please consult the WOAH web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on infection with yellow head virus genotype 1

NB: FIRST ADOPTED IN 1995 AS YELLOWHEAD DISEASE. MOST RECENT UPDATES ADOPTED IN 2019.

Annex 38. Item 11.2.1. – Section 2.2.1. and 2.2.2. of Chapter 2.4.5. Infection with *P. marinus*

CHAPTER 2.4.5.

INFECTION WITH *PERKINSUS MARINUS*

[…]

2.2. Host factors

2.2.1. Susceptible host species

~~Eastern oyster, *Crassostrea virginica;* Pacific oyster, *C. gigas;* suminoe oyster, *C. ariakensis;* mangrove oyster, *C. rhizophorae;* Cortez oyster, *C. corteziensis* (Andrews 1996; Calvo *et al.,* 1999; Calvo *et al.,* 2001; Villalba *et al.,* 2004; Cáceres-Martínez *et al.,* 2008); softshell clam, *Mya arenaria;* Baltic macoma, *Macoma balthica* (Dungan *et al.,* 2007).~~

Species that fulfil the criteria for listing as susceptible to infection with *Perkinsus marinus* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: American cupped oyster (*Crassostrea virginica*), Ariake cupped oyster (*Magallana* [Syn. *Crassostrea*] *ariakensis*), Cortez oyster (*Crassostrea corteziensis*) and palmate oyster (*Saccostrea palmula*).

2.2.2. ~~Susceptible stages of the host~~ Species with incomplete evidence for susceptibility

~~All stages after settlement are susceptible.~~

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *P. marinus* according to Chapter 1.5. of the *Aquatic Code* are: Gasar cupped oyster (*Crassostrea tulipa*), mangrove cupped oyster (*Crassostrea rhizophorae),* and Pacific cupped oyster (*Magallana* [Syn. *Crassostrea*] *gigas*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Columbia black oyster (*Crassostrea columbiensis*), soft shell clam (*Mya arenaria*), and stone oyster (*Striostrea prismatica*).

[…]

Annex 39. Item 14.1.2. – Innocreate Bioscience WSSV RP Rapid Test Kit

WOAH Procedure for Registration of Diagnostic Kits
Validation Studies Abstract

Name of the diagnostic kit: Innocreate Bioscience WSSV RP Rapid Test Kit
Manufacturer: Innocreate Bioscience Co., Ltd.
Procedure /Approval number: *082132*
Date of Registration: May 2023
Disease: Infection with white spot syndrome virus in shrimp
Pathogen Agent: Whispovirus, White Spot Syndrome Virus (WSSV)
Type of Assay: Lateral flow immunochromatographic assay (LFIA)

Purpose of Assay

The Innocreate Bioscience WSSV RP Rapid Test Kit is a qualitative detection kit for WSSV infection in shrimp. The lateral flow immunoassay device is designed for the following purposes:

1. Field based confirmatory diagnosis of clinical cases (includes confirmation of suspect cases and a positive screening test)
2. Estimate the prevalence of infection to facilitate risk analysis in production system shrimp farms to aid in management practices. (The test kit should not be used to estimate prevalence in broodstock or post larvae shrimp for risk analysis prior to translocation to other farms or across borders).
3. For use in conjunction with other tests or diagnostic procedures as an aid in the diagnosis or other clinical or epidemiological assessments.

Species and Specimens： 2-3 small pieces of shrimp gill.

1. Information on the kit

Please refer to the kit insert available on the WOAH Registry web page or contact manufacturer at:
Website: https://www.innocreatebio.com/
Email: info@innocreatebio.com

2. Summary of validation studies

Analytical specificity

Conclusion:

AHPND-caused Vibrio parahaemolyticus, Infectious hypodermal and hematopoietic necrosis virus (IHHNV), EHP (*Enterocytozoon hepatopenaei*), Monodon Baculovirus (MBV), Yellow head virus (YHV) and Taura syndrome virus (TSV) infected shrimp were tested. All infected samples showed negative results.

Analytical sensitivity

Conclusion:

Serial dilution of recombinant WSSV target protein with homogenized shrimp tissues was used to estimate the limit of detection (LOD). The LOD was estimated to be 0.4 ng/ test.

Repeatability

***Conclusion*:**

Within run repeatability was assessed using quadruplicates of 6 samples with various levels of infection, which were tested by the same operator on 5 separate days. Between run repeatability was performed by testing of the 6 samples by 3 different operators using 3 different lots of kits on 5 days. The results for both the within- and the between-runs were reproducible with kappa values of 1.0.

Diagnostic characteristics

Threshold determination and Diagnostic sensitivity (DSe) and specificity (DSp) estimates:

Threshold determination:

Innocreate Bioscience WSSV RP Rapid Test Kit is an immunochromatographic assay designed for the qualitative detection of WSSV infection in shrimp. The pink purple band needs to be observed at both the test line (T) and control line (C), to indicate that the shrimp was infected with WSSV. If the pink purple band appears only on the control line (C), this indicates that there is no infection of WSSV or light infection beyond the sensitivity of the kit. The threshold is determined by the analytical sensitivity as 0.4 ng of the target protein.

Diagnostic sensitivity (DSe) and specificity (DSp) estimates:

1. Diagnostic sensitivity and specificity estimates – with defined reference animals

Two hundred and fifty two specific pathogen free shrimp were either used as negative controls (n=105) or challenged (n= 147) with an injection of 100 ul of WSSV infected hemolymph to determine the diagnostic sensitivity and specificity in samples from defined reference animals. Of the 147 challenged and WOAH TaqMan real time PCR (Durand & Lightner, 2002) positive samples, 125 tested positive by the WSSV RP Rapid Test Kit, while 22 samples were considered false-negative. The infected level of these 22 samples was considered very light (Ct>32.5). From the 105 WOAH TaqMan real time PCR Negative non-challenged samples, no false-positive results were observed.

Reference Animal:

Innocreate Bioscience WSSV RP Rapid Test Kit		**Target Species/Specimen: Gill**	
		WOAH TaqMan real time PCR method	
		Ct<32.5 considered positive	**Ct<40 considered positive**
Diagnostic sensitivity	N DSe CI	*(126)* *(99.21%)* *(99.66% - 99.98%)*	*(147)* *(85.03%)* *(78.22% - 90.38%)*
Diagnostic specificity	N DSp	*(105)* *(100%)*	*(105)* *(100%)*

	CI	*(96.55-100%)*	*(96.55-100%)*

2. **Diagnostic sensitivity and specificity estimates – Production shrimps**

A total of 465 shrimp from 4 batches of production systems were tested, of which 45 of 465 shrimp were classified as symptomatic, and 64 of 465 were classified as positive (Ct<40) on qPCR testing by the WOAH TaqMan real time PCR method (Durand & Lightner, 2002).

Compared with the WOAH TaqMan real time PCR method, the overall DSe of the WSSV RP Rapid Test was 92.50% when Ct<32.50 is considered as positive, 84.00% when Ct<36 is considered as positive, or 65.62 % if Ct<40 is considered as positive; DSp was 100%.

As for diagnostic performance in symptomatic production system shrimp, DSe was 93.33%, and DSp was 100% when using Ct<40 as the cut-off. Positive predictive value (PPV) and Negative predictive value (NPV) were 100% and 99.3%. For samples with Ct<32.5 (Moderate to High infection, >≈100 copies), the DSe was 92.50%. Overall the Innocreate Bioscience WSSV Rapid test kit shows high agreement on diagnosis.

Production shrimps:

Innocreate Bioscience WSSV RP Rapid Test Kit		**Target Species/Specimen: Gill**			
		WOAH TaqMan real time PCR method			
		Symptomatic	**Ct<32.5 considered positive**	**Ct<36 considered positive**	**Ct<40 considered positive**
Diagnostic sensitivity	N DSe CI	*(45)* *(93.33%)* *(95.66%-99.98%)*	*(40)* *(92.50%)* *(79.61%-98.43%)*	*(50)* *(84.00%)* *(95.66%-99.98%)*	*(64)* *(65.62%)* *(52.70%-77.05%)*
Diagnostic specificity	N DSp CI	*(420)* *(100%)* *(99.13%-100%)*	*(425)* *(100%)* *(99.14%-100%)*	*(415)* *(100%)* *(70.89%-92.83%)*	*(401)* *(100%)* *(99.08%-100%)*

Conclusion:

The WSSV Rapid test kit is fit-for-its defined purposes and demonstrates high overall sensitivity in identifying moderate and heavy levels of WSSV infection or when used on samples from symptomatic shrimp, and the test has very high specificity. The high PPV and NPV of the assay and fast turn-around time (15-30 min onsite vs. >4hrs plus shipping time) make it a powerful tool for identifying potential outbreaks. We recommend users apply the test to shrimp, that present with behavioural changes (lethargy, decreased or absence of feed consumption, and abnormal swimming behaviours such as slow swimming, swimming on side, swimming near the water surface, or gathering around edges of rearing units) either on a regular basis or when environmental stress, such as rapid changes in salinity, or when suspecting a WSSV outbreak.

Reproducibility

Analytical reproducibility

Conclusion:

Analytical reproducibility evaluation was carried out by 2 laboratories. Six samples with various levels of infection (2 Light, 2 Moderate, 1 Heavy and 1 Negative infection) as determined by the WOAH TaqMan real time PCR method (Durand & Lightner, 2002) were selected and provided 'blinded' to the 2 laboratories. Testing was repeated on 4 occasions and a Kappa value was calculated on the results of the 24 repeated assays. There was no misclassification in all assays (20 positive and 4 negative). The agreement of the two methods was 100%, and Kappa =1.0.

Diagnostic reproducibility

Diagnostic reproducibility was conducted by the five laboratories in Taiwan and Thailand, including one WOAH reference laboratory. The test panel consisted of 25 samples (with various virus-infected levels, comprising 5 'known' samples (3 positive with target protein concentrations of 1.6, 0.8 or 0.4 ng and 2 negative) and 20 unknown 'blind' samples. The participating laboratories followed the procedures described in the instruction manual of the Innocreate Bioscience WSSV RP Rapid Test Kit.

***Conclusion*:**

Samples were analysed by each of the 5 laboratories using Innocreate Bioscience WSSV RP Rapid Test Kit. Results show high reproducibility. The 5 laboratories showed 100% agreement on the 5 known samples. Four of the 5 laboratories showed 100% agreement on all of the 'blinded' samples, whilst results from one laboratory showed a slight discrepancy for one sample. Chi-square test for homogeneity was conducted to analyse the experimental results from the five labs. Independent Chi-squared test p-value = 0.998 (Hsu et al., 2022).

Reference

1) Durand, S., & Lightner, D. V. (2002). Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *Journal of Fish Diseases*, *25*(7), 381-389.
2) Hsu, J. C.-K., Hsu, T.-K., Kannan, J., Wang, H.-C., Tassanakajon, A., & Chen, L.-L. (2022). Diagnostic performance of a Rapid Test Kit for white spot syndrome virus (WSSV). *Aquaculture*, *558*, 738379. https://doi.org/https://doi.org/10.1016/j.aquaculture.2022.738379