Organisation Mondiale de la Santé Animale

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REPORT OF THE MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 15–19 February 2016

EU comment

The EU would like to commend the OIE Aquatic Animal Health Standards Commission for its work and for having taken into consideration EU comments on the Aquatic Code and Manual submitted previously.

A number of general comments on this report of the February 2016 meeting of the Aquatic Animals Commission as well as the intended positions of the EU on the draft Aquatic Code and Manual chapters proposed for adoption at the 84th OIE General Session are inserted in the text below, while specific comments are inserted in the text of the respective annexes of the report.

The EU would like to stress again its continued commitment to participate in the work of the OIE and to offer all technical support needed by the Aquatic Animals Commission and its ad hoc groups for future work on the Aquatic Code and Manual.

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at OIE Headquarters in Paris from 15 to 19 February 2016. The list of participants is attached as Annex 1.

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts circulated after the Commission's October 2015 meeting: Argentina, Australia, Canada, Chile, China (People's Rep. of), Chinese Taipei, Japan, Mexico, New Zealand, Norway, Panama, Switzerland, Thailand, the United States of America (USA) and the Member States of the European Union (EU).

The Aquatic Animals Commission reviewed comments that Member Countries had submitted prior to 15 January 2016 and amended texts in the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (the *Aquatic Manual*) where appropriate. The amendments are shown in the usual manner by 'double underline' and 'strikethrough' and may be found in the Annexes to this report. The amendments made at the February 2016 meeting are highlighted with a coloured background in order to distinguish them from those made at the October 2015 meeting.

All Member Countries' comments were considered by the Aquatic Animals Commission. However, the Commission was not able to prepare a detailed explanation of the reasons for accepting or not accepting every proposal received.

The Commission encourages Member Countries to refer to previous reports when preparing comments on longstanding issues. The Commission also draws the attention of Member Countries to the reports of *ad hoc* Groups, which include important information and encourages Member Countries to review these reports together with the report of the Commission, where relevant.

The table below summarises the texts as presented in the Annexes. Member Countries should note that texts in Annexes 3 to 9 are proposed for adoption at the 84th General Session in May 2016; Annexes 10 to 28 are presented for Member Countries' comments; Annexes 29 to 31 are presented for information.

The Commission strongly encourages Member Countries to participate in the development of the OIE's international standards by submitting comments on this report. Comments should be submitted as specific proposed text changes, supported by a scientific rationale. Proposed deletions should be indicated in 'strikethrough' and proposed additions with 'double underline'. Member Countries should not use the automatic 'track-changes' function provided by word processing software as such changes are lost in the process of collating Member Countries' submissions into the Commission's working documents.

Comments on Annexes 10 to 28 of this report must reach OIE Headquarters by the **5th August 2016** to be considered at the September 2016 meeting of the Aquatic Animals Commission.

All comments should be sent to the OIE International Trade Department at: trade.dept@oie.int

Texts proposed for adoption:	Annex number
Aquatic Code:	
Glossary Part A	Annex 3
Revisions to Articles 1.4.8., 1.5.2., 2.1.4., 4.2.3. and 4.6.3.	Annex 4
Notification of diseases and provision of epidemiological information (Chapter 1.1.)	Annex 5
General recommendations on disinfection (Chapter 4.3.)	Annex 6
General obligations related to certification (Chapter 5.1.)	Annex 7
Infection with yellow head virus (Chapter 9.2.)	Annex 8
Aquatic Manual:	
Infection with yellow head virus genotype 1 (Chapter 2.2.8.)	Annex 9
Texts for Member Countries' comment:	**Annex number**
Aquatic Code:	
Glossary Part B	Annex 10
Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)	Annex 11 A and B
Diseases listed by the OIE (Chapter 1.3.)	Annex 12
Acute hepatopancreatic necrosis disease (new Chapter 9.X.)	Annex 13
Revised Article X.X.8. (clean text and track changes text)	Annex 14 A and B
Chapter 4.4. Recommendations for disinfection of salmonid eggs	Annex 15
Crayfish plague *(Aphanomyces astaci)* (Chapter 9.1.)	Annex 16
Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)	Annex 17
Infectious myonecrosis (Chapter 9.4.)	Annex 18
Necrotising hepatopancreatitis (Chapter 9.5.)	Annex 19
Taura syndrome (Chapter 9.6.)	Annex 20
White tail disease (Chapter 9.8.)	Annex 21
Aquatic Manual:	
Acute hepatopancreatic necrosis disease (new draft Chapter 2.2.X.)	Annex 22

Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.)	Annex 23
Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.2.)	Annex 24
Infectious myonecrosis (Chapter 2.2.3.)	Annex 25
Necrotising hepatopancreatitis (Chapter 9 2.2.4.)	Annex 26
Taura syndrome (Chapter 2.2.5.)	Annex 27
White tail disease (Chapter 9.8. and 2.2.7.)	Annex 28
Annexes for Member Countries' information:	
Assessment for listing for *Batrachochytrium salamandrivorans*	Annex 29
OIE *ad hoc* Group report on susceptibility of crustacean species to infection with OIE listed diseases (October 2015)	Annex 30
Aquatic Animal Health Standards Commission Work Plan for 2016/2017	Annex 31

A. MEETING WITH THE DIRECTOR GENERAL

The Aquatic Animals Commission met with Dr Monique Eloit, Director General, on 17 February 2016. Dr Eloit welcomed the Commission members and thanked them for their support and commitment to achieving OIE objectives.

Among other matters, Dr Eloit discussed the implementation of the key objectives of the sixth strategic plan, and how that may impact the work of the Commission.

Dr Eloit also noted the importance of OIE procedures for standards development. She explained that key steps to be implemented in the near future, concerning the Aquatic Animals Commission and other Specialist Commissions, include:

1)   the creation of a single department to serve as the Secretariat of all four Specialist Commissions with the aim of facilitating closer collaboration among the Commissions, and easier document sharing through common support services;

2)   the development of an internal staff training programme to strengthen the skills of this scientific secretariat;

3)   refurbishment of the OIE website to provide easier access to various technical meeting reports, and improve the transparency of OIE work in general to enhance Member Countries' participation in standard development.

Dr Eloit also explained the plan to improve the election process for membership of the Specialist Commissions. The aim is to better inform the voting Delegates on the scientific expertise and credentials of candidates standing for election. In the context of strengthening scientific excellence, Dr Eloit also highlighted the need for closer and stronger relationships with relevant scientific communities, including new fields of science, and the next generation of scientists.

Dr Ingo Ernst, President of the Aquatic Animals Commission, thanked Dr Eloit for her support. He explained that the Aquatic Animals Commission is developing a work programme that takes into account Member Countries' comments and sets achievable goals within the Commission's 3 year term.

B ADOPTION OF THE AGENDA

The draft agenda circulated prior to the meeting was discussed, and several new agenda items were added. The adopted agenda of the meeting is attached as Annex 2.

C MEETING WITH THE PRESIDENT OF
THE TERRESTRIAL ANIMAL HEALTH STANDARDS COMMISSION

The President of the Terrestrial Animal Health Standards Commission (Code Commission) met with the President of the Aquatic Animals Commission on several occasions during the week when both Commissions where meeting, to discuss issues of mutual interest, notably:

− proposed new glossary definitions for OIE Standard and OIE Guideline;

− proposed revised Chapters 1.1. of the *Aquatic* and *Terrestrial Codes*;

− proposed revised Chapters 1.2. of the *Aquatic* and *Terrestrial Codes*;

− proposed restructuring of Section 4 of the *Aquatic* and *Terrestrial Codes*;

− proposed development of a Guidance document for *ad hoc* Groups in the application of the listing criteria.

The Aquatic Animals Commission agreed that the alignment of the Aquatic Animals Commission and Code Commission meetings to enable the Presidents to meet, whilst each Commission was meeting, was a good initiative and should be continued in the future to facilitate the harmonisation of relevant chapters when under review by the respective Commissions.

D AQUATIC ANIMAL HEALTH CODE – EXAMINATION OF MEMBER COUNTRIES COMMENTS AND WORK OF RELEVANT AD HOC GROUPS

Item 1 General comments

Comments were received from Chile and Switzerland.

In response to a Member Country's comment regarding the transit of commodities not listed in Article X.X.3. of disease-specific chapters (importation or transit of aquatic animals and aquatic animal products for any purpose), the Aquatic Animals Commission reminded Member Countries that this is addressed in points 2 and 3 of Article X.X.3.

The Aquatic Animals Commission noted some Member Countries' comments to change the generic names for some penaeid shrimp species. The Commission agreed to investigate the issue further and discuss it at its next meeting.

In line with a recommendation being made by the Code Commission, the Aquatic Animals Commission agreed that Delegates be notified when the new edition of the *Aquatic Code* is uploaded onto the OIE website and recommended that OIE Headquarters send a letter to Delegates to notify them of this. The Code Commission also suggested that the Delegates be provided with a list of chapters that had been amended.

In line with a recommendation being made by the Code Commission, the Aquatic Animals Commission agreed with a Member Country's request that the convention of strike out and underline for text amendments in revised draft chapters be applied to whole words rather than crossing out and adding syllables, to facilitate Member Countries' review.

Item 2 Glossary

Comments were received from China (People's Rep. of), EU, Japan, Mexico, New Zealand, Norway, Thailand and the United States of America.

The Aquatic Animals Commission considered Member Countries' comments and amendments being proposed by the Code Commission to relevant definitions in the Glossary in the *Terrestrial Code*. In addition, the President of the Aquatic Animals Commission met with the President of the Code Commission on two occasions during Commission meetings to discuss harmonisation of definitions for OIE Standard and OIE Guideline between the two Codes.

OIE Standard

In response to a Member Country's comment, the Aquatic Animals Commission re-iterated that both an OIE Standard and an OIE Guideline may include recommendations, and that the definition of an OIE Standard is intended to distinguish standards by the process of adoption of texts at the OIE General Session.

The President of the Aquatic Animals Commission and the President of the Code Commission discussed edits proposed by each of the Commissions. The Commissions agreed on several edits that ensured consistency of the definitions proposed for the *Aquatic Code* and *Terrestrial Code*.

The Aquatic Animals Commission agreed that any definition in the glossary of the *Aquatic Code* must be suitable for the purposes of the *Aquatic Code* and provide clear understanding of relevant articles within the *Aquatic Code*. For this reason, the Aquatic Animals Commission agreed that the definition should refer to the *Aquatic Code* or *Aquatic Manual,* which are both defined terms. The President of the Aquatic Animals Commission met with the Terrestrial Code Commission to discuss whether common definitions for 'OIE Standard' and 'OIE Guideline' that refer to "Codes and Manuals" could be used in both the *Terrestrial* and *Aquatic Codes*. An alternative approach would be to refer to the respective defined terms '*Aquatic Code* or *Aquatic Manual'* and '*Terrestrial Code* or *Terrestrial Manual'* in the definitions for each Code. The relevance of these defined terms for purposes beyond of each of the Codes was also discussed. These issues are to be forwarded to the OIE Council. In the meantime slightly different definitions are presented in the Aquatic Animals Commission and Code Commission reports.

EU comment

The EU insists that the definitions of "OIE Standard" and "OIE Guideline" must be identical in all OIE Standards, i.e. in the Terrestrial and Aquatic Codes and Manuals. Different definitions of these terms in the individual OIE Standards would be unacceptable for the EU.

The Aquatic Animals Commission acknowledged Member Countries' comments highlighting the need to review the use of 'OIE Standard' throughout the *Aquatic Code* and align it with the new definition once adopted.

OIE Guideline

The Aquatic Animals Commission re-arranged text in this definition to follow the structure of the definition used for OIE Standard, and made several amendments in response to comments from Member Countries' and the Code Commission to improve clarity.

The Aquatic Animals Commission acknowledged Member Countries' comments highlighting the need to review the use of this term throughout the *Aquatic Code* and align it with the new definition once adopted.

Vector

The Aquatic Animals Commission considered Member Countries' comments and replaced the word 'organism' with 'pathogenic agent' given this is a defined term.

Fallowing

The Aquatic Animals Commission proposed that the word 'carrier' be replaced by 'vector' to align with the new proposed definition for vector.

Aquatic animals

The Aquatic Animals Commission recognised that there is an inconsistent use of the terms 'aquatic animals' and 'live aquatic animals' in the *Aquatic Code* and that the current definition for 'aquatic animals' only refers to live aquatic animals. The Commission proposed a revision to the definition for

aquatic animals to make this point explicit and also deleted text regarding the end uses of aquatic animals as this was considered to be unrelated to the definition of an aquatic animal.

The Aquatic Animals Commission noted that, once adopted, where the words 'live aquatic animals' appear in the *Aquatic Code* the word 'live' will be deleted.

Glossary Part B (revised definition for 'aquatic animals' and new definitions for 'OIE Standard' and 'OIE Guideline') is attached as <u>Annex 10</u> for Member Countries' comments.

EU comment

The EU thanks the OIE and in general support the proposed changes to the glossary. Comments are inserted in the text of Annex 10.

Glossary Part A (new definition for 'vector' and the revised definition for 'fallowing') is attached as <u>Annex 3</u> to be presented for adoption at the 84th General Session in May 2016.

EU position

The EU thanks the OIE and in general supports the adoption of this modified glossary. A comment is inserted in the text of Annex 3.

Item 3   Proposed revisions to Articles 1.4.8., 1.5.2., 2.1.4., 4.2.3. and 4.6.3   as a consequence of the proposed definition of 'vector'

Comments were received from the EU, Mexico, New Zealand, Norway and the United States of America.

Member Countries' comments supported the proposed amendments to Articles 1.5.2. and 4.2.3.

In addition to the proposed amendments to Articles 1.5.2. and 4.2.3., the Aquatic Animals Commission noted that the term 'carrier' should be replaced by 'vector' in Articles 1.4.8., 2.1.4. and 4.6.3. given the new proposed definition for vector.

The Aquatic Animals Commission did not address Member Countries' comments related to text that was not being proposed for amendment.

The revised Articles 1.4.8., 1.5.2., 2.1.4., 4.2.3. and 4.6.3. are attached as <u>Annex 4</u> to be presented for adoption at the 84th General Session in May 2016.

EU position

The EU in general supports the adoption of these modified articles. Comments are inserted in the text of Annex 4.

Item 4   Notification of diseases and provision of epidemiological information (Chapter 1.1.)

The Aquatic Animals Commission reminded Member Countries that, given the importance of standardisation of this chapter with the corresponding chapter in the *Terrestrial Code,* amendments proposed by the Aquatic Animals Commission during their October 2015 meeting had been provided to the Code Commission for their consideration at their February 2016 meeting.

The Aquatic Animals Commission reviewed the amendments being proposed by the Code Commission to the corresponding chapter in the *Terrestrial Code* and considered these when amending Chapter 1.1. of the *Aquatic Code*.

The Aquatic Animals Commission agreed to make minor amendments in order to standardise text between the two Code chapters except where different terminology is necessary given differences in glossary definitions between the two Codes.

The revised Chapter 1.1. Notification of diseases and provision of epidemiological information is presented at Annex 5 for adoption at the 84th General Session in May 2016.

EU position

The EU in general supports the adoption of this modified chapter. Comments are inserted in the text of Annex 5.

Item 5   Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)

Comments were received from Australia, Canada, EU, Japan, Malaysia, Mexico, New Zealand, Norway, Thailand and the United States of America.

The Aquatic Animals Commission considered Member Countries' comments and amendments being proposed by the Code Commission to the corresponding chapter in the *Terrestrial Code*, and made relevant amendments. In addition, the Presidents of the Aquatic Animals Commission and Code Commission met to consider points where text differed. They agreed to harmonise text where possible noting that some differences are necessary due to the different circumstances of aquatic and terrestrial animal diseases.

The Aquatic Animals Commission noted that to achieve the objective of listing, the criteria in the *Aquatic Code* must be responsive and flexible for the dynamic circumstances of aquatic animal diseases, such as the rapid growth and expansion of aquaculture, high volumes of trade, diversity of species, the frequent emergence of aquatic animal diseases and the difficulty in achieving eradication. During the last 20 years, 19 new aquatic animal diseases have been added to the OIE list. In addition 16 aquatic animal diseases have been de-listed since 2005.

The Aquatic Animals Commission did not accept Member Countries' comments to amend the reference, in Article 1.2.1., to the *Aquatic Manual* Chapter 1.1.2. regarding principles and methods of validation of diagnostic tests as they considered the text as written reflected the subject matter of the Manual chapter and they did not consider it necessary to include the title in full.

The Aquatic Animals Commission agreed with Member Countries' comments to include 'vectors' in point 1 of Article 1.2.2.

The Aquatic Animals Commission did not accept a Member Country's comment to align Article 1.2.2. Criterion 1 with text used in the *Terrestrial Code*, i.e. to insert 'has been proven' rather than 'is likely'. The Commission reiterated that the objective of listing is to '*prevent the transboundary spread of important diseases of aquatic animals through transparent, timely and consistent reporting*'. The Commission emphasised that it would be contrary to the objective of listing to wait for the 'international spread of an agent' to be proven when scientific evidence and international trade patterns indicate that spread is likely. This is important for aquatic animal diseases given the factors described above and in particular the challenge of successfully eradicating aquatic animal diseases once they have spread.

The Aquatic Animals Commission did not accept a Member Country's comment to replace 'precise' with 'accurate' in point 3 of Article 1.1.2. as they considered that the use in this context did not apply to measurement and the word precise was more appropriate.

Proposed criterion 4b and 4c

The Aquatic Animals Commission did not accept a Member Country's comment to include reference to 'severity of the clinical signs' in these points as a measure of impact because clinical signs are not always useful indicators of the consequences of disease in aquatic animals.

Some Member Countries commented on the need for examples in these criteria. The Aquatic Animals Commission noted that the examples given are not exhaustive and provide useful guidance to the types of consequences when considering a disease for listing against these criteria. In addition the Commission noted that this approach was consistent with that used in the corresponding articles of the *Terrestrial Code*.

The Aquatic Animals Commission did not accept Member Countries' comments to include the word 'direct' with regard to production and economic losses because indirect losses can be important when considering consequences of disease.

In response to some Member Countries' comments the Aquatic Animals Commission considered it important to differentiate impacts on farmed and wild aquatic animals in two different criteria (i.e. 4b and 4c) because half of global aquatic animal production originates from wild aquatic animals. Further, the Commission agreed to include the term productivity in 4c as this is an important measure of aquatic animal disease impacts on fisheries resources.

The Aquatic Animals Commission noted that in Chapters 1.2. of the current editions of the *Aquatic Code* and *Terrestrial Code* there is substantial divergence between the criteria for listing diseases. The proposed amendments to the criteria in Chapter 1.2. of the *Aquatic Code* will result in improvements to, and closer alignment of, the listing criteria between the two Codes. The minor differences that remain are considered necessary to meet the objective of listing described in Article 1.2.1. of the *Aquatic Code*. The Commission encourages Member Countries to review previous explanations regarding the proposed criteria in its October 2015 report.

Member Countries' supported the Aquatic Animals Commission suggestion to use the 'Explanatory notes' that are being proposed for deletion from Chapter 1.2., in the development of a document to guide *ad hoc* Groups in the application of the listing criteria. The Aquatic Animals Commission agreed to work with the Code Commission in the development of this document.

The proposed Chapter 1.2. Criteria for the inclusion of diseases in the OIE list is presented at <u>Annex 11A</u> for Member Countries' comments. Annex 11A shows edits made to the clean text version that was presented to Member Countries in the Commission's October 2015 report. Annex 11B shows edits made to the track changes text version that was presented to Member Countries in the Commission's October 2015 report.

EU comment

The EU thanks the OIE and supports the proposed changes to this chapter.

Item 6   Diseases listed by the OIE (Chapter 1.3.)

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

The Aquatic Animals Commission reviewed the names used for all listed crustacean diseases in Article 1.3.3. and made changes in line with the accepted convention: 'infection with pathogenic agent X'. They noted that when this naming convention is applied for diseases commonly recognised by the name of the disease (rather than the pathogen), then the disease name would be retained in brackets in the relevant chapter title, e.g. 'Infection with *Aphanomyces astaci* (Crayfish plague)'. In Article 1.3.3. the following names are proposed:

– Infection with *Aphanomyces astaci* (crayfish plague);

– Infection with yellow head virus genotype 1;

– Infection with infectious hypodermal and haematopoietic necrosis virus;

– Infection with infectious myonecrosis virus;

– Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis);

– Infection with Taura syndrome virus;

– Infection with white spot syndrome virus;

– Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease).

The Aquatic Animals Commission noted that this convention will also be applied to all disease-specific chapters for crustacean diseases in the *Aquatic Code* and *Manual* (except Chapter 9.7. White spot disease which will be done after the review of the list of susceptible species for this disease) (see Item 13).

Some Member Countries requested that the name 'Infection with HPR-deleted or HPR0 infectious salmon anaemia virus' in Chapter 1.3. be amended to 'infection with infectious salmon anaemia virus'. The Aquatic Animals Commission reminded Member Countries that the naming of this disease is for the purpose of notification and is to emphasise the fact that infection with both HPR-deleted and HPR0 forms of the infectious salmon anaemia virus are notifiable to the OIE. The Aquatic Animals Commission noted that the name of the listed disease is consistent with the scope of Chapter 10.4.

A Member Country requested that the Aquatic Animals Commission review the listing of red sea bream iridoviral disease as to whether it still meets the criteria for listing. The Commission was not aware of any new information that would alter an assessment of this disease against the listing criteria. The Commission would welcome receipt of any such new information. The Commission recognises that there are some issues with the diagnostic methods described in the *Aquatic Manual* for this disease and noted that these will be addressed by a new *ad hoc* Group that will be working on several aspects of the *Aquatic Manual*.

A Member Country requested the delisting of Infectious hypodermal and haematopoietic necrosis because they did not consider that it met the current criterion number 7. The Aquatic Animals Commission did not agree noting that several countries may be declared free of this disease.

The Aquatic Animals Commission proposed that the revised names for these crustacean diseases be circulated for Member Countries' comments.

Item 6.1. Assessment of *Marteilia cochilia* for listing

The Aquatic Animals Commission reviewed their assessment for *Marteilia cochilia* developed in accordance with Article 1.2.2.

The Aquatic Animals Commission concluded that there was insufficient evidence, at this time, to meet criterion 6 and 7 (of Article 1.2.2. in the 2015 *Aquatic Code*) and to support listing of this disease.

Item 6.2. Assessment of *Batrachochytrium salamandrivorans* for listing

The Aquatic Animals Commission reviewed their assessment for listing for *Batrachochytrium salamandrivorans* developed in accordance with Article 1.2.2.

The Aquatic Animals Commission concluded that *B. salamandrivorans* meets the criteria for listing in Chapter 1.3. and should be proposed for listing under Article 1.3.4.

The assessment for *Batrachochytrium salamandrivorans* is presented at <u>Annex 29</u> for Member Countries' information.

Item 6.3. Infection with Ranavirus

The Aquatic Animals Commission noted that the spelling of 'ranavirus' is incorrect in Chapters 1.3. and 8.2. and they proposed that the listed name be corrected to 'Infection with *Ranavirus*'. Once this revision is adopted the Commission propose to make relevant changes in Chapter 8.2. for the next edition of the *Aquatic Code*.

The revised Chapter 1.3. Diseases listed by the OIE is presented at <u>Annex 12</u> for Member Countries' comments.

EU comment

> **The EU in general supports the proposed changes to this chapter, except for the proposed listing of *Batrachochytrium salamandrivorans*. Comments in this regard are included in Annex 12.**

Item 7   General recommendations on disinfection (Chapter 4.3.)

Comments were received from Argentina, Australia, Chile, China (People's Rep. of), EU, Mexico, New Zealand, Norway and Thailand.

The Aquatic Animals Commission considered Member Countries' comments and made relevant amendments.

The Aquatic Animals Commission noted the strong support of Member Countries for this draft chapter and noted that the majority of comments were not of a technical nature but rather provided to improve readability of the text. Many of these suggestions were accepted by the Commission.

The Aquatic Animals Commission noted that some Member Countries commented that the draft chapter had an apparent bias towards finfish aquaculture; however, no specific text was provided to address this issue. The Commission did not accept Member Countries' comments that were more related to biosecurity rather than disinfection, noting that biosecurity will be addressed in a new chapter as proposed by the Commission for the restructuring of Section 4 of the *Aquatic Code* (see item 8).

The revised Chapter 4.3. Disinfection of aquaculture establishments and equipment is presented at Annex 6 for adoption at the 84th General Session in May 2016.

> **EU position**
>
> **The EU thanks the OIE and in general supports the adoption of this modified chapter. Comments are inserted in the text of Annex 6.**

Item 8   Proposed restructure of Section 4: Disease prevention and control

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

The Aquatic Animals Commission considered Member Countries' comments and appreciated the strong support for the proposed restructure. The Commission prioritised revision and development of chapters in Section 4 in the context of its three-year work plan. In line with Member Countries' comments the Commission agreed that the first priority is to finalise and adopt Chapter 4.3. Disinfection of aquaculture establishments and equipment. In order of priority other chapters that will be addressed include: a new chapter on biosecurity, revision of Chapters 4.1. and 4.2. on zoning and compartmentalisation, and a new chapter on emergency disease preparedness.

The Aquatic Animals Commission noted that some Member Countries' suggested that additional text be developed on disinfection of eggs of other aquatic animal species. The Commission requested that Member Countries provide suggestions on the species for which disinfection of eggs is a priority to prevent disease transmission based on industry practice and trade. The Aquatic Animals Commission urge Member Countries' to provide relevant scientific information including any existing validated protocols for egg disinfection. The Commission will consider the development of new text based on Member Countries' responses.

Item 9   General obligations related to certification (Chapter 5.1.)

Comments were received from Australia, Canada, EU, New Zealand, Norway and Thailand.

The Aquatic Animals Commission considered Member Countries' comments and made relevant amendments.

The Aquatic Animals Commission agreed with a Member Country's comment to re-introduce text at point 2 of Article 5.1.4. This text addresses the need for the Competent Authority of an exporting

country to inform the importing country of the result of an investigation that was initiated following the detection of disease associated with importation of aquatic commodities.

The revised Chapter 5.1. General obligations related to certification is attached at <u>Annex 7</u> to be presented for adoption at the 84th General Session in May 2016.

EU position

The EU supports the adoption of this modified chapter.

Item 10 Infection with yellow head virus (Chapter 9.2.)

Comments were received from the EU, Canada, China (People's Rep. of), Malaysia, New Zealand, Norway and Thailand.

The Aquatic Animals Commission considered Member Countries' comments and made relevant amendments.

In view of a recent publication, a Member Country suggested the addition of red claw crayfish *(Cherax quadricarinatus)* to Article 9.2.2. as a susceptible species for yellow head virus genotype 1 (YHV1). The Aquatic Animals Commission sought advice from the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases who concluded that it did not meet the criteria for susceptibility (as described in Article 1.5.7.) because there was insufficient evidence to indicate that that infection had occurred. The *ad hoc* Group recommended that this species be included in Chapter 2.2.8. of the *Aquatic Manual* in Section 2.2.2. 'Species with incomplete evidence for susceptibility'.

The Aquatic Animals Commission amended the title in several articles to reflect the amended title name of Chapter 9.2 which includes only genotype 1.

The Aquatic Animals Commission did not agree with a Member Country's comment to merge Articles 9.2.4. and 9.2.5., as the conditions described are subtly different.

In response to a Member Country's suggestion to expand the scope of the chapter to include all genotypes of yellow head virus, the Aquatic Animals Commission reminded Member Countries that the scope of this chapter has not changed and includes only yellow head virus genotype 1, the pathogenic agent that causes yellow head disease. The proposed revisions are intended to make this scope explicit and ensure consistency throughout the chapter.

The Aquatic Animals Commission agreed with Member Countries' comments to amend the title of Article 9.2.3. to align with the intended purpose of this article, i.e. the importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with YHV1 status of the exporting country, zone or compartment. The Aquatic Animals Commission proposed, that once adopted, this amendment would be made in Article X.X.3. of all disease-specific chapters in the *Aquatic Code*.

The revised Chapter 9.2. Infection with yellow head virus genotype 1 is attached as <u>Annex 8</u> to be presented for adoption at the 84th General Session in May 2016.

EU position

The EU thanks the OIE and in general supports the adoption of this modified chapter. A comment is inserted in the text of Annex 8.

Item 11 New chapter on Acute hepatopancreatic necrosis disease (Chapter 9.X.)

Comments were received from Australia, Canada, China (People's Rep. of), EU, Japan, Mexico, New Zealand, Norway, Thailand and the United States of America.

In response to Member Countries' comments, the Aquatic Animals Commission amended the scope and aligned this section in both the proposed *Aquatic Code* and *Aquatic Manual* chapters for Acute hepatopancreatic necrosis disease (AHPND).

In response to several Member Country's comments on the lists of commodities in Articles 10.X.3. and 10.X.11. the Aquatic Animals Commission reminded Member Countries that these sections have been placed 'under study' (as shown by square brackets [...]) until assessments have been conducted on a range of commodities commonly traded internationally against the criteria provided in Chapter 1.5. The Commission requested that these assessments be undertaken by the *ad hoc* Group on Susceptible species when they next meet later this year and to report back to the Commission's September 2016 meeting so that relevant articles can be updated.

Member Countries requested that *P. chinensis* (fleshy prawn) be listed as a susceptible species in Article 9.X.2. as it is reported to be a susceptible species in a number of publications e.g. by the Food and Agricultural Organization of the United Nations (FAO) and the Network of Aquaculture Centres in Asia-Pacific (NACA). The Aquatic Animals Commission noted that the *ad hoc* Group on Susceptibility of crustacean species considered that there is incomplete evidence to fulfil the criteria for listing a species as susceptible to AHPND in accordance with Chapter 1.5. (see details in Annex 30).

The Aquatic Animals Commission agreed with a Member Country's comment to use alternative terminology for the word 'risk' and made amndments to the text where the intended meaning of the word risk did not match the definition in the glossary.

The Aquatic Animals Commission also made some minor amendments to improve readability, as shown below. As similar text appears in all disease-specific chapters the Commission proposed that, once adopted, the following changes be made in all disease-specific chapters:

1)   in points 4b) in Articles 9.X.4. and 9.X.5.: replace '*risk*' with 'likelihood';

2)   in Article 9.X.5.: *Competent Authority(ies)* of the country(ies);

3)   in point 3 of Article 9.X.3.: delete the words 'of spread' after *risk*.

Given the proposal to amend Article X.X.8. (see Item 12), the Aquatic Animals Commission noted that the proposed text for Article 9.X.8. is currently aligned with other disease-specific chapters in the *Aquatic Code*, and will be revised accordingly to align with any future amendments that are adopted for this article.

The Aquatic Animals Commission requested that the OIE Technical Fact Sheet on acute hepatopancreatic necrosis disease be removed from the OIE website given that new chapters on AHPND for the *Code* and *Manual* are under development.

The new Chapter 9.X. Acute hepatopancreatic necrosis disease is presented at <u>Annex 13</u> for Member Countries' comments.

EU comment

The EU thanks the OIE and supports this new chapter.

Item 12 Revised Article X.X.8.

The Aquatic Animals Commission reviewed the cross reference to the ICES Code in points 2 and 3 of Article X.X.8. in all disease-specific chapters (or Article 10.4.12. for Infection with infectious salmon anemia), and agreed it was not appropriate for the *Aquatic Code* and that specific requirements should be described. The Commission developed some revised text for this article which more adequately describes the requirements for the importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from *disease X*.

The Aquatic Animals Commission noted that when any proposed amendments are adopted these changes will be made in all disease-specific chapters of the *Aquatic Code*.

The revised Article X.X.8. is presented as 'clean' text at Annex 14A and in 'track changes' at Annex 14B for Member Countries' comments.

EU comment

The EU in general supports the proposed changes to this article. Comments are inserted in the text of Annex 14B.

Item 13 *Ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases

The Aquatic Animals Commission reviewed the report of the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases and commended the *ad hoc* Group for their substantial work.

The Aquatic Animals Commission reviewed the *ad hoc* Group proposed changes to the list of susceptible species in Article X.X.2 of the *Aquatic Code* for Crayfish plague (*Aphanomyces astaci*), Infectious hypodermal and haematopoietic necrosis, Infectious myonecrosis, Necrotising hepatopancreatitis, Taura syndrome, White tail disease and Acute hepatopancreatic necrosis disease (the new draft chapter).

The Aquatic Animals Commission noted that the application of the new criteria for listing species as susceptible to infection with a specific pathogen (described in Chapter 1.5.) resulted in an amended list of susceptible species in Article X.X.2. of these disease-specific chapters (see details in the *ad hoc* Group report at Annex 30).

The Aquatic Animals Commission noted that application of the criteria to diseases with a wide host range may result in a substantial change to the susceptible species that are listed in Article X.X.2. for the relevant disease-specific chapters. For example, based on application of the criteria by the *ad hoc* Group, the crayfish plague chapter would apply to only 10 species, rather than the current scope of several hundred species within the families Cambaridae, Astacidae and Parastacidae. The Commission agreed that this would have implications for the application of measures for safe trade, particularly where susceptibility of traded species is likely but has not been demonstrated because species have not been exposed to natural infection or experimental challenge has not been performed. Further, in some cases, there may be no evidence that any of the species tested, within a taxonomic group (e.g. genus or family) are refractory to infection. The Commission noted that this issue would have implications for other diseases with wide host susceptibility, such as white spot disease and epizootic ulcerative syndrome, and invited Member Countries to provide comment on how these circumstances should be addressed.

The Aquatic Animals Commission agreed to circulate the proposed amendments to the list of susceptible species in Articles 9.1.2., 9.3.2., 9.4.2., 9.5.2, 9.6.2, 9.8.2. and 9.X.2. (AHPND) for Member Countries' comments.

In addition, the Aquatic Animals Commission proposed to amend the disease-specific chapters for these crustacean diseases to align with proposed amendments to disease names, i.e. infection with pathogen X'. (see item 6).

The Aquatic Animals Commission also recommended that the *ad hoc* Group continue its work to review the list of susceptible species for white spot disease.

The Aquatic Animals Commission also recommended that a new *ad hoc* Group be convened to review the list of susceptible species for OIE listed fish diseases.

The report of the OIE *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases is attached as Annex 30 for Member Countries' information.

The revised Chapters 9.1 Crayfish plague (*Aphanomyces astaci*), 9.3. Infectious hypodermal and haematopoietic necrosis, 9.4. Infectious myonecrosis, 9.5. Necrotising hepatopancreatitis, 9.6. Taura syndrome and 9.8. White tail disease are attached as Annexes 16–21 for Member Countries' comments.

> **EU comment**
>
> **The EU in general supports the proposed changes to these chapters. One comment is inserted in the text of Annex 16.**

Note also similar amendments to Chapter 9.X. Acute hepatopancreatic necrosis disease in <u>Annex 13</u> (see Item 11).

Item 14 Chapter 4.4. Recommendations for disinfection of salmonid eggs

Comments were received from Chile, Norway and the United States of America.

Several Member Countries provided detailed comments with supporting rationale for amendments to the text of Chapter 4.4. Recommendations for disinfection of salmonid eggs. The Aquatic Animals Commission considered these comments and amended the text where relevant.

The revised Chapter 4.4. Recommendations for disinfection of salmonid eggs is presented at <u>Annex 15</u> for Member Countries' comments.

> **EU comment**
>
> **The EU supports the proposed changes to this chapter.**

E OTHER CODE ISSUES

Item 15 Commodity assessment document

The Aquatic Animals Commission was informed that a document that includes all of the aquatic animal product assessments undertaken using the criteria described in Chapter 5.4. 'Criteria to assess the safety of aquatic animal commodities' for aquatic animal products that are included in the disease-specific chapters, in Article X.X.3. 'products for any purpose' and in Article X.X.11. (or 10.4.15. for infection with infectious salmon anaemia) 'products for retail trade for human consumption' will be uploaded onto the OIE website at:

http://www.oie.int/en/international-standard-setting/specialists-commissions-groups/aquatic-animal-commission-reports/other-reports/

The Aquatic Animals Commission noted that this document, which provides the scientific rationale for determining which aquatic animal products are considered safe, is a valuable resource for Veterinary Authorities and other Competent Authorities responsible for the certification of aquatic animal products for international trade.

F. *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS* – EXAMINATION OF MEMBER COUNTRY COMMENTS AND WORK OF RELEVANT *AD HOC* GROUPS

Item 16 Infection with yellow head virus genotype 1 (Chapter 2.2.8.)

Comments were received from Australia, Canada, China (People's Rep. of), Chinese Taipei, European Union, Japan, Norway, Malaysia, Mexico, Switzerland, Thailand and the United States of America.

The Aquatic Animals Commission reviewed all Member Countries' comments and amended the text accordingly.

The Aquatic Animals Commission amended the title in several articles to reflect the amended chapter title name that includes genotype 1.

In response to a Member Country's suggestion to expand the scope of the chapter to include all genotypes of yellow head virus, the Aquatic Animals Commission reminded Member Countries that the scope of this chapter has not changed and includes only yellow head virus genotype 1 (YHV1), the

pathogenic agent that causes yellow head disease. The proposed revisions are intended to make this scope explicit and ensure consistency throughout the chapter.

In view of a recent publication, a Member Country suggested the addition of red claw crayfish (*Cherax quadricarinatus*) as a susceptible species for YHV1. The Aquatic Animals Commission sought advice from the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases, which concluded that it did not meet the criteria for susceptibility susceptibility (as described in Article 1.5.7.) because there was insufficient evidence to indicate that infection had occurred in this species. The *ad hoc* Group recommended that *Cherax quadricarinatus* be included in Section 2.2.2. 'Species with incomplete evidence for susceptibility' of the *Aquatic Manual* chapter.

The Aquatic Animals Commission sought the advice of the Reference Laboratory expert for YHV on a number of comments of a technical nature and amended the text accordingly.

In response to a Member Country's comment that the case definition could be improved, the Aquatic Animals Commission requested that the new *ad hoc* Group on the *Aquatic Manual*, which will meet in April 2016, address this issue.

The revised Chapter 2.2.8. is presented as <u>Annex 9</u> for adoption at the 84th General Session in May 2016.

EU position

The EU in general supports the adoption of this modified chapter. However, comments are inserted in the text of Annex 9, the last one of which (on Section 7.2.) is important and should be taken into account before adoption.

Item 17 Acute hepatopancreatic necrosis disease (new draft chapter 2.2.X.)

The Aquatic Animals Commission reviewed the new draft chapter on Acute hepatopancreatic necrosis disease (new draft chapter 2.2.X.) amended by an electronic *ad hoc* Group, which had considered the large number of Member Countries' comments received. The Commission thanked the members of this *ad hoc* Group for their substantial work on this chapter.

As the chapter has been substantially revised since it was circulated for Member Countries' comments in March 2015, only a clean version has been provided.

The revised chapter on Acute hepatopancreatic necrosis disease (Chapter 2.2.X.) is attached as <u>Annex 22</u> for Member Countries' comments.

EU comment

The EU supports this draft new chapter.

Item 18 *Ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases

The Aquatic Animals Commission reviewed the *ad hoc* Group's report and noted that the application of the new criteria for listing species as susceptible to infection with a specific pathogen (described in Chapter 1.5.), resulted in amendments to Section 2.2. 'Host factors' of the *Aquatic Manual* chapters for the following pathogens:

– Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.);

– Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.2.);

– Infectious myonecrosis (Chapter 2.2.3.);

– Necrotising hepatopancreatitis (Chapter 2.2.4.);

– Taura syndrome (Chapter 2.2.5.);

– White tail disease (Chapter 2.2.7.); and

–   Acute hepatopancreatic necrosis disease (new draft Chapter X.X.X.) (see also Item 17).

The Aquatic Animals Commission noted that proposed amendments to the *Aquatic Manual* chapters had been circulated to relevant Reference Laboratory experts for each *Aquatic Manual* chapter requesting them to review Section 2.2. in line with the recommendations of the *ad hoc* Group report.

The Aquatic Animals Commission reviewed recommendations of the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases and comments from relevant Reference Laboratory experts, and amended the relevant crustacean disease chapters accordingly.

The Commission proposed text for Article 2.2.2 of the Crayfish plague chapter to indicate that it is likely that other species, as yet untested or naturally exposed to *A. astaci*, may meet some or all of the criteria for susceptibility.

In addition the Aquatic Animals Commission:

–   amended the Section 1 'Scope', where relevant, to align with the corresponding chapter of the *Aquatic Code*;

–   amended the title and text throughout the chapter to follow the naming convention 'infection with disease X';

–   removed references in Sections 2.2.1. and 2.2.2.

The report of the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases is provided at Annex 30 for Member Countries' information.

The revised chapters for Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.), Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.2.), Infectious myonecrosis (Chapter 2.2.3.), Necrotising hepatopancreatitis (Chapter 2.2.4.), Taura syndrome (Chapter 2.2.5.) and White tail disease (Chapter 2.2.7.) are attached as Annexes 23–28 for Member Countries' comments.

EU comment

The EU in general supports the proposed changes to these chapters. Comments are inserted in the text of Annexes 23 and 27.

G. OIE REFERENCE CENTRES

Item 19 Applications for OIE Reference Centre status or changes of experts

The Aquatic Animals Commission recommended acceptance of the following application for OIE Reference Centre status:

OIE Reference Laboratory for infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, AZ 85721, United States of America Tel.: (+1-520) 621.44.38; Fax: (+1-520) 626.56.02; E-mail: fengjyu@u.arizona.edu Website: http://acbs.cals.arizona.edu/aqua Designated Reference Expert: Dr Kathy Tang-Nelson.

Item 20 Withdrawal of OIE Reference Laboratory status

The Aquatic Animals Commission noted the request from the OIE Reference Laboratory for Infection with Abalone Herpesvirus at the National Taiwan University in Taipei (Chinese Taipei) to be removed from the list following the retirement of the designated expert Dr Pen Heng Chang. The Commission thanked Dr Chang for his valuable contribution to the OIE over the past eight years.

Item 21 Annual reports of Reference Centres activities for 2015 and other specific issues related to Reference Centres

Dr Min-Kyung Park, Scientific and Technical Department of the OIE, joined the meeting for this agenda item. Dr Park presented an analysis of activities based on the annual reports submitted by the OIE Reference Centres for aquatic animals. As of 16 February 2016, 40 out of 41 Reference Laboratories and one of two Collaborating Centres had submitted annual reports to the OIE.

The Aquatic Animals Commission expressed its on-going appreciation for the support and expert advice provided by the OIE by the Reference Centres. The Commission welcomed the increasing number of OIE Reference Laboratories with internationally recognised quality management systems in place. With reference to the recommendation from the 3rd Global Conference of OIE Reference Centres, that: *"OIE Reference Centres achieve or maintain accreditation to the ISO 17025 or equivalent quality management system in their diagnostic laboratories"*, the Commission noted that the deadline to have these systems in place is fast approaching (i.e. the end December 2017). The Commission agreed that there is a need to develop a procedure outlining how to review and manage Reference Laboratories that do not meet this requirement by the deadline.

The Aquatic Animals Commission was also informed of relevant discussions and outcomes of the recent Biological Standards Commission meeting. The Aquatic Animals Commission agreed with the Biological Standards Commission's recommendation that applications to become an OIE Reference Laboratories should be submitted a minimum of 45 days prior a scheduled Aquatic Animals Commission meeting to be considered at that meeting. That time period is required to allow sufficient time for the OIE Headquarters to screen, translate when necessary, and process the dossiers, and for the members of the relevant Commission to fully evaluate the applications prior to their meeting.

The Aquatic Animals Commission also agreed that two critical points to consider for the evaluation of a laboratory's performance are: i) the laboratory's annual report and ii) progress toward achievement of accreditation to ISO 17025 or an equivalent quality management system. The Commission agreed that any OIE Reference Laboratory that does not meet the requirements specified in their Terms of Reference for these points could be recommended for de-listing.

The Aquatic Animals Commission noted the importance of cooperation with the Biological Standards Commission on issues of mutual interest and agreed to strengthen the working relationship between the two Commissions. The Aquatic Animals Commission requested that OIE Headquarters organise a tele-conference call between the Presidents of the two Commissions prior to their next meetings to discuss relevant agenda items.

Item 22 Update on OIE activities on antimicrobial resistance

Dr Elisabeth Erlacher-Vindel, Deputy Head of the Scientific and Technical Department, updated the Aquatic Animals Commission on OIE activities concerning antimicrobial resistance, including the current work of the OIE *ad hoc* Group on Antimicrobial Resistance. The Aquatic Animals Commission agreed to follow the work of the OIE on this important topic, and update the *Aquatic Code* and *Manual* as relevant.

H. AQUATIC ANIMALS COMMISSION WORK PLAN FOR 2015/2016

The Aquatic Animals Commission reviewed and updated its work programme, taking into account Member Countries' comments, Headquarters' comments, and completed work. The work plan includes significant new activities including prioritised activities for the restructuring of section 4.

The revised work programme is attached as Annex 31 for Member Countries' information.

I. AQUATIC ANIMALS COMMISSION ACTIVITIES

The Aquatic Animals Commission agreed that it is important to inform Member Countries of activities that Commission members undertake in their role as Commission members.

Since October 2015, the members of the Commission have participated in the following activities:

Dr Ingo Ernst was invited to participate in the 14th meeting of the Asia Regional Advisory Group on Aquatic Animal Health which was convened by the Network of Aquaculture Centres in Asia Pacific (Bangkok, 23–25 November 2015). Details of the meeting are provided at item 24 below.

Dr Edmund Peeler was invited to participate in the European Commission expert working group meeting in Brussels (8 December 2015) to coordinate the EU response to the October 2015 report of the Aquatic Animals Commission. Dr Peeler provided background information to the report and answered questions. He also elaborated on the future work plans of the Commission.

Dr Alicia Gallardo and Dr Joanne Constantine were invited to participate in a Regional Commission of the America's meeting (December 2015) to discuss the October 2015 report of the Aquatic Animals Commission. They presented an overview of the report and discussed possible amendments to text circulated for comment and the Commission's work plan.

J. COLLABORATION

Item 23 Food and Agricultural Organization Update

Melba Reantaso, representing the Food and Agricultural Organization, joined the Aquatic Animals Commission meeting by tele-conference and provided an update on relevant FAO Technical Cooperation Programmes underway, in particular those focused on acute hepatopancreatic necrosis disease in Asia and Latin America and epizootic ulcerative syndrome in Africa. Dr Ernst provided an update on relevant activities of the Aquatic Animals Commission.

The members of the Commission welcomed this update noting the importance of the relationship with FAO.

Item 24 NACA

Dr Ernst provided the Commission with a report on the 14th meeting of NACA's Asia Regional Advisory Group on Aquatic Animal Health which was held in Bangkok on 23–25 November 2015.

The Advisory Group was established by NACA to provide advice to NACA members on aquatic animal health management, including through: evaluating disease trends and emerging threats; identifying developments with global aquatic animal disease standards of importance to the region; reviewing the regional reporting programme for aquatic animal diseases; and providing guidance and leadership on regional strategies to improving management of aquatic animal health. Members of the Advisory Group include invited aquatic animal disease experts, the OIE, FAO and collaborating regional organisations.

Dr Ernst provided presentations to the Advisory Group on OIE aquatic animal health standards adopted at the 83rd General Session in May 2015 and outcomes of the October 2015 meeting of the Aquatic Animals Commission. The reports of Advisory Group meetings are available on the NACA website (www.enaca.org).

K. NEXT MEETING

Future meetings of the Aquatic Animals Commission are scheduled for 12-16 September 2016 and 20–24 February 2017, inclusive.

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…/Annexes

MEETING OF THE OIE

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 15–19 February 2016

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MEETING OF THE OIE

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 15–19 February 2016

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Adopted agenda

A) MEETING WITH THE DIRECTOR GENERAL

B) ADOPTION OF THE AGENDA

C) MEETING WITH THE PRESIDENT OF THE TERRESTRIAL HEALTH STANDARDS COMMISSION

D) EXAMINATION OF MEMBER COUNTRY COMMENTS AND WORK OF RELEVANT *AD HOC* GROUPS

1) General comments

2) Glossary

3) Proposed revisions to Articles 1.5.2. and 4.2.3. as a consequence of the proposed definition of 'vector'

4) Notification of diseases and provision of epidemiological information (Chapter 1.1.)

5) Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)

6) Diseases listed by the OIE (Chapter 1.3.)

    6.1. Assessment of *Marteilia cochilia* for listing

    6.2. Assessment of *Batrachochytrium salamandrivorans* for listing

    6.3. Infection with Ranavirus

7) General Recommendations on disinfection (Chapter 4.3.)

8) Proposed restructure of Section 4: Disease prevention and control

9) General obligations related to certification (Chapter 5.1.)

10) Infection with yellow head virus (Chapter 9.2.)

11) New chapter Acute hepatopancreatic necrosis disease (Chapter 9.X.)

12) Revised Article X.X.8

13) *Ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases

14) Chapter 4.4. Recommendations for disinfection of salmonid eggs

Annex 2 (contd)

E) **OTHER** *AQUATIC ANIMAL HEALTH CODE* **ISSUES**

15. Commodity assessment document

F) *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS*

16) Review of Member Country comments on Chapter 2.2.8. Infection with yellow head virus genotype 1 (YHD1)

17) Review of draft chapter on acute hepatopancreatic necrosis disease

18) Ad hoc Group on Susceptibility of crustacean species to infection with OIE listed diseases

G) **OIE REFERENCE CENTRES**

19) Applications for OIE Reference Centre status or changes of experts

20) Withdrawal of OIE Reference Laboratory status

21) Annual reports of Reference Centres activities for 2015 and other specific issues related to Reference Centres

22) Update on OIE activities on antimicrobial resistance

H) **AQUATIC ANIMALS COMMISSION WORK PLAN FOR 2015/2016**

I) **AQUATIC ANIMALS COMMISSION ACTIVITIES**

J) **COLLABORATION**

23) FAO

24) NACA

K) **NEXT MEETING**

GLOSSARY (PART A)

EU position

The EU thanks the OIE and in general supports the adoption of this modified glossary. A comment is inserted in the text below.

For the purpose of the *Aquatic Code*:

VECTOR

> means any living organism that transports ~~an infectious agent~~ *a pathogenic agent* to a susceptible *aquatic animal* ~~individual~~ or its food or immediate surroundings. The ~~organism~~ *pathogenic agent* may or may not pass through a development cycle within the vector.

FALLOWING

> means, for *disease* management purposes, an operation where an *aquaculture establishment* is emptied of *aquatic animals* susceptible to a *disease* of concern or known to be capable of transferring the *pathogenic agent*, and, where feasible, of the carrying water. For *aquatic animals* of unknown susceptibility and those agreed not to be capable of acting as ~~carriers~~ vectors of a *disease* of concern, decisions on *fallowing* should be based on a *risk assessment*.

EU comment

In the definition of fallowing above, the EU suggests putting the word "vectors" in italics as soon as the definition of "vector" above has been adopted.

- - - - - - - - - - - - -

— Text deleted.

**PROPOSED REVISIONS TO ARTICLES 1.4.8., 1.5.2., 2.1.4., 4.2.3. AND 4.6.3.
AS A CONSEQUENCE OF THE PROPOSED NEW DEFINITION OF 'VECTOR'**

EU position

The EU in general supports the adoption of these modified articles. Comments are inserted in the text below.

CHAPTER 1.4.

AQUATIC ANIMAL HEALTH SURVEILLANCE

[..]

Article 1.4.8.

Therefore, it should be chosen taking into account factors such as ~~carriers~~, reservoirs, vectors, immune status, genetic resistance and age, sex, and other host criteria.

[..]

EU comment

In Article 1.4.8., the EU suggests putting the word "vectors" in italics as soon as the definition of "vector" has been adopted.

— Text deleted.

CHAPTER 1.5.

CRITERIA FOR LISTING SPECIES AS SUSCEPTIBLE
TO INFECTION WITH A SPECIFIC PATHOGEN

[…]

Article 1.5.2.

Scope

Susceptibility may include clinical or non-clinical *infection* but does not include ~~mechanical vectors (i.e.~~ species that may carry the *pathogenic agent* without replication~~)~~.

[...]

- - - - - - - - - - - - -

— Text deleted.

CHAPTER 2.1.

IMPORT RISK ANALYSIS

[..]

Article 2.1.4.

[..]

2. Exposure assessment

[..]

b) Country factors
– Presence of potential vectors or intermediate hosts
– *Aquatic animal* demographics (e.g. presence of known susceptible ~~and carrier~~ species, distribution).

[..]

- - - - - - - - - - - - -

— Text deleted.

CHAPTER 4.2.

APPLICATION OF COMPARTMENTALISATION

[...]

Article 4.2.3.

[...]

2. Infrastructural factors

Structural aspects of an *establishment* or *establishments* within a *compartment* contribute to the effectiveness of its *biosecurity*. Consideration should be given to:

a) water supply;
b) effective means of physical separation;
c) facilities for people entry including access control;
d) *vehicle* and vessel access including washing and *disinfection* procedures;
e) unloading and loading facilities;
f) isolation facilities for introduced *aquatic animals*;
g) facilities for the introduction of material and equipment;
h) infrastructure to store *feed* and veterinary products;
i) disposal of *aquatic animal* waste;
j) measures to prevent exposure to fomites~~, mechanical~~ or ~~biological~~ vectors;

EU comment

In point j) above, the EU suggests putting the word "vectors" in italics as soon as the definition of "vector" has been adopted.

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    k)   feed supply/source.

- - - - - - - - - - - - -

— Text deleted.

CHAPTER 4.6.

FALLOWING IN AQUACULTURE

[..]

Article 4.6.3.

Technical parameters for the implementation of a statutory fallowing plan

Fallowing of a farm should start immediately after:

1)   removal of all *susceptible species* of *aquatic animals* for the *disease* of concern; and

2)   removal of all species capable of acting as ~~carriers~~ <u>vectors</u> of the *disease* of concern; and

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EU comment

In point 2) above, the EU suggests putting the word "vectors" in italics as soon as the definition of "vector" has been adopted.

[..]

- - - - - - - - - - - - -

— Text deleted.

CHAPTER 1.1.

NOTIFICATION OF DISEASES, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

EU position

The EU in general supports the adoption of this modified chapter. Comments are inserted in the text below.

Article 1.1.1.

For the purposes of the *Aquatic Code* and in terms of Articles 5, 9 and 10 of the OIE Organic Statutes, Member Countries every Member Country of the OIE shall recognise the right of the *Headquarters* to communicate directly with the *Competent Authority* of its *territory* or *territories*.All *notifications* and all information sent by the OIE to the *Competent Authority* shall be regarded as having been sent to the country concerned and all *notifications* and all information sent to the OIE by the *Competent Authority* shall be regarded as having been sent by the country concerned.

Article 1.1.2.

1) Member Countries shall make available to other Member Countries, through the OIE, whatever information is necessary to minimise the spread of important *diseases* of *aquatic animals* and their *pathogenic agents* and to assist in achieving better world-wide worldwide control of these *diseases*.

2) To achieve this, Member Countries shall comply with the *notification* requirements specified in Articles 1.1.3. and 1.1.4.

3) For the purposes of this chapter an 'event' means, a single *outbreak* or a group of epidemiologically related *outbreaks* of a given *disease* that is the subject of a *notification*. An event is specific to a pathogen and strain, when appropriate, and includes all related outbreaks reported from the time of the immediate *notification* through to the final report. Reports of an event include susceptible species, number and geographical distribution of affected *aquatic animals* and *epidemiological units*.

EU comment

The EU supports the new text in point 3 above. However, we would like to point out that it is almost impossible to provide numbers in case of wild animals. This should be kept in mind also in the upcoming revision of WAHIS.

3~~4~~) To assist in the clear and concise exchange of information, reports shall conform as closely as possible to the current OIE *disease* reporting format.

4~~5~~) The detection of the *pathogenic agent* of a *listed disease* in an *aquatic animal* should be reported, even in the absence of clinical signs. Recognising that scientific knowledge concerning the relationship between *pathogenic agents* and clinical *disease* is constantly developing and that the presence of an infectious agent does not necessarily imply the presence of clinical *disease*, Member Countries shall ensure through their reports that they comply with the spirit and intention of point 1 above.

5~~6~~) In addition to notifying findings in accordance with Article 1.1.3. and 1.1.4., Member Countries shall also provide information on the measures taken to prevent the spread of *diseases*. Information shall include possible *quarantine* measures and restrictions on applied to the movement of *aquatic animals*, *aquatic animal products*, *biological products* and other miscellaneous objects which could by their nature be responsible for transmission of *disease*. In the case of *diseases* transmitted by vectors, the measures taken against such vectors shall also be specified.

EU comment

In point 6) above, the EU suggests putting the word "vectors" in italics as soon as the glossary definition of "vector" has been adopted. This should be done throughout the OIE Aquatic Code whenever the word "vector" is used.

Article 1.1.3.

The *Competent Authority* shall, under the responsibility of the Delegate, send to the *Headquarters* ~~of the OIE~~:

1) in accordance with relevant provisions in the *disease*-specific chapters, *notification*, through the World Animal Health Information System (WAHIS) or by fax or e-mail within 24 hours of any of the following events:

   a) first occurrence of a *listed disease* in a country, a *zone* or a *compartment*;

   b) ~~re-occurrence~~ recurrence of a *listed disease* in a country, a *zone* or a *compartment* following the final report that declared the *outbreak* ended;

   c) first occurrence of a new strain of a *pathogenic agent* of a *listed disease* in a country, a *zone* or a *compartment*;

   d) a sudden and unexpected change in the distribution or increase in incidence or virulence of, or morbidity or mortality caused by, the *pathogenic agent* of a *listed disease*, present within a country, a *zone* or a *compartment*;

   e) occurrence of a *listed disease* in a new host species.

   ~~In deciding whether findings justify immediate *notification* (within 24 hours), Member Countries must ensure that they comply with the obligations of Chapters 5.1. and 5.2. (especially Article 5.1.1.), to report developments that may have implications for *international trade*;~~

2) weekly reports subsequent to a *notification* under point 1 above, to provide further information on the evolution of the event which justified the *notification*. These reports should continue until the *disease* has been eradicated or the situation has become sufficiently stable so that six-monthly reporting under point 3 will satisfy the obligation of the Member Country. ~~to the OIE; for~~ For each event notified, a final report ~~on the event~~ should be submitted;

3) six-monthly reports on the absence or presence and evolution of *listed diseases* and information of epidemiological significance to other Member Countries;

4) annual reports concerning any other information of significance to other Member Countries.

Article 1.1.4.

Competent Authorities shall, under the responsibility of the Delegate, send to the *Headquarters*:

1) a *notification* through WAHIS or by fax or ~~e-mail~~ email, when an *emerging disease* event has occurred ~~been detected~~ in a country, a *zone* or a *compartment*;

2) periodic reports subsequent to a *notification* of an *emerging disease*~~, as described under point 1. These~~ should continue ~~until~~:

   a) for the time necessary to have reasonable certainty that:

      ~~b~~i) the *disease* has been eradicated; or

      ~~c~~ii) the situation has becomes ~~sufficiently~~ stable; ~~or~~

EU comment

Please replace "becomes" by "become" in point ii) above (grammar).

OR

b)   until sufficient scientific information is available to determine whether it meets the criteria for ~~listing~~ inclusion in the OIE list as described in Chapter 1.2.

3)   a final report once requirements in point 2) a) or b) are met.

Article 1.1.5.

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

2)   ~~An *infected zone* or *compartment* for a particular *disease* shall be considered as such until a period exceeding the *infective period* specified in the *Aquatic Code* has elapsed after the last reported *case* and when full prophylactic and appropriate *aquatic animal* health measures have been applied to prevent possible reappearance or spread of the *disease*. These measures will be found in detail in various *disease*-specific chapters of the *Aquatic Code*.~~

2~~3~~) A ~~Member Country~~ country, *zone* or *compartment* may be considered to have ~~regain~~ regained freedom from a specific *disease* when all relevant conditions given in the *Aquatic Code* have been fulfilled.

3~~4~~) The *Competent Authority* of a Member Country which ~~sets up~~ 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.

1)   Although Member Countries are only required to notify *listed diseases*, and *emerging diseases*, they are encouraged to provide ~~inform~~ the OIE with ~~of~~ other important *aquatic animal* health ~~events~~ information.

2)   The *Headquarters* shall communicate by email ~~e-mail~~ or through the interface of the World Animal Health Information ~~Database~~ System (~~WAHID~~ WAHIS) to *Competent Authorities* all *notifications* received as provided in Articles 1.1.2. to 1.1.5. and other relevant information.

———————————

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—   Text deleted.

CHAPTER 4.3

DISINFECTION OF AQUACULTURE ESTABLISHMENTS AND EQUIPMENT

> **EU position**
>
> **The EU thanks the OIE and in general supports the adoption of this modified chapter. Comments are inserted in the text below.**

Article 4.3.1.

Purpose

To provide recommendations on planning and implementation of *disinfection* procedures to prevent the introduction or spread of *pathogenic agents*.

Article 4.3.2.

Scope

This chapter describes recommendations on for *disinfection* procedures of for *aquaculture establishments* and equipment used during routine biosecurity activities and for emergency response. Guidance is provided on general principles, planning and implementation of *disinfection* activities.

For specific methods of pathogen inactivation refer to the *disease*-specific chapters in the *Aquatic Manual.*

Article 4.3.3.

Introduction

Disinfection is commonly employed as a *disease* management tool in *aquaculture establishments* as part of a *biosecurity plan*. *Disinfection* is used to prevent entry or exit of target *pathogenic agents* to or from an *aquaculture establishment* or *compartment*, as well as the spread of *pathogenic agents* within *aquaculture establishments*. *Disinfection* may be used during emergency disease response to support the maintenance of *disease* control *zones* and for *disease* eradication (stamping-out procedures) from affected *aquaculture establishments*. The specific objective of *disinfection* will determine the strategy used and how it is applied.

Where When possible, the spread of *pathogenic agents* should be prevented by avoiding risk transmission pathways rather than attempting to manage them through *disinfection*. For example, high risk and difficult to disinfect items (e.g. gloves, dive and harvest equipment, ropes and nets) should be dedicated to a specific site specific rather than disinfecting and moving these items moved between production units or and *aquaculture establishments* after *disinfection*.

Article 4.3.4.

General principles

Disinfection is a structured process that uses physical and chemical procedures to destroy or inactivate target *pathogenic agents*. The process should include planning and implementation stages that take into account potential options, efficacy and *risks*.

The *disinfection* process may vary depending on whether the overall objective is *disease* prevention, control or eradication or *disease* control. Procedures addressing eradication will generally involve destocking of all *aquatic animals* as well as *disinfection* of *aquaculture establishments* and equipment, whereas *disease* control aims at limiting the spread of *disease* between or within *aquaculture establishments*. Although different approaches may be used to achieve the identified objective, the general principles described below should be applied in all cases.

Annex 6 (contd)

1) The *disinfection* process should include the following phases:

   a) <u>Cleaning and washing</u>

> **EU comment**
>
> **The EU suggests inserting a new sentence at the beginning of this article, as follows:**
>
> **"<u>Cleaning is an important part of the disinfection process and particular attention should be paid to this aspect to ensure successful disinfection.</u>"**
>
> **Indeed, the importance of thorough cleaning before disinfection should be emphasised further as an introduction to this article.**

Cleaning and washing of surfaces and equipment should always precede the application of *disinfectants*. It is necessary to remove solid waste, organic matter (including biofouling) and chemical residues as these may reduce the ~~effectiveness~~ efficacy of *disinfectants*. The detergent used should be compatible with the *disinfectant* and the surface being treated. ~~Waste produced during this phase should be disposed of in a safe manner because it may contain viable *pathogenic agents* that have the potential to spread *infection* if not controlled.~~ After cleaning procedures, any excess water should be drained before application of *disinfectants*.

Where treatment of water is required, the presence of suspended solids may also reduce the ~~capacity~~ efficacy of some *disinfectants*. Removal of suspended solids through various processes such as filtration, sedimentation, coagulation or flocculation should be performed.

Biofilms, often referred to as slime, are a thin film of microorganisms and extracellular polymeric substances that adhere to surfaces. Biofilms physically protect embedded microorganisms against *disinfectants*. In order to achieve effective *disinfection*, biofilms should be removed during the cleaning and washing stage prior to the application of *disinfectants*.

<u>All waste produced should be disposed of in a biosecure manner because it may contain viable *pathogenic agents* that have the potential to spread *infection* if not controlled.</u>

   b) <u>Application of disinfectants</u>

This phase involves the application of chemical compounds or physical processes that are appropriate to inactivate the ~~target~~ *pathogenic agent*.

The application of *disinfectants* should take into account the type of material requiring *disinfection* and how *disinfectants* should be applied. Hard non-permeable materials ~~such as~~ (e.g. polished metal surfaces, plastics and painted concrete) can be cleaned thoroughly and allow contact with the *disinfectant* because there is little opportunity for infective material to lodge in crevices. *Disinfection* efficacy will decrease if the surface is corroded, pitted or paint is flaking, so proper maintenance of equipment is essential. For permeable surfaces and materials (e.g. woven material, nets and soil), a higher *disinfectant* concentration and a longer contact time is required because the surface area is greater, chemicals cannot penetrate easily and residual organic matter may be present.

The choice of the application method should ensure all surfaces come into contact with the agent for the required period of time. The application of *disinfectants* should be undertaken methodically (e.g. using a grid pattern) to ensure that complete coverage and adequate contact times are achieved. Each phase should start from the highest point and proceed downwards, commencing from the least contaminated areas. However for some equipment, rinsing of surfaces with the *disinfectant* may be sufficient. When *disinfectants* are applied to vertical surfaces, care should be taken to ensure that the required contact time is maintained before the *disinfectant* drains away. Vertical surfaces may need retreatment or the addition of compatible foaming agents to prolong adherence to surfaces.

For pipes and biofilters, complete filling with the *disinfectant* solution should be done to ensure contact with all surfaces. Difficult to access and complex areas may require fumigation or use of misting equipment.

c)   Removal or inactivation of the disinfectant

Removal or inactivation of chemical residues is important to avoid toxicity to *aquatic animals*, corrosion of equipment and environmental impacts. Processes that may be employed for the removal or inactivation of chemical residues may include: rinsing of surfaces, dilution to acceptable levels, treatment to inactivate chemical agents or, time to allow deactivation or dissipation of the active compound. These processes may be used in isolation or in combination.

2)   *Disinfectants* should be used in accordance with relevant legislation. *Disinfectants* may present *risks* to the health of <u>people</u> ~~users~~, *aquatic animals* and the environment. Chemical *disinfectants* should be stored, used and disposed of in accordance with regulations and manufacturer's instructions.

3)   *Disinfection* should be monitored to ensure appropriate dose of *disinfectant* and *disinfection* efficacy. Depending on the application process and the ~~pathogen~~ *pathogenic agent* of concern, this may be done in different ways. Examples include measurement of the active agent (e.g. residual chlorine levels), indirect measurement of the active agent by an indicator process (e.g. monitoring oxygen reduction potential), and measuring efficacy using indicator bacteria (e.g. heterotrophic bacteria plate counts).

In facilities that have undergone destocking and *disinfection*, the use of a sentinel population prior to restocking may be considered. The sentinel population should be susceptible to the pathogen of concern and exposed to conditions that would be conducive to the expression of clinical *disease* should viable pathogen remain.

4)   *Aquaculture establishments* should keep records of the *disinfection* processes applied. The records should be sufficient to allow evaluation of the *disinfection* plan.

Article 4.3.5.

Planning

A *disinfection* plan should be developed that incorporates an assessment of the ~~risk~~ <u>transmission</u> pathways, the type of material to be disinfected, the *pathogenic agent*s to be inactivated<u>, the health and safety precautions and control measures required,</u> and the environment in which the process is to be undertaken. The *disinfection* plan should be regularly reviewed and include a mechanism for determining efficacy. Any changes to the *disinfection* plan should also be documented.

The planning process should assess the critical control points where *disinfection* will be most effective. *Disinfection* priorities should be developed by considering potential pathways for spread of *pathogenic agents* and the relative <u>likelihood</u> ~~risk~~ of contamination. For effective *disinfection* of facilities containing vectors (e.g. ponds) the vectors should be excluded, removed or destroyed as part of the *disinfection* process.

EU comment

The EU suggests removing the words "excluded, removed or" before the word "destroyed" in the paragraph above. Indeed, not destroying the vectors might constitue a risk of further transfer of pathpogens. Therefore, these vectors should preferably destroyed.

An inventory of all items requiring *disinfection* should be developed <u>when practical.</u> ~~and include an~~ <u>An</u> assessment <u>should be made</u> of <u>the</u> materials used in construction, <u>their</u> surface porosity~~, access to areas~~ and resistance to chemical damage<u>, and accessibility for *disinfection*</u>. Then, the appropriate *disinfection* method should be decided for each item.

The level of cleaning required prior to *disinfection* should be assessed for each type of equipment. If heavy soiling with solids and particulate matter is present, specific attention should be given to the cleaning process and the resources required. The physical or chemical cleaning process should be compatible with the *disinfectant* chosen.

Personnel, equipment and materials to be *disinfected* should be assessed taking into account the type and number of items to be treated and how waste material will be managed.

The ability to control water flow and water volumes should be considered at the planning stage and will depend on the enterprise type (recirculation, flow-through and open systems). Water may be disinfected using a variety of methods as described in Article 4.3.11.

Article 4.3.6.

Disinfection in an emergency response

Disinfection is essential part of any emergency response to support *disease* control activities such as *quarantine* of affected *aquaculture establishments* and stamping-out procedures. The conditions associated with an emergency response require different approaches for *disinfection* to those used in routine biosecurity. These conditions include a high level of *disease risk* (due to the significance of the *disease*), high pathogen loading, potential high volumes of infected *aquatic animals* and waste, large areas requiring *disinfection* and large volumes of contaminated water. Planning should consider these circumstances, incorporate an evaluation of *risks* and include methods for monitoring efficacy.

In an emergency response it may be preferable to avoid ~~disease~~ *risk* <u>transmission</u> pathways rather than relying on *disinfection*. Equipment should not be moved from an infected ~~premise~~ <u>*aquaculture establishment*</u> unless effective *disinfection* has been achieved. In some circumstances, ~~destruction of high~~ *risk* equipment or material <u>that is difficult to disinfect or has a high likelihood of contamination may need to be disposed of in a biosecure manner rather than be disinfected.</u> ~~in a way that inactivates the pathogen (e.g. incineration) will be required~~.

Article 4.3.7.

Types of disinfectants

Types of *disinfectants* commonly used in *aquaculture* include the following:

1. <u>Oxidising agents</u>

    The majority of oxidising agents are relatively fast acting and are effective *disinfectants* for a large range of micro-organisms. These compounds are inactivated by organic matter and therefore should be used following an effective cleaning stage. Organic matter consumes oxidising agents and the initial concentration (loading dose) may drop rapidly, making effective dosing levels (residual dose) difficult to predict. Therefore, residual dose levels should always be monitored to ensure that they remain above the minimum effective concentration for the required time period.

    Oxidising agents may be toxic to *aquatic animals* and therefore should be removed or inactivated.

    Common oxidising agents include chlorine compounds, chloramine-T, iodophores, peroxygen compounds, chlorine dioxide and ozone.

2. <u>pH modifiers (alkalis and acids)</u>

    pH modifiers consist of either alkalis or acid compounds used to modify ambient pH. They have the advantage that they are not inactivated by organic matter and therefore can be used in areas where an effective cleaning phase is not possible such as in pipes and biofilters.

EU comment

The EU suggests inserting the words "<u>the concentration of the effective dose is easy to measure, and that</u>" after the words "They have the advantage that".

3. <u>Aldehydes</u>

    Aldehydes act by denaturing protein. Two aldehyde compounds that may be used during decontamination of *aquaculture establishments* are formaldehyde and glutaraldehyde. They are highly effective against a wide range of organisms but require long exposure times. Aldehydes maintain their activity in the presence of organic matter and are only mildly corrosive. ~~Formalin~~ <u>Glutaraldehyde is used in the liquid form as a cold steriliant, particularly for heat-sensitive equipment. Formaldehyde may be used as a mist or a</u> ~~can also be used to produce formaldehyde~~ gas for fumigation.

4. <u>Biguanides</u>

    Of the many biguanides available, chlorhexidine is the most commonly used. However they are not effective in hard or alkaline water and are less effective against many *pathogenic agents* compared to other groups of *disinfectants*. These compounds are comparatively non-corrosive and relatively safe, thus they are commonly used in the *disinfection* of ~~people~~ <u>skin surfaces</u> and delicate equipment.

5.   Quaternary ammonium compounds (QACs)

The biocidal efficacy of QACs is variable and selective. They are effective against some vegetative bacteria and some fungi, but not all viruses. QACs are most active against gram-positive bacteria; action against gram-negative bacteria is slow, with some strains showing resistance. These compounds are not effective against spores. The advantages of QACs are that they are noncorrosive and have wetting properties that enhance contact with surfaces. QACs may be toxic to *aquatic animals* and should be removed from surfaces following *disinfection* procedures.

6.   Ultraviolet (UV) irradiation

UV irradiation is a viable option for the treatment of water entering or leaving *aquaculture establishments* where there is some control of water flows in recirculation or flow-through systems. UV irradiation should be used following effective filtration because suspended solids reduce UV transmission and the effectiveness of this method.

7.   Heat treatment

~~The effectiveness of heat treatment is dependent on the combination of temperature and exposure time.~~ Susceptibility of *pathogenic agents* to heat treatment varies significantly, therefore, the characteristics of the target *pathogenic agent* should be taken into consideration. Under most conditions, moist heat is more effective than dry heat.

8.   Desiccation

Desiccation may be an effective *disinfectant* for susceptible *pathogenic agents* and may be used in circumstances where other *disinfection* methods are impractical or as an ancillary method to other *disinfection* methods.

Desiccation can be considered to be a *disinfection* method if complete drying of the item is achieved because the absence of water will kill many *pathogenic agents*. However, moisture content may be difficult to monitor in some circumstances. The effectiveness will vary depending on environmental conditions such as temperature and humidity.

9.   Combined disinfection methods

Combined *disinfection* methods should be considered wherever they are synergistic and provide a higher assurance of effective *pathogenic agent* inactivation. Some examples include:

a) direct sunlight and ~~drying~~ desiccation as a combined *disinfection* method provides three potential *disinfection* actions, i.e. UV irradiation, heating and desiccation. It has no operational cost and may be used subsequent to other methods;

> **EU comment**
>
> **In point a) above, the EU suggests removing the reference to operational costs, as this is not usually included anywhere else in the Aquatic Code.**

b) ozone and UV irradiation are often combined in series as they provide back-up systems and different modes of action. UV irradiation also has the advantage of removing ozone residues from treated water.

Antagonistic effects may occur when chemical agents or detergents are combined.

Article 4.3.8.

Selection of a disinfectant

The *disinfectant* should be selected considering the following:

–   ~~effectiveness~~ efficacy against the *pathogenic agents*;

–   effective concentration and exposure time;

–   ability to measure efficacy;

–   nature of the items to be disinfected and the potential for them to be damaged;

– compatibility with the available water type (e.g. fresh water, hard water or seawater);

– availability of the *disinfectant* and equipment;

– ease of application;

– the ability to remove organic matter;

– cost;

– impacts of residues on *aquatic animals* and the environmental; and

– user safety.

Article 4.3.9.

Types of aquaculture establishments and equipment

Aquaculture establishments and equipment differ widely in their characteristics. This section presents some considerations for effective *disinfection* of different types of *aquaculture establishments* and equipment.

1. Ponds

   Ponds are generally large and may be earthen based or be fitted with plastic liners. These characteristics together with the large volumes of water make cleaning prior to decontamination difficult and high organic loads may affect many chemical *disinfectants*. Ponds should be drained of water and have as much organic matter as possible removed prior to *disinfection*. Water and organic matter should be disinfected or disposed of in a biosecure manner. Earthen ponds should be dried thoroughly and lime compounds applied to raise pH and aid the inactivation of *pathogenic agents*. Cultivation Scraping, ploughing or tiling of the base of unlined ponds will also aid in incorporation of liming compounds and drying.

2. Tanks

   Tank construction material (e.g. fibreglass, concrete or plastic) will determine the type of *disinfection* method used. Bare concrete tanks are susceptible to corrosion by acids and potential damage by high pressure sprayers. They are also porous and therefore require longer application of chemicals to ensure *disinfection*. Plastic, painted and fibreglass tanks are more easily disinfected because they have smooth, non-porous surfaces that facilitate thorough cleaning and are resistant to most chemicals.

   Tanks should be drained of water and have as much organic matter as possible removed prior to *disinfection*. Water and organic matter should be disinfected or disposed of in a biosecure manner. Prior to *disinfection, water should be drained from tanks.* Tank equipment should be removed for separate cleaning and *disinfection*, and all organic waste and debris removed. Tank surfaces should be washed using high-pressure sprayers or mechanical scrubbing with detergent to remove fouling such as algae and biofilms. Heated water may be used to enhance the cleaning process. Any excess water should be drained before application of *disinfectants*.

   When *disinfectants* are applied to vertical surfaces, care should be taken to ensure that adequate contact time is maintained before the *disinfectant* is drained. Following *disinfection*, tanks should be rinsed to remove all residues and allowed to dry completely.

3. Pipes

   Disinfection of pipes may be difficult due to lack of access. Pipe construction material should be taken into consideration when selecting the *disinfection* method.

   Pipes can be cleaned effectively through the use of alkaline or acid solutions, or foam projectile pipe cleaning systems. Effective *disinfection* in pipes requires the removal of biofilms, followed by flushing of the resulting particulate matter and thorough rinsing.

   Once pipes are cleaned, chemical *disinfectants* or circulation of heated water can be used. All steps require pipes to be fully filled so that internal surfaces are treated.

4. Cage nets and other fibrous materials

EU comment

> **The EU suggests adding "and other materials" after the word "Nets" in the paragraph below. Indeed, as suggested by the title of this point 4, it is important to take into consideration also other materials.**
>
> **Furthermore, in order to manage the environmental risks, disinfection activities should be performed in a dedicated area for disinfection.**

Nets used in cage culture are often large, difficult to handle, have significant levels of biofouling and are usually made from fibrous materials that trap organic matter and moisture. ~~Due to the difficulty associated with disinfecting~~ large Nets should be dedicated to a single *aquaculture establishment* or area ~~nets and their close contact with fish populations they~~ because they have a high likelihood of contamination and may be difficult to disinfect. ~~are considered to be high *risk* items that should be dedicated to a single *aquaculture establishment* or area~~.

Once the net has been removed from the water, it should be transferred directly to the net washing site. Nets should be thoroughly cleaned prior to *disinfection* to remove organic matter and aid in the penetration of chemical *disinfectants*. Cleaning of nets is best achieved by first removing gross biofouling and then washing with a detergent solution.

Following cleaning, nets may be disinfected by complete immersion in chemical *disinfectants* or heated water. Treatment duration should be sufficient to allow penetration into net material. The treatment method should be chosen considering the potential to weaken or damage nets. Following *disinfection*, nets should be dried before storage. If rolled nets are not completely dry they will retain moisture which may enhance survival of the *pathogenic agent*.

> **EU comment**
>
> **For reasons of clarity, the EU suggests replacing the proposed new sentence in the paragraph above by the following:**
>
> **"Treatment may have a detrimental impact upon the strength of nets and ropes. This must be considered when deciding upon the treatment method to be applied to ensure net integrity is not compromised.".**

Other fibrous materials such as wood, ropes and dip nets have characteristics similar to cage nets and they require special consideration. Wherever possible, it is recommended that equipment is site specific if it includes fibrous material.

5.   Vehicles

The *risk* ~~associated with~~ likelihood of *vehicle* contamination ~~vehicles~~ will be determined by their use, e.g. transportation of mortalities, live *aquatic animals*, harvested *aquatic animals*. All potentially contaminated internal and external surfaces should be disinfected. Special consideration should be given to ~~high *risk*~~ areas likely to be contaminated such as the internal surface of *containers*, pipes, transportation water and waste. The application of ~~Corrosive~~ corrosive *disinfectants* to *vehicles* ~~Corrosive *disinfectants*~~ should be avoided or if used, corrosive residues removed following *disinfection* by thorough rinsing. Oxidative compounds such as chlorines are the most commonly used *disinfectants* for *vehicles*.

6.   Buildings

Aquaculture establishments include buildings for culture, harvesting and processing of *aquatic animals*, and other buildings associated with storage of feed and equipment.

The approach to *disinfection* may vary depending on the structure of the building and degree of contact with contaminated material and equipment.

Buildings should be designed to allow effective cleaning and thorough application of *disinfectants* to all internal surfaces. Some buildings will contain complex piping, machinery and tank systems that may be difficult to disinfect. Wherever possible, buildings should be cleared of debris and emptied of equipment, prior to *disinfection*.

Misting or foaming agents are options for *disinfection* of complex areas and vertical surfaces. Fumigation can be considered for large or difficult to access areas if buildings can be adequately sealed.

7.   Containers

Containers range from simple plastic bins used to transport harvested *aquatic animal products* or dead *aquatic animals* through to complex tank systems used for the transport of live *aquatic animals*.

Containers are generally manufactured using smooth non-porous material (i.e. plastic, steel) which can be easily disinfected. They should be considered high *risk* items because they are in close contact with *aquatic animals* or their *products* (e.g. blood, diseased *aquatic animals*). In addition the need to move them between locations makes them potential fomites for the spread of *pathogenic agents*. In the case of transport of live *aquatic animals, containers* may also have pipes and pumping systems and confined spaces that should also be disinfected.

All water should be drained from the *container* and any *aquatic animals*, faecal matter and other organic material removed by flushing with clean water <u>and disposed of in a biosecure manner</u>. All pipes and associated pumps should also be inspected and flushed. *Containers* should then be washed using appropriate chemical detergents combined with high-pressure water cleaners or mechanical scrubbing.

All internal and external surfaces of *containers* should be treated using an appropriate *disinfection* method. They should then be rinsed and inspected to ensure there are no organic residues and stored in a manner that allows them to drain and dry quickly.

8.   Boats

All boats should undergo routine *disinfection* to ensure that they do not transfer *pathogenic agents*. The level of contamination of boats will be determined by their use. Boats used to harvest or to remove dead *aquatic animals* from *aquaculture* sites should be considered as <u>highly likely to be contaminated</u> ~~high~~ *~~risk~~*. Organic material should be regularly removed from decks and work areas.

As part of the *disinfection* planning process, an assessment should be made to identify ~~high~~ *~~risk~~* areas <u>likely to be contaminated</u> such as in and around machinery, holding tanks, bilges and pipes. All loose equipment should be removed prior to *disinfection*. Additional procedures should be developed for well-boats because of their potential to transfer *pathogenic agents* through the discharge of contaminated water. ~~Where there is a~~ <u>Contaminated</u> ~~risk of pathogenic agent,~~ effluent water ~~from well-boats~~ should be disinfected prior to discharge (refer to Article 4.3.10.).

Where possible, boats should be placed on land for *disinfection* in order to limit waste water entering the aquatic environment and to allow access to hull areas. Biofouling organisms that may act as ~~mechanical carriers~~ <u>vectors and fomites</u> ~~or intermediate hosts~~ should be removed.

Where boats cannot be removed to land, a *disinfection* method should be chosen that minimises the discharge of toxic chemicals into the aquatic environment. Divers should be used to inspect and clean hulls. Where appropriate, mechanical methods such as high-pressure sprayers or steam cleaners should be considered as an alternative to chemical *disinfection* for cleaning above and below the water-line. Fumigation may also be considered for large areas if they can be adequately sealed.

9.   Biofilters

Biofilters associated with closed or semi-closed production systems are an important control point for *disease*. Biofilters are designed to maintain a colony of beneficial bacteria used to enhance water quality. The conditions that support these bacteria <u>may</u> also enhance survival of some *pathogenic agents* should they be present. It is normally not possible to disinfect biofilters without also destroying beneficial bacteria. Therefore potential water quality issues should be taken into account when planning strategies for *disinfection* of biofilters.

EU comment

In the paragraph above, the EU suggests adding the words "<u>and must be disinfected or replaced as necessary</u>" at the end of the first sentence. Indeed, it is important to sanitise or replace filters regularly.

When disinfecting biofilters <u>and their substrates,</u> the system should be drained, organic residues removed and surfaces cleaned. ~~All filters should be removed for separate *disinfection*.~~

Disinfection of biofilter systems can be undertaken by modifying water pH levels (using either acid or alkaline solutions). Where this is undertaken, the pH levels must be sufficient to inactivate the ~~target pathogen~~ *pathogenic agent*, but should not be corrosive to pumps and equipment within the biofilter system. Alternatively, the biofilter can be completely dismantled, including removal of biofiter substrate, and the components cleaned and *disinfectants* applied separately. In the case of emergency *disease* response, the latter procedure is recommended. The biofilter substrate should be replaced if it cannot be effectively disinfected. Biofilter systems should be thoroughly rinsed before re-stocking.

10. Husbandry equipment

Aquaculture establishments will normally have a range of husbandry equipment items that come into close contact with *aquatic animals* and have potential to act as fomites. Examples include graders, automatic vaccinators and fish pumps.

The general principles described in Article 4.3.4. should be applied to *disinfection* of husbandry equipment. Each item should be examined to identify areas that come into close contact with *aquatic animals* and where organic material accumulates. If required, equipment should be dismantled to allow adequate cleaning and application of *disinfectants.*

Article 4.3.10.

Personal equipment

Disinfection of personal equipment should consider the level of ~~risks~~ contamination associated with previous use. Where possible, personal equipment should be site specific to avoid the need for regular *disinfection.*

Equipment should be chosen which is non-absorbent and easy to clean. All staff entering a production area should use protective clothing that is clean and uncontaminated. On entry and exit of production areas boots should be cleaned and disinfected. When footbaths are used they should incorporate a cleaning procedure to remove accumulations of organic material and mud, be sufficiently deep to cover boots, use a *disinfectant* solution that is ~~resistant~~ not inactivated by ~~to~~ organic matter and be regularly refreshed with a new solution.

~~High~~ *risk* Highly contaminated equipment such as dive equipment requires special attention ~~because it may be exposed to very high levels of contaminated material and~~ is often ~~susceptible~~ prone to chemical corrosion. Frequent rinsing of equipment will assist in reducing build-up of organic matter and make *disinfection* more efficient. Equipment should be allowed to dry thoroughly to ensure that moist microenvironments that may harbour ~~pathogens~~ *pathogenic agents* are minimised.

Article 4.3.11.

Disinfection of water

Aquaculture establishments may need to disinfect intake and effluent water~~, as a general biosecurity measure for intake water, to exclude entry of target *pathogenic agents*, or~~ to eliminate ~~pathogens~~ *pathogenic agents* ~~in effluent water~~. The most appropriate *disinfection* method will differ depending on the *disinfection* objective and the characteristics of the water to be disinfected.

Exclusion of *aquatic animals* and removal of suspended solids from the water to be treated are essential prior to the application of *disinfectants*. Pathogens are known to adhere to organic and inorganic matter and removal of suspended solids can significantly reduce loading of *pathogenic agents* in water. Removal of suspended solids can be achieved by filtration or settlement of suspended material. The most suitable filtration system will depend on the initial quality of water, volumes to be filtered, capital and operating costs and reliability.

Physical (e.g. UV irradiation) and chemical (e.g. ozone, chlorine and chlorine dioxide) *disinfectants* are commonly used to disinfect water. Suspended solids should be removed prior to the application of these *disinfectants* because organic matter may inhibit oxidative *disinfection* processes and suspended solids inhibit UV transmission and reduce efficacy of UV irradiation by shielding *pathogenic agents*. A combination of methods may be beneficial where they are synergistic or where a level of redundancy is required.

It is essential to monitor the efficacy of water *disinfection*. This can be achieved by direct testing for ~~pathogens~~ *pathogenic agents* of concern, indirect monitoring of indicator organisms or monitoring of residual levels of *disinfectants*.

Management of chemical residues is important to avoid toxic effects on *aquatic animals*. For example, residuals formed between ozone and seawater such as bromide compounds are toxic to early life stages of *aquatic animals*

and may be removed using charcoal filtration. Residual chlorine should be removed from water by chemical deactivation or off gassing.

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— Text deleted.

C H A P T E R   5 . 1 .

GENERAL OBLIGATIONS RELATED TO CERTIFICATION

EU position

The EU supports the adoption of this modified chapter.

[…]

Article 5.1.4.

Responsibilities in case of an incident related to importation

1) *International trade* involves a continuing ethical responsibility. Therefore, if within a reasonable period subsequent to an export taking place, the *Competent Authority* becomes aware of the appearance or reappearance of a *disease* that has been specifically included in the *international aquatic animal health certificate* or other *disease* of potential epidemiological importance to the *importing country* there is an obligation for the *Competent Authority* to notify the *importing country*, so that the imported *commodities* may be inspected or tested and appropriate action be taken to limit the spread of the *disease* should it have been inadvertently introduced.

2) ~~If a *disease* condition appears in imported *aquatic animals* within a reasonable period after importation, the *Competent Authority* of the *exporting country* should be informed so as to enable an investigation to be made, because this may be the first available information on the occurrence of the *disease* in a previously free *aquatic animal* population. The *Competent Authority* of the *importing country* should be informed of the result of the investigation because the source of *infection* may not be in the *exporting country*.~~

2~~3~~) If a *disease* appears in *aquatic animals* in an *importing country* and is associated with importation of *commodities*, the *Competent Authority* of the *exporting country* should be informed. This will enable the *exporting country* to investigate as this may be the first available information on the occurrence of the *disease* in a previously free *aquatic animal* population. ==The *Competent Authority* of the *exporting country* should inform the *importing country* of the result of the investigation because further action may be required if the source of the *infection* did not originate in the *exporting country*.==

3~~4~~) In case of suspicion, on reasonable grounds, that an *international aquatic animal health certificate* may be fraudulent, the *Competent Authorit~~y~~ies* of the *importing country* and *exporting country* should conduct an investigation. Consideration should also be given to notifying any third country==(ies)== ~~(ies)~~ that may have been implicated. All associated consignments should be kept under official control, pending the outcome of the investigation. *Competent Authorities* of all countries involved should fully cooperate with the investigation. If the *international aquatic animal health certificate* is found to be fraudulent, every effort should be made to identify those responsible so that appropriate action can be taken in accordance with the relevant legislation.

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— Text deleted.

CHAPTER 9.2.

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

EU position

The EU thanks the OIE and in general supports the adoption of this modified chapter. A comment is inserted in the text below.

Article 9.2.1.

For the purposes of the *Aquatic Code*, infection with yellow head virus genotype 1 (YHD) means *infection* with yellow head virus genotype 1 (YHV1). YHV is classified as a species in of the genus Genus *Okavirus*, in the family Family *Roniviridae* and order the Order *Nidovirales*.

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

Article 9.2.2.

Scope

The recommendations in this chapter apply to the following *susceptible species* which meet the criteria for listing species as susceptible in Chapter 1.5.: giant tiger prawn *(Penaeus monodon),* white leg shrimp *(Penaeus vannamei),* blue shrimp *(Penaeus stylirostris),* dagger blade grass shrimp *(Palaemonetes pugio)* and Jinga shrimp *(Metapenaeus affinis)* giant tiger prawn *(Penaeus monodon),* brown tiger prawn *(P. esculentus)* and Kuruma prawn *(P. japonicus).* These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 9.2.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with YHV1 status of the exporting country, zone or compartment from a country, zone or compartment not declared free from infection with yellow head virus

1) *Competent Authorities* should not require any conditions related to infection with YHDYHV1, regardless of the infection with YHDYHV1 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 9.2.2. which are intended for any purpose and which comply with Article 5.4.1.:

   a) heat sterilised hermetically sealed crustacean products (i.e. a heat treatment at 121°C for at least 3.6 minutes or equivalent);

   b) cooked crustacean products that have been subjected to heat treatment at 60°C for at least 15 minutes (or any time/temperature equivalent which has been demonstrated to inactivate YHDYHV1);

   c) pasteurised crustacean products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent which has been demonstrated to inactivate YHDYHV1);

   d) crustacean oil;

   e) crustacean *meal*;

   f) chemically extracted chitin.

2)   When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.2.2., other than those referred to in point 1 of Article 9.2.3., *Competent Authorities* should require the conditions prescribed in Articles 9.2.7. to 9.2.11. relevant to the infection with ~~YHD~~YHV1 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 9.2.2. but which could reasonably be expected to pose a *risk* of <mark>spread of</mark> infection with ~~YHD~~YHV1, 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.

Article 9.2.4.

Country free from infection with yellow head virus <mark>genotype 1</mark>

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with ~~YHD~~YHV1 if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with ~~YHD~~YHV1 (see Article 9.2.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with ~~YHD~~YHV1 if:

1)   none of the *susceptible species* referred to in Article 9.2.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.2.2. are present and the following conditions have been met:

a)   there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression, as described in the corresponding chapter of the *Aquatic Manual*; and

b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with ~~YHD~~YHV1;

OR

4)   it previously made a *self-declaration of freedom* from infection with ~~YHD~~YHV1 and subsequently lost its *disease* free status due to the detection of infection with ~~YHD~~YHV1 but the following conditions have been met:

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

b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the <mark>~~risk~~ likelihood</mark> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in the *Aquatic Manual*) have been completed; and

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

d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with ~~YHD~~YHV1.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.2.5.

Article 9.2.5.

Zone or compartment free from infection with yellow head virus genotype 1

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from infection with ~~YHD~~YHV1 if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from infection with ~~YHD~~YHV1 may be declared free by the *Competent Authority~~(ies)~~* of the country~~(ies)~~ concerned if:

1) none of the *susceptible species* referred to in Article 9.2.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.2.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a) there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of infection with ~~YHD~~YHV1;

OR

4) it previously made a *self-declaration of freedom* from infection with ~~YHD~~YHV1 for a *zone* and subsequently lost its disease status due to the detection of infection with ~~YHD~~YHV1 in the *zone* but the following conditions have been met:

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in the *Aquatic Manual*) have been completed; and

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

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with ~~YHD~~YHV1.

Annex 8 (contd)

Article 9.2.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with ~~YHD~~YHV1 following the provisions of points 1 or 2 of Articles 9.2.4. or 9.2.5. (as relevant) may maintain its status as free from infection with ~~YHD~~YHV1 provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with ~~YHD~~YHV1 following the provisions of point 3 of Articles 9.2.4. or 9.2.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from infection with ~~YHD~~YHV1 provided that conditions that are conducive to clinical expression of infection with ~~YHD~~YHV1, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with ~~YHD~~YHV1, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.2.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from infection with yellow head virus genotype 1

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.2.2. from a country, *zone* or *compartment* declared free from infection with ~~YHD~~YHV1, 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 e*xporting country* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.2.4. or 9.2.5. (as applicable) and 9.2.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with ~~YHD~~YHV1.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.2.3.

Article 9.2.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with yellow head virus genotype 1

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with ~~YHD~~YHV1, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and all effluent and waste materials in a manner that ensures inactivation of YHV1.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for YHV1, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for ~~infection with YHD~~ YHV1 and perform general examinations for pests and general health/disease status;

g)   if ~~infection with~~ ~~YHD~~ YHV1 is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as free from infection with ~~YHD~~YHV1 ~~free~~ or specific pathogen free (SPF) for infection with ~~YHD~~YHV1;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.2.3.

EU comment

The EU reiterates its editorial comment made previously on the sentence above. Indeed, since aquatic animals are not listed in point 1 of Article 9.2.3., the words "aquatic animals" should be replaced by the words "commodities" (as is the case e.g. in the relevant sentence of Art. 9.2.7.).

Article 9.2.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with yellow head virus genotype 1

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with ~~YHD~~YHV1, 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 9.2.3., or products described in point 1 of Article 9.2.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of YHV1 or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.2.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with yellow head virus <mark>genotype 1</mark>

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with ~~YHD~~YHV1, the *Competent Authority* of the *importing country* should require that:

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

2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of YHV1.

This Article does not apply to *commodities* referred to in point 1 of Article 9.2.3.

Article 9.2.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from infection with yellow head virus <mark>genotype 1</mark>

1) *Competent Authorities* should not require any conditions related to infection with ~~YHD~~YHV1, regardless of the infection with ~~YHD~~YHV1 status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp or decapod crustacea (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

   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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with ~~YHD~~YHV1, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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CHAPTER 2.2.8.

INFECTION WITH
YELLOW HEAD VIRUS <u>GENOTYPE 1</u>

EU position

The EU in general supports the adoption of this modified chapter. However, comments are inserted in the text below, the last one of which (on Section 7.2.) is important and should be taken into account before adoption.

1. Scope

~~For the purpose of this chapter,~~ <u>Infection with</u> yellow head <u>virus genotype 1</u> ~~disease (YHD)~~ <u>means</u> ~~is considered to be~~ infection with yellow head virus genotype 1 (YHV1) <u>of</u> ~~YHV is classified as a species in~~ the genus *Okavirus*, Family *Roniviridae* and Order *Nidovirales*.

2. Disease information

2.1. Agent factors
2.1.1. Aetiological agent, agent strains

Yellow head virus genotype 1 (YHV1) is one of ~~six~~ <u>eight</u> known genotypes in the yellow head complex of viruses and is the only known agent causing YHD. <u>YHV1 and other genotypes in the yellow head complex are</u> ==formally== <u>classified by the International Committee on Taxonomy of Viruses as a single species (*Gill-associated virus*) in the genus *Okavirus*, Family *Roniviridae*, Order *Nidovirales* (Cowley *et al.,* 2012). Yellow head virus genotype 2 is commonly known as</u> gill-associated virus (GAV~~) is designated as genotype 2~~. ~~GAV and~~ Four other ~~known~~ genotypes in the complex (genotypes 3–6) 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). <u>Recently, two new YHV-complex genotypes have been reported, one designated YHV7 was detected in diseased *P. monodon* in Australia (Mohr *et al.,* 2015) and an eighth genotype was detected in ==*Penaeus Fenneropenaeus* chinensis== suspected of suffering from acute hepatopancreatic necrosis disease (Liu *et al.,* 2014).</u> There is evidence of genetic recombination between genotypes (Wijegoonawardane *et al.,* 2009).

EU comment

The EU is of the opinion that the wording in the paragraph above as regards "gill-associated virus" is confusing, as from the text as proposed it seems associated with both genotype 1 and 2. This should preferably be clarified.

Furthermore, the EU notices a discrepancy as regards the number of genotypes between Article 2.1.1. above and Article 4.3.1.2.3.1. below. Indeed, whereas the former mentions an eighth genotype, the latter mentions only seven.

YHV<u>1</u> 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.

2.1.2. Survival outside the host

YHV<u>1</u> remains viable in aerated seawater for up to 72 hours (Flegel *et al.,* 1995b).

2.1.3. Stability of the agent (effective 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).

2.1.4. Life cycle

High multiplicity YHV1 infections in cell culture have not been reported. Infection at a multiplicity of infection of 0.001 in primary cultures of lymphoid organ cells has indicated that maximum viral titres are obtained 4 days post-infection (Assavalapsakul *et al.*, 2003). Clinical signs of ==infection with YHV1== ~~YHD~~ occur in *P. monodon* within 7–10 days of exposure. 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).

2.2. Host factors
2.2.1. Susceptible host species

~~YHD outbreaks have been reported in the~~ Species that fulfil the criteria for listing a species as susceptible to infection with YHV1 according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) include ~~black~~ giant tiger prawn (*P. monodon*), ~~and the~~ white leg ~~Pacific~~ shrimp (*P. vannamei*) (Chantanachookin *et al.,* 1993; Senapin *et al.,* 2010), ~~The Pacific~~ blue shrimp ~~prawn~~ (*P. stylirostris*), ~~the~~ daggerblade grass shrimp (*Palaemonetes pugio*), and ~~the~~ Jinga shrimp (*Metapenaeus affinis*). ~~also fulfil the criteria required for listing a species susceptible to infection with YHV1 according to Chapter 1.5 of Aquatic Animal Health Code. Natural infections have also been detected in the kuruma prawn (P. japonicus), white banana prawn (P. merguiensis), Pacific blue prawn (P. stylirostris), white prawn (P. setiferus), red endeavour prawn (Metapenaeus ensis), mysid shrimp (Palaemon styliferus) and krill (Acetes sp.). Other species of penaeid and palemonid shrimp and prawns and krill that have been reported to be susceptible to experimental infection include: brown tiger prawn (P. esculentus), brown prawn (P. aztecus); pink prawn, hopper and brown spotted prawn (P. duorarum), greentail prawn (Metapenaeus bennettae), Sunda river prawn (Macrobrachium sintangense), barred estuarine shrimp (Palaemon serrifer), the paste prawn (Acetes sp.) and the daggerblade grass shrimp (Palaemonetes pugio) (Ma et al., 2009). There are variations in the susceptibility of different species to disease. Laboratory trials have shown that YHV can cause high mortality in P. monodon, P. vannamei, P. stylirostrus, P. aztecus, P. duorarum, M. sintangense, P. styliferus and P. serrifer (Lightner et al., 1998; Longyant et al., 2005; 2006; Ma et al., 2009). A survey of 16 crab species collected from the vicinity of shrimp farms in Thailand detected no evidence of either natural infection or experimental susceptibility (Longyant et al., 2006). A critical review of susceptibility of crustaceans to yellow head disease and implications of inclusion in European legislation has been conducted (Stentiford et al., 2009). GAV has been detected in P. monodon and P. esculentus (Walker et al., 2001). To date, infections by other genotypes in the YHV complex have been detected only in P. monodon (Wijegoonawardane et al., 2008a). Metapenaeus brevicornis and P. aztecus also fulfil some of the criteria required for listing as susceptible but evidence was lacking to either confirm the identity of the pathogen under study as YHV1, to`~~

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing a species as susceptible to infection with YHV1 according to Chapter 1.5 of the *Aquatic Code* include: Sunda river prawn (*Macrobrachium sintangense*), yellow shrimp (*Metapenaeus brevicornis*), Carpenter prawn (*Palaemon serrifer*), Pacific blue prawn (*Palaemon styliferus*), northern brown shrimp (*Penaeus aztecus*), northern pink shrimp (*Penaeus duorarum*), kuruma prawn (*Penaeus japonicus*), banana prawn (*Penaeus merguiensis*) and northern white shrimp (*Penaeus setiferus*). Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is YHV1, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

2.2.3.~~2.~~ Susceptible stages of the host

Penaeus monodon are susceptible to YHV1 infection beyond PL15 (Khongpradit *et al.*, 1995). ~~Experimental infections with GAV indicate that larger (~20 g) P. japonicus are less susceptible to disease than smaller (~6–13 g) shrimp of the same species (Spann et al., 2000).~~

2.2.4.~~3.~~ Species or subpopulation predilection (probability of detection)

YHV1 ~~(genotype 1)~~ infections are usually detected only when disease is evident and whilst they do not occur commonly in healthy *P. monodon*, infections have been detected in healthy wild populations of *P. stylirostris* (Castro-Longoria *et al.,* 2008). During ~~YHD~~ outbreaks in aquaculture ponds, the YHV1 infection prevalence can be assumed to be high. 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), but there is little information available on the natural prevalence. ~~Viruses in yellow head complex genotypes 2–6 are only known to occur commonly (prevalence up to 100%) in *P. monodon*, which appears to be the natural host (Walker *et al.,* 2001; Wijegoonawardane *et al.,* 2008a; 2009).~~

2.2.5.4.   Target organs and infected tissue

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).

2.2.6.5.   Persistent infection ~~with lifelong carriers~~

~~GAV persists as a chronic infection for at least 50 days in *P. esculentus* that survive experimental challenge (Spann *et al.,* 2003). The high prevalence of subclinical or chronic infection often found in healthy *P. monodon* infected with GAV (genotype 2) and genotypes 3–6 from postlarval stages onwardssuggests that these infections can perrsist for life (Walker *et al.,* 2001; Wijegoonawardane *et al.,* 2008a). There is also evidence that YHV (genotype 1) can persist in survivors of experimental infection (Longyant *et al.,* 2005; 2006).~~

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).

2.2.7.6.   Vectors

There are no known vectors of YHV1.

2.2.8.7.   ~~Known or suspected wild aquatic animal carriers~~

~~There are no known documented or suspected wild aquatic animal carriers of YHV1. Infection susceptibility and long-term persistence indicate the potential for a wide range of wild penaeid and palaemonid shrimp to act as carriers.~~

2.3.   Disease pattern

2.3.1.   Transmission mechanisms

YHV1 infection can be transmitted horizontally by injection, ingestion of infected tissue, immersion in sea water containing tissue extracts filtered to be free of bacteria, or by co-habitation of naive shrimp with infected shrimp (Flegel *et al.,* 1995b; Lightner, 1996). Infection of shrimp has also been established by injection of extracts of paste prawns (*Acetes* sp.) collected from infected ponds (Flegel *et al.,* 1995a). ~~For GAV, vertical transmission of infection to progeny has been shown to occur from both male and female parents, possibly by surface contamination or infection of tissue surrounding fertilised eggs (Cowley *et al.,* 2002).~~ The dynamics of how YHV1 infection spreads within aquaculture ponds have not been studied. However, the rapid accumulation of mortalities during disease outbreaks suggests that horizontal transmission occurs very effectively.

2.3.2.   Prevalence

The infection prevalence of yellow head complex viruses in healthy *P. monodon* (as detected by reverse transcription-nested PCR [RT-nPCR])) can be high (50–100%) in farmed and wild populations in Australia, Asia and East Africa as well as in ~~L~~*P. vannamei* farmed in Mexico (~~Castro-Longoria *et al.,* 2008;~~ Cowley *et al.,* 2004; Sanchez-Barajas *et al.,* 2009; Walker *et al.,* 2001; Wijegoonawardane *et al.,* 2008a). The prevalence of individual genotypes varies according to the geographical origin of the shrimp. The use of detection methods less sensitive than nested PCR (e.g. histology, immunoblot, dot-blot, *in-situ* hybridisation), is likely in most cases to result in the real infection prevalence amongst populations of shrimp being underestimated.

EU comment

In the sencond line of the paragraph above, the nucleic acid detection method is referred to as "reverse transcription-nested PCR" and abbreviated "RT-nPCR". Thus, that abbreviation should be used also in the 6th line (i.e. "nested PCR" should be replaced by "RT-nPCR"). That term or abbreviation should be used consistently across the chapter whenever reference is made to the nested RT-PCR.

Furthermore, as a general rule, whenever reference is made to "PCR" in this chapter, this should be replaced by "RT-PCR", as a reverse transcription step prior to the amplification of the nucleic acid will always be necessary in case of RNA viruses.

2.3.3. Geographical distribution

YHV1 has been reported in Chinese Taipei, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam (Walker *et al.*, 2001). GAV and other genotypes in the yellow head complex have been detected in healthy *P. monodon* from Australia, Chinese Taipei, India, Indonesia, Malaysia, Mozambique, the Philippines, Thailand and Vietnam (Wijegoonawardane *et al.*, 2008a). YHV1 has also been detected in *P. vannamei* cultured in Mexico (Castro-Longoria *et al.,* 2008; Sanchez-Barajas *et al.*, 2009).

2.3.4. Mortality and morbidity

With *P. monodon* being farmed in ponds, disease caused by YHV1 (genotype 1) can cause up to 100% mortality within 3–5 days of the first appearance of clinical signs (Chantanachookin *et al.,* 1993). Whilst mortalities can easily be induced by experimental exposure of *P. monodon* to YHV1 or GAV, bioassays have identified YHV1 to be far more virulent (~10^6-fold by lethal dose [LD_{50}] 50% end-point analysis) (Oanh *et al.,* 2011). Genotypes 3, 4, 5 and 6 have not yet been associated with disease (Wijegoonawardane *et al.*, 2008a).

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). The much higher virulence of YHV compared with GAV and other genotypes appears to ensure that the infection threshold required to cause disease is reached far more easily.

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods have been developed.

2.4.2. Chemotherapy

No effective commercial anti-viral product is yet available.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Resistance breeding

Not reported.

2.4.5. Restocking with resistant species

All marine shrimp species farmed commercially appear to be susceptible to YHV1.

2.4.6. Blocking agents

Injection of shrimp with double-stranded (ds) RNA homologous to ORF1a/1b gene regions of YHV1 or GAV (thus targeting the genome length viral RNA) can inhibit viral replication and prevent mortalities following experimental challenge. The antiviral action of the dsRNA appears to involve the RNA interference (RNAi) pathway (Tirasophon *et al.,* 2007).

2.4.7. Disinfection of eggs and larvae

Not reported.

2.4.8. General husbandry practices

Specific pathogen free (SPF) or PCR-negative seedstock and biosecure water and culture systems may be used to reduce the risk of disease.

EU comment

Please replace "PCR-negative" by "RT-nPCR-negative" in point 2.4.8. above.

3. Sampling

3.1.   Selection of individual specimens

For diagnosis during a disease outbreak, moribund shrimp collected from pond edges are the preferred source of material for examination. Apparently normal shrimp should also be collected from the same ponds. For surveillance for evidence of infection in populations of apparently healthy shrimp, life stages from mysis onwards (mysis, postlarvae [PL], juveniles or adults) can provide tissue sources useful for testing.

3.2.   Preservation of samples for submission

Moribund shrimp (or tissue from moribund shrimp) should be snap-frozen on-site in a dry ice and alcohol slurry and preserved frozen in dry ice, liquid nitrogen or in a –80°C freezer. Freezing at or above –20°C is unsuitable.

Tissue samples for PCR screening should be preserved in a minimum 3 fold excess of 80–90% analytical/reagent-grade (absolute) ethanol. At least 10 times the volume of ethanol to tissue should be used. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. Commercial RNA preservatives (e.g. RNAlater) may also be used.

EU comment

In the paragraph above, please replace "80-90%" by "80-95%". Indeed, in point 4.3.1.2.3.1. below, "80-95%" is used.

Furtermore, "(absolute)" should be deleted, as it is not necessary.

Tissue samples for histology should be sampled from fresh shrimp and preserved in Davidson's fixative. Formalin (10%) in seawater may be a useful alternative. At least 10 times the volume of fixative to tissue should be used.

Tissues for electron microscopy should be sampled from live shrimp.

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

3.3.   Pooling of samples

For detecting YHV1 infection in large populations of shrimp, pooling of tissue samples is acceptable for screening or surveillance of batches of mysis to PL from a hatchery tank or batches of juvenile shrimp in a pond. For PCR analysis, pool size should be determined by tissue mass that can be processed without compromise in a single test. The total numbers of shrimp sampled, either as a single pool or as multiple smaller pools, are selected based on the infection prevalence expected and the required confidence limits of detection. Typically in populations comprising more than a 100,000 shrimp, if the prevalence of infection exceeds 5%, a total of 60 individuals tested in appropriate pool sizes will be required to detect YHV1 at a 95% confidence limit. However, definitive detection may be compromised if the YHV1 loads in the infected shrimp are very low or if tests less sensitive than two-step RT-PCR or real-time RT-PCR are employed. See also Chapter 2.2.0.

3.4.   Best organs or tissues

In moribund shrimp suspected to be infected with YHV1, lymphoid organ and gill are the most suitable sample tissues. For screening or surveillance of juvenile or adult shrimp that appear grossly normal, lymphoid organ is preferred. Gills or haemolymph can be used for non-sacrificial sampling.

3.5.   Samples or tissues that are not suitable

Not determined.

4. Diagnostic methods

4.1.   Field diagnostic methods
4.1.1.   Clinical signs

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 caused by the underlying yellow hepatopancreas, which may be exceptionally soft when compared with the brown hepatopancreas of a healthy shrimp. In many cases, the total loss of a pond crop occurs within a few days of the first appearance of shrimp showing gross signs of YHV1D (Chantanachookin *et al.,* 1993). Cessation of feeding and congregation of moribund shrimp at pond edges are always seen in YHD outbreaks. However, these disease features are not particularly distinctive for YHD, and in the absence of other more pathognomonic gross signs are not reliable even

for preliminary diagnosis of YH<mark>V1</mark>D. ~~Gross signs of GAV disease include swimming near the surface and at the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow discoloration of the gills (Spann *et al.*, 1997). However, these signs can occur commonly in response to various stressors and thus are not considered pathognomonic for GAV disease. Shrimp chronically infected with YHV or GAV display normal appearance and behaviour.~~

4.1.2. Behavioural changes

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).

4.2. Clinical methods

4.2.1. Gross pathology

See Section 4.1.

4.2.2. Clinical chemistry

None described.

4.2.3. Microscopic pathology

Fix the cephalothorax tissues of moribund shrimp suspected to be affected by YH<mark>V1</mark>D 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.

4.2.4. Wet mounts

Fix whole shrimp or gill filaments overnight in Davidson's fixative (Lightner, 1996). After fixation, wash some gill filaments thoroughly with tap water to remove the fixative and stain with H&E (Lightner, 1996). After staining and dehydration, when the tissue is in xylene, place a gill filament on a microscope slide in a drop of xylene and, using a fine pair of needles (a stereo microscope is helpful), break off several secondary filaments. Replace the main filament in xylene where it can be stored indefinitely in a sealed vial as a permanent reference. Being careful not to let the xylene dry, tease apart the secondary filaments and remove any large fragments or particles that would thicken the mount unnecessarily. Add a drop of mounting fluid and a cover-slip and use light pressure to flatten the mount as much as possible. This procedure may also be used with thin layers of subcuticular tissue. Examine under a light microscope using a ×40 objective lens. For samples from YH<mark>V1</mark>D-affected shrimp, moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller) will be observed (Flegel *et al.,* 1997). Evidence of such pathology should be used to support results from haemolymph smears (see below) in making a presumptive diagnosis of YH<mark>V1</mark>D. As for the fixed tissues and gill filaments preserved in xylene, these whole-mount slides can be preserved as a permanent record.

If rapid results are required, the fixation step can be shortened to only 2 hours by replacing the acetic acid component of Davidson's fixative with a 50% dilution of concentrated HCl. For good fixation, this fixative should not be stored for more than a few days before use. After fixation, wash thoroughly to remove the fixative and check that the pH has returned to near neutral before staining. Do not fix for longer periods or above 25°C as this may result in excessive tissue damage that will make it difficult or impossible to identify specific pathology.

4.2.5. Electron microscopy/cytopathology

For transmission electron microscopy (TEM), the most suitable tissues of shrimp suspected to be infected with YHV<u>1</u> infection are lymphoid organ and gills. For screening or surveillance of grossly normal shrimp, the most suitable tissue is lymphoid organ.

Stun live shrimp by immersion in iced water until just immobilised or kill by injection of fixative. Quickly dissect and remove small portions of target tissue (no larger than a few mm in diameter) and fix in a volume of 6% glutaraldehyde at least 10 time greater than the volume of tissue, held at 4°C and buffered with sodium cacodylate ($Na[CH_3]_2AsO_2 \cdot 3H_2O$) solution (8.6 g Na cacodylate, 10 g NaCl, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl) or phosphate solution (0.6 g $NaH_2PO_4 \cdot H_2O$, 1.5 g Na_2HPO_4, 1 g NaCl, 0.5 g sucrose, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl). Fix for at least 24 h prior to processing. For long-term storage in fixative at 4°C, reduce glutaraldehyde to 0.5–1.0%. Processing involves post-fixation with 1% osmium tetroxide, dehydration, embedding, sectioning and staining with uranyl acetate and lead citrate according to standard TEM reagents and methods (Lightner, 1996).

In the cytoplasm of cells infected with YHV<u>1</u>, both nucleocapsid precursors and complete enveloped virions are observed. Nucleocapsid precursors appear as long filaments approximately 15 nm in diameter that can vary markedly in length (80–450 nm) and that can sometimes be packed densely in

paracrystalline arrays. Virions appear as rod-shaped, enveloped particles 40–50 nm × 150–180 nm with rounded ends and prominent projections (8–11 nm) extending from the surface. In the cell cytoplasm, virions are commonly seen to be localised or packed densely within intracellular vesicles. Virions may also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV1 by TEM.

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. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Although primary shrimp cell culture methods are available, they are not recommended to isolate and identify YHV1 as a routine diagnostic method because of the high risk of them becoming contaminated with adventitious agents. No continuous cell lines suitable for YHV1 culture are yet available.

4.3.1.2.2. Antibody-based antigen detection methods

Reagents and protocols for detecting YHV1 proteins with antibodies have been published (Loh *et al.* 1998; Lu *et al.* 1994). Virions purified from haemolymph of experimentally infected shrimp have been used to produce antiserum in New Zealand white rabbits. From this antiserum, immunoglobulin (IgG) was purified using protein-G-linked columns and cross-reacting normal shrimp antigens were removed by adsorption to acetone-dried, ground shrimp muscle tissue and haemolymph. To detect YHV1 proteins by Western blotting, dilute 0.1 ml haemolymph collected from a live shrimp in an equal volume of citrate buffer and either run immediately or store at –80°C until used. Clarify 200 µl of the sample at 8000 *g* for 5 minutes and then pellet virions from the clarified supernatant by ultracentrifugation at 140,000 *g* for 5 minutes. Resuspend pellets in 100 µl 2 × loading buffer (2.5 ml 0.5 mM Tris/HCl pH 6.8, 4 ml 10% sodium dodecyl sulphate [SDS], 2 ml glycerol, 1 µl β-mercaptoethanol, 0.5 ml deionised distilled water) and heat at 95°C for 5 minutes. Load 10 µl sample onto a 5% SDS polyacrylamide gel and electrophorese at 200 V. Blot the gel onto a 0.1 mm pore size nitrocellulose membrane in blotting buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol per litre) at 100 V for 1 hour. Rinse the membrane with phosphate buffered saline (PBS pH 7.4), block in 5% skim milk (in PBS) for 1 hour, and rinse with PBS for 5 minutes. Soak the membrane in a 1/1000 dilution of the anti-YHV1 antibody (IgG) for 1 hour, rinse three times with PBS for 5 minutes, and then soak for 1 hour in a 1/2500 dilution of goat anti-rabbit IgG-horseradish-peroxidase (HRP) conjugate. Rinse membrane three times with PBS for 5 minutes and then soak in HRP substrate 3,3',5,5'-tetramethylbenzidine , until blue-purple colour develops. Stop the reaction by soaking the membrane in distilled water. All incubations should be carried out at 25°C ± 2°C. Use a purified viral preparation as a positive control to identify positions of the YHV1 116 kDa, 64 kDa and 20 kDa structural proteins. The Western blot YHV1 detection sensitivity is approximately 0.4 ng YHV1 protein ($\approx 10^6$ virions).

Annex 9 (contd)

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1 Reverse-transcription polymerase chain reaction (RT-PCR)

Three RT-PCR protocols are described. The first is a 1-step RT-PCR adapted from Wongteerasupaya *et al.* (1997) that can be used to detect YHV1 in affected shrimp ~~affected by YHD~~. This protocol will detect YHV1 (highly virulent genotype first detected in Thailand in association with YHD) but not GAV or any of the other ~~three~~ genotypes currently recognised. The second is a more sensitive multiplex nested RT-PCR protocol adapted from Cowley *et al.* (2004). It can be used to differentiate YHV1 from GAV in diseased shrimp or for screening healthy carriers. ~~This test will not detect all six known genotypes and genotype 3 may generate a PCR product indistinguishable in size from that generated with GAV (genotype 2).~~ The first stage or step of the multiplex nested RT-PCR (primary RT-PCR) detected YHV7 (Mohr *et al.,* 2015). Both the RT-PCR and the nested PCR (second stage or step) detected the novel YHV genotype from China (Liu *et al.,* 2014). The test is available in a suitably modified form from a commercial source (YHV/GAV IQ2000, GeneReach Biotechnology Corp., Chinese Taipei). However, this kit is not currently listed as having completed the OIE's formal process for validating and certifying commercial tests (a list of certified test kits and manufacturers is available on the OIE website: http://www.oie.int/en/our-scientific-expertise/registration-of-diagnostic-kits/background-information/). The third is a sensitive multiplex nested RT-PCR protocol described by Wijegoonawardane *et al.* (2008b). This test can be used for screening ~~healthy~~ shrimp for any of the six genotypes of the yellow head complex of viruses ~~(including YHV and GAV)~~, but will not discriminate between genotypes. Assignment of genotype can be achieved by nucleotide sequence analysis of the RT-PCR product.

Sample preparation: For juvenile or adult shrimp, lymphoid organ, gill tissue or haemolymph may be used to prepare total RNA. Fresh tissue is preferred. Lymphoid organ and gill tissue preserved in 80% to 95% analytical-grade ethanol ~~or RNAlater (various manufacturers),~~ or stored frozen at –70°C are also suitable for total RNA preparation. Disrupt 10–20 mg lymphoid organ or gill tissue or 50 µl haemolymph in 500 µl Trizol^{TM1} reagent and extract total RNA according to the product manual. Resuspend RNA in 25 µl water treated with DEPC (diethyl-pyrocarbonate)-, heat at 55°C for 10 minutes, cool on ice and use immediately or store at –70°C until required. ~~Ideally, a 1/200 dilution (i.e. 2.5 µl RNA in 500 µl DEPC-treated water) should be prepared, and UV absorbances at A_{260} nm and A_{280} nm (a UV spectrophotometer is required) should be determined to quantify and check the quality of the RNA (ratio approximately 2:1).~~ RNA yield will vary depending on the type and freshness of tissues, quality of the preservative used, and the length of time tissue has been preserved. However, RNA yields from fresh tissues would be expected to vary from 0.2 to 2.0 µg µl^{-1} and about half these amounts from alcohol-preserved tissues. Tissues can also be homogenised by bead beating and extracted using commercially available kits (e.g. QIAmp Viral RNA Mini Kit) (Mohr *et al.,* 2015).

From a nursery tank or hatchery tank containing 100,000 PL or more, sample approximately 1000 PL from each of 5 different points. Pool the samples in a basin, gently swirl the water and then select samples of live PL that collect at the centre of the basin. Choose numbers of PL to be pooled and tested according to the assumed or infection prevalence. Homogenise tissue samples in an appropriate volume of Trizol^{TM} reagent and extract RNA according to the product manual. Based on the standard Trizol^{TM} extraction procedure, tissue masses equivalent to 25–30 × PL5, 15 × PL10 and 5 × PL15 are accommodated and produce high quality total RNA free of protein contamination.

For each set of RNA samples to be tested, DEPC-treated water and extracts known to contain YHV1 RNA and/or GAV RNA (as appropriate to the test) should be included as negative and positive controls, respectively.

Protocol 1: RT-PCR for specific detection of YHV1 in diseased shrimp

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: Template (2 µl) is added to 23 µl reaction mixture containing 12.5 µl 2x reaction mix, 1 µl Superscript III RT/Platinum Taq mix (Invitrogen),180 nM of each primer and molecular grade water. ~~To synthesise cDNA, mix 2 µl RNA in 20 µl PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV (Moloney murine leukaemia virus) reverse transcriptase, 1.0 U ribonuclease inhibitor, 0.75 µM antisense primer 144R, 1 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM MgCl$_2$, and incubate at 42°C for 15 minutes. Incubate the mixture at 100°C for~~

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

5 minutes to inactivate the reverse transcriptase and allow the mixture to cool to 5°C. Add the PCR master mix containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2.5 U *Taq* DNA polymerase, 2 mM MgCl₂ and 0.75 µM of sense primer 10F to give a final volume of 100 µl. Unless the instrument is fitted with a heated lid, overlay the tubes with 100 µl of mineral oil and Conduct After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, PCR amplification consists of 40 cycles at of 94°C for 30 seconds, 58°C for 30 45 seconds, 72 68 C for 30 45 seconds, and finishing followed by at 72 68 C for 10 7 minutes. Alongside a suitable DNA ladder, apply a 20 µl aliquot of the PCR to a 2 1.5% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gel containing 0.5 µg ml⁻¹ ethidium bromide SYBR-safe and following electrophoresis, detect the 135 bp DNA band expected for YHV using a UV blue-light transilluminator.

The sensitivity of the PCR is approximately 0.01 pg of purified YHV1 RNA ($\approx 10^3$ genomes).

PCR primer sequences:
10F: 5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'
144R: 5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

Protocol 2: Nested RT-PCR for differential detection of YHV1 and GAV in healthy or diseased shrimp

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: For the primary PCR, 2 µl template is added to 23 µl reaction mixture containing 12.5 µl 2x reaction mix, 1 µl Superscript lll RT/Platinum Taq mix (Invitrogen),180 nM of each GY1 and GY4 primer and molecular grade water. For cDNA synthesis, 2 µl RNA (ideally 1.0 µg total RNA, if quantified),0.7 µl 50 pmol µl⁻¹ primer GY5 and DEPC treated water are added to 6 µl total, the mixture , incubated at 70°C for 10 minutes and chilled on ice. Add 2 µl Superscript II buffer × 5 (250 mM Tris/HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 1 µl 100 mM DTT and 0.5 µl 10 mM dNTP stock mixture (i.e. 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP) and mix gently. Preheat to 42°C for 2 minutes, add 0.5 µl 200 U µl⁻¹ reverse transcriptase and incubate at 42°C for 1 hour. Heat the reaction at 70°C for 10 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, prepare a 50 µl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton X 100), 1.5 mM MgCl₂, 35 pmol of each primer GY1 and GY4, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube. Overlay the reaction mixture with 50 µl liquid paraffin, Heat at 85°C for 2 3 minutes and then add 1 µl cDNA. After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, Conduct PCR amplification using consists of 35 cycles at of 95°C for 30 seconds, 66°C for 30 seconds, and 72 68° C for 45 seconds, followed by final extension at 72 68°C for 7 minutes. For the second, nested PCR step, prepare a 50 25 µl reaction mixture containing 2 µl of the first step PCR product, 1 × *Taq* buffer (above), 1.5 mM MgCl₂, 12.5 µl HotStarTaq Master Mix (Qiagen), 360 nM 35 pmol of each primer GY2, Y3 and G6, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube and overlay with liquid paraffin and molecular grade water. Conduct PCR using amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes conditions as described above. Alongside a suitable DNA ladder, apply a 20 µl aliquot of the PCR to a 1.5% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gel containing SYBR-safe and following electrophoresis, detect amplicons using a blue-light transilluminator. Apply a 10 µl aliquot of the PCR to 2% agarose/TAE gels containing 0.5 µg ml⁻¹ ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

If the viral load is sufficiently high, a 794 bp DNA will be amplified from either GAV or YHV1 in the first PCR step. 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 detection sensitivity of the second-step PCR is ~1000-fold greater than the first-step PCR and GAV or YHV1 RNA can be detected to a limit of 10 fg lymphoid organ total RNA. The nested PCR can be run as two separate assays specific for YHV1 or GAV by omitting either the G6 or Y3 primer, respectively. The primer contains a mismatch for GAV but is specific for YHV1. 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 sequences of RT-PCR primers generic for GAV and YHV (GY) or specific for GAV (G) or YHV (Y) are as follows:
GY1: 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'
GY2: 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'

GY4:   5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'
GY5:   5'-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3'
Y3:   5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3'
G6:   5'-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3'

NB: 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: Nested *RT*-PCR for detection of all currently ~~known~~ characterised genotypes in the yellow head complex ~~(including~~ YHV1 ~~and~~ ti YHV7 ~~GAV)~~

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: For the primary PCR, 2 µl template is added to 23 µl reaction mixture containing 12.5 µl 2x reaction mix, 1 µl Superscript III RT/Platinum Taq mix (Invitrogen), 180 nM of each YC-F1ab and YC-R1ab primer pools and molecular grade water. ~~For cDNA synthesis, mix 2 µl RNA (ideally 1.0 µg total RNA, if quantified), 50 ng random hexamer primers and 1.0 µl 10 mM dNTP and make up to a total volume of 14 µl in sterile DEPC-treated water, incubate at 65°C for 5 minutes and chill on ice. Add 4.0 µl Superscript III buffer × 5, 1.0 µl 100 mM DTT, 1.0 µl 40 U µl^{-1} RNaseOUT^{TM} (Invitrogen) and 1.0 µl 200 U µl^{-1} reverse transcriptase and mix gently. Incubate at 25°C for 5 minutes and then at 42°C for 55 minutes, stop the reaction by heating at 70°C for 15 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, add 1 µl cDNA to a total 25 µl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1% Triton X 100), 1.5 µl 25 mM MgCl$_2$, 0.35 µl primer mix containing 25 pmol µl^{-1} of each primer pool (see below) YC-F1ab and YC-R1ab, 0.5 µl 10 mM dNTP mix and 0.25 µl 5 U µl^{-1} *Taq* DNA polymerase.~~ After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, ~~Conduct~~ PCR amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 68°C for 45 seconds, followed by final extension at 68°C for 7 minutes. For the second, nested PCR step, prepare a 25 µl reaction mixture containing 2 µl of the first step PCR product, 12.5 µL HotStarTaq Master Mix (Qiagen), 180 nM of each YV-F2ab and YC-R2ab primer pool, and molecular grade water. PCR amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. Alongside a suitable DNA ladder, apply a 20 µl aliquot of the PCR to a 1.5% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gel containing SYBR-safe and following electrophoresis, detect amplicons using a blue-light transilluminator. ~~using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final extension at 72°C for 7 minutes. For the second PCR step, use 1 µl of the first PCR product in the reaction mixture as prepared above but substituting primer pools YC-F2ab and YC-R2ab. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Apply an 8 µl aliquot of the PCR to 2% agarose/TAE gels containing 0.5 µg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.~~

If the viral load is sufficiently high, a 358 bp DNA is amplified in the first PCR step. The second (nested) PCR step amplifies a 146 bp product. The detection of these products indicates detection of one of the ~~six~~ seven genotypes in the yellow head complex. Further assignment of genotype (if required) is possible by nucleotide sequence analysis of either PCR product followed by comparison with sequences of the known genotypes by multiple sequence alignment and phylogenetic analysis. The detection sensitivity limits of the first PCR step and nested PCR step are 2,500 and 2.5 RNA templates, respectively.

PCR primer sequences (each primer comprises a pool of equal quantities of two related oligonucleotide sequences):

YC-F1ab pool: 5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3'
                5'-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3'
YC-R1ab pool: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3'
                5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'
YC-F2ab pool: 5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3'
                5'-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3'
YC-R2ab pool: 5'-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3'
                5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'
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).

4.3.1.2.4. *In-situ* hybridisation

The protocol of Tang *et al.* (2002) described 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). Process the fixed tissue using standard histological methods and prepare 4 µm thick sections on Superfrost Plus slides (Fisher Scientific, Pennsylvania, USA). Prior to hybridisation, incubate sections at 65°C for 45 minutes, remove paraffin with Hemo-De (Fisher Scientific, Pennsylvania, USA), and rehydrate through a reducing ethanol concentration series to water. Digest sections with proteinase K (100 µg ml^{-1}, in 50 mM Tris/HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) for 15 minutes at 37°C, followed by post-fixation in 0.4% formaldehyde for 5 minutes. Rinse in 2 × SSC (standard saline citrate), pre-hybridise with 500 µl pre-hybridisation solution (4 × SSC, 50% formamide, 1 × Denhardt's, 0.25 mg ml^{-1} yeast RNA, 0.5 mg m^{-1} sheared salmon sperm DNA, 5% dextran sulphate) at 42°C for 30 minutes. For hybridisation, overlay the sections with 250 µl hybridisation solution containing a digoxigenin-labelled DNA probe (20–40 ng ml^{-1}) at 42°C overnight. The next day, wash the sections as follows: 2 × SSC once for 30 minutes at room temperature; 1 × SSC twice for 5 minutes at 37°C; 0.5 × SSC twice for 5 minutes at 37°C. Incubate the sections with sheep anti-digoxigenin-alkaline phosphatase conjugate (Roche) at 37°C for 30 minutes. Wash with 0.1 M Tris/HCl pH 7.5, 0.15 M NaCl twice for 10 minutes at room temperature and rinse with 0.1 M Tris/HCl pH 9.5, 0.1 M NaCl. Incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in the dark for 1–2 h for colour development. Counterstain with Bismarck Brown Y (0.5%), dehydrate through a series of ethanol and Hemo-De, add Permount (Fisher Scientific, Pennsylvania, USA) and cover with a cover-slip. YHV-infected cells give a blue to purple-black colour against the brown counter stain. Include positive controls of YHV-infected tissue and negative controls of uninfected shrimp tissue. 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'

4.3.1.2.5 Agent purification

A YHV1 purification method based on density gradient ultracentrifugation is described (Wongteersupaya *et al.* 1995). Approximately 250 healthy juvenile *P. monodon* (or P. vannamei) shrimp (approximately 10 g) should ideally be used as a source of virus for purification. After acclimatising for several days in 1500 litre tanks (approximately 80 shrimp/tank) at a salinity of 3.5 parts per thousand (mg ml^{-1}), inoculate each shrimp intramuscularly with 100 µl of a 1/100 gill extract suspension prepared from YHV-infected shrimp. At 2 days post-infection, harvest moribund shrimp showing typical signs of YH1D. Use a syringe to draw haemolymph from the sinuses at the base of the walking legs and mix carefully on ice with the same volume of lobster haemolymph medium (LHM) (486 mM NaCl, 15 mM CaCl$_2$, 10 mM KCl, 5 mM MgCl$_2$, 0.5 mM Na$_2$HPO$_4$ 8.1 mM MgSO$_4$, 36 mM NaHCO$_3$, 0.05% dextrose in Minimal Eagle's Medium, adjusted pH 7.6 with 1 N NaOH). Centrifuge the mixture at 480 *g* for 30 minutes at 4°C to remove cellular debris. Ultracentrifuge the supernatant at 100,000 *g* for 1 hour at 4°C. Discard the supernatant and gently resuspend the pellet overnight at 4°C in 1 ml LHM. Layer this suspension over a continuous gradient of 20–40% Urografin and ultracentrifuge at 100,000 *g* for 1 hour at 4°C. After centrifugation, collect the viral band by using a Pasteur pipette and dilute with NTE buffer (0.02 M EDTA, 0.2 M NaCl, 0.2 M Tris/HCl [pH 7.4]) to a final volume of 12 ml. Ultracentrifuge the suspension at 100,000 *g* for 1 hour at 4°C and resuspend the pellet (purified virus) in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA [pH 7.4]) and store in 20 µl aliquots at –80°C until required.

4.3.1.2.6 Bioassay

The bioassay procedure is based on that described by Spann *et al.* (1997), but similar procedures have been described by several other authors (Lu *et al.,* 1994). The bioassay should be conducted in susceptible shrimp (see Section 2.2 above) ideally that have been certified as SPF and have been obtained from a biosecure breeding facility. Alternatively, susceptible wild or farmed shrimp to be used for bioassay should be screened by nested RT-PCR using RNA extracted from tissue or haemolymph to confirm the absence of pre-existing chronic infections with YHV1, GAV YHV complex or related viruses. Throughout the procedure, shrimp should be maintained under optimal conditions for survival of the species in laboratory tank systems.

Collect moribund shrimp from a YH1D-affected ponds or shrimp suspected of being carriers of infection and maintain at 4°C or on ice. Remove and discard the tail and appendages. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at –80°C or in liquid nitrogen until required. Thaw stored samples rapidly in a 37°C water bath within two snap-seal

plastic bags and then maintain at 4°C or on ice during all procedures. Remove the carapace and calciferous mouth-parts. Suspend the remaining tissues in six volumes of TN buffer (0.02 M Tris/HCl, pH 7.4, 0.4 M NaCl) and homogenise in a tissue grinder to form a smooth suspension. Clarify the homogenate at 1300 *g* for 20 minutes at 4°C. Remove the supernatant fluid below the lipid layer and pass through a 0.45 µm filter. Maintain the filtrate at 4°C for immediate use or snap-freeze and store in aliquots at –80°C or in liquid nitrogen. Thaw the filtrate rapidly at 37°C and maintain on ice prior to use.

Inject at least 12 juvenile (1–5 g) shrimp of a known susceptible species (*P. monodon*, *P. esculentus*, *P. japonicus*, *P. merguiensis*, *P. vannamei*, *P. stylirostris*), with 5 µl of filtrate per gram body weight into the second abdominal segment using a 26-gauge needle. Inject two equivalent groups of at least 12 shrimp with TN buffer and a filtered tissue extract prepared from uninfected shrimp. One additional group of at least 12 shrimp should be injected last with a known and calibrated positive control inoculum from shrimp infected with YHV1 or GAV (as required). Maintain each group of shrimp in a separate covered tank with a separate water supply for the duration of the bioassay. Ensure no inadvertent transfer of water between tanks by good laboratory practice. Observe the shrimp and record mortalities for at least 21 days or until the test and positive control groups reach 100% mortality. Collect at least one moribund shrimp from each of the four groups for examination by histology, TEM, *in situ* nucleic acid hybridisation, and PCR or Western-blot analysis to confirm the presence of YHV1 or GAV (as required) in the sample (refer to the Sections above for test procedures).

NOTE: shrimp to be tested that are suspected of being carriers of low level chronic infections may produce an inoculum containing a very low dose of virus. In bioassay, such an inoculum may not necessarily cause mortalities, gross signs of disease or histology characteristic of a lethal infection. In this event, molecular tests (PCR or ISH) or TEM must be applied to the bioassay shrimp.

4.3.2. Serological methods

Not applicable.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of YHV1D are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Bioassay	d	d	d	d	c	b
Direct LM	d	d	d	d	a	d
Histopathology	d	d	c	c	a	d
Transmission EM	d	d	c	c	d	b
Antibody-based assays	d	d	c	c	a	b
In-situ DNA probes	d	d	c	c	b	a
PCR	a	a	a	a	a	a

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Sequence	a	a	a	a	d	a

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

EU comment

In the first column of Table 5.1., "PCR" should be replaced by "RT-PCR". Consequently, the words "PCR = polymerase chain reaction" underneath the table should be replaced by "RT-PCR = reverse transcription polymerase chain reaction".

Furthermore, the word "Sequence" should be replaced by "Sequencing", as this is the term used to designate the method (note that this comment is valid for all Aquatic Manual chapters).

6. Test(s) recommended for targeted surveillance to declare freedom from infection with yellow head virus genotype 1

Nested RT-PCR (Section 4.3.1.2.3.1; Protocol 3) followed by confirmatory sequencing of the amplified PCR product is the prescribed method for declaring freedom. Two-step PCR negative results are required. ~~The very rare case when a two step PCR positive result cannot be confirmed by sequencing is also considered to be a negative result.~~

EU comment

The term "Two-step PCR negative results" is confusing. Indeed, what is meant is the nested RT-PCR, which is indeed a two-step reaction (reverse transcription followed by nested PCR). However, the term "two-step" is usually only used for RT-PCR (reverse transcription followed by PCR). Therefore, the EU suggests replacing the words "Two-step PCR" by "Reverse transcription-nested PCR" or "RT-nPCR".

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case of infection with YHV1 ~~genotype 1~~ is defined as a disease outbreak in marine shrimp with rapidly accumulating mortalities (up to 100%) in the early to late juvenile stages, which may be preceded by cessation of feeding and congregation of shrimp at pond edges. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas. Histological examination of fixed lymphoid organ tissues should reveal moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller).

7.2. Definition of confirmed case

YHV1 may be confirmed by the detection of high levels of disseminated infection in tissues of ectodermal and mesodermal origin by *in situ* hybridisation in conjunction with the detection of amplified products of the prescribed size using discriminatory RT-PCR assays and sequencing, as described in Section 4.3 of this chapter. ~~As low level chronic infections with yellow head complex viruses are common in some regions, detection of the presence of virus is not, in itself, evidence of aetiology.~~

EU comment

The EU requests a clarification as to what is meant by the words "in conjunction with". Indeed, that wording is confusing, as it is not clear whether both *in situ* hybridisation and RT-PCR followed by sequencing is required, or if either the former or the latter

> **alone are sufficient. Since this is crucial for the definition of a confirmed case, that clarification should be made before the chapter is submitted for adoption.**

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* *

NB: There is an OIE Reference Laboratory for <u>infection with</u> yellow head ~~disease~~ <u>virus genotype 1</u>
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on <u>infection with</u> yellow head ~~disease~~
<u>virus genotype 1</u>

G L O S S A R Y (P A R T B)

EU comment

The EU thanks the OIE and in general support the proposed changes to the glossary. Comments are inserted in the text below.

For the purpose of the *Aquatic Code*:

AQUATIC ANIMALS

means all <u>viable</u> life stages (including *eggs* and *gametes*) of fish, molluscs, crustaceans and amphibians originating from *aquaculture establishments* or ~~removed~~ from the wild~~, for farming purposes, for release into the environment, for human consumption or for ornamental purposes~~.

EU comment

The EU is of the opinion that it would be important to clearly distinguish between domestic and wild aquatic animals in the Aquiatic Code, like is already the case in the Terrestrial Code. This would for example be relevant for the information to be provided in WAHIS, which distinguishes between the two.

OIE STANDARD~~S~~

means a text that has been ~~formally~~ adopted by the OIE World Assembly of Delegates, published by the OIE~~, in the *Aquatic Code* or *Aquatic Manual* and that provides requirements, recommendations, specifications and characteristics that should be used consistently~~ intended to ensure the maintenance or improvement of *aquatic animal* health and welfare, or veterinary public health ~~and animal welfare~~ worldwide.

EU comment

For clarity reasons and for consistency with the proposed definition of "OIE Guideline", the EU suggests inserting the words "<u>and that is</u>" before the words "intended to ensure".

Furthermore, the EU insists that the definitions of "OIE Standard" and "OIE Guideline" must be identical in all OIE Standards, i.e. in the Terrestrial and Aquatic Codes and Manuals. Different definitions of these terms in the individual OIE Standards would be unacceptable for the EU.

OIE GUIDELINE~~S~~

means a text ~~an OIE publication that provides advice to improve animal health, veterinary public health and animal welfare worldwide and~~ that has been endorsed by an OIE Specialist Commission or the OIE Council~~,~~ but ~~has~~ not ~~been formally~~ adopted by the OIE World Assembly of Delegates, and that provides advice for the maintenance ~~intended to maintain~~ or improvement of *aquatic animal* health and welfare, or~~,~~ veterinary public health ~~or animal welfare~~ worldwide.

EU comment

With reference to the proposed deletion of the words "an OIE publication" in the above definition, the EU is of the opinion that OIE Guidelines should in any event continue to be published, either as a specific OIE publication or on the OIE website. This should be reflected in the definition.

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— Text deleted.

CHAPTER 1.2.

CRITERIA FOR THE INCLUSION OF DISEASES IN THE OIE LIST

EU comment

The EU thanks the OIE and supports the proposed changes to this chapter.

Article 1.2.1.

Introduction

This chapter describes the criteria for the inclusion of ~~listing~~ *diseases* in Chapter 1.3.

The objective of listing *diseases* is to support Member Countries by providing information needed to take appropriate action to prevent the transboundary spread of important *diseases* of *aquatic animals*. This is achieved by transparent, timely and consistent *notification*.

~~For the *diseases* listed in accordance with Article 1.2.2., the corresponding *disease* specific chapters~~ Each *listed disease* usually has a corresponding chapter that assists Member Countries in the harmonisation of *disease* detection, prevention and control, and provides standards for safe international trade in *aquatic animals* and *aquatic animal products*.

The requirements for *notification* ~~of *listed diseases*~~ are detailed in Chapter 1.1.

Principles and methods of validation of diagnostic tests are ~~provided~~ described in Chapter 1.1.2 of the *Aquatic Manual*.

Article 1.2.2.

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

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

AND

2) At least one country ~~or a country with a *zone*~~ 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* exist.

AND

4)

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

    OR

b)   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 and or mortality at a *zone* or country level.

OR

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

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—   Text deleted.

CHAPTER 1.2.

CRITERIA FOR LISTING AQUATIC ANIMAL ~~THE INCLUSION OF DISEASES IN THE OIE~~ LIST

Article 1.2.1.

Introduction

This chapter describes the criteria for the inclusion of ~~listing~~ *diseases* in Chapter 1.3.

The objective of listing *diseases* is to support Member Countries' by providing information needed to take appropriate action ~~efforts~~ to prevent the transboundary spread of important *diseases* of *aquatic animals*. This is achieved by ~~through~~ transparent, timely and consistent ~~reporting~~ *notification*.

~~For the *diseases* listed in accordance with Article 1.2.2., the corresponding *disease* specific chapters~~ in the ~~*Aquatic Code*~~ Each *listed disease* usually has a corresponding chapter that assists Member Countries in the harmonisation of *disease* detection, prevention and control and provide standards for safe *international trade* in *aquatic animals* and *aquatic animal* ~~their~~ *products*.

The requirements for *notification* ~~of *listed diseases*~~ are detailed in Chapter 1.1.

Principles and methods of~~or~~ validation ~~selection~~ of diagnostic tests are provided ~~described~~ described in Chapter 1.1.2. of the *Aquatic Manual*.

Article 1.2.2.

The c~~C~~riteria for the inclusion of a ~~listing an aquatic animal~~ *disease* in the OIE list are as follows:

~~*Diseases* proposed for listing should meet the relevant criteria as set out in A. Consequences, B. Spread and C. Diagnosis. Therefore, to be listed, a *disease* should have the following characteristics: 1 or 2 or 3; and 4 or 5; and 6; and 7; and 8. Such proposals should be accompanied by a *case definition* for the *disease* under consideration.~~

~~No.~~		~~Criteria for listing~~	~~Explanatory notes~~
		A. Consequences	
1 OR	b.	The *disease* has been shown to affect ~~cause a significant production losses at a national or multinational (zonal or regional) level~~ ~~impact on the health of cultured *aquatic animals* at the~~ level of a country or a *zone* ~~taking into account the occurrence and severity of the clinical signs,~~ resulting in significant consequences ~~impacts~~, e.g. production losses, morbidity ~~and~~ or mortality at a *zone* or country level. ~~including direct production losses and mortality~~.	~~There is a general pattern that the disease will lead to losses in susceptible species, and that morbidity or mortality are related primarily to the infectious agent and not management or environmental factors. (Morbidity includes, for example, loss of production due to spawning failure.) The direct economic impact of the disease is linked to its morbidity, mortality and effect on product quality.~~
2 OR	c. Or	The *disease* has been shown to, or scientific evidence indicates that it ~~is likely to~~ would, affect ~~cause a significant impact on~~ the health of ~~morbidity or mortality in~~ *wild aquatic animals* resulting in significant consequences, e.g. morbidity ~~and~~ or mortality at a population level. reduced productivity or ~~and~~ ecological impacts. ~~populations taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats~~.	~~Wild aquatic animal populations can be populations that are commercially harvested (wild fisheries) and hence are an economic asset. However, the asset could be ecological or environmental in nature, for example, if the population consists of an endangered species of aquatic animal or an aquatic animal potentially endangered by the disease.~~

Annex 11B (contd)

No.		Criteria for listing	Explanatory notes
AND			
3.4.	a.Or	The agent is of public health concern. Natural transmission to humans has been proven, and human infection is associated with severe consequences.	
And B. Spread			
4.	-	Infectious aetiology of the disease is proven.	-
5.	Or	An infectious agent is strongly associated with the disease, but the aetiology is not yet known.	Infectious diseases of unknown aetiology can have equally high risk implications as those diseases where the infectious aetiology is proven. Whilst disease occurrence data are gathered, research should be conducted to elucidate the aetiology of the disease and the results be made available within a reasonable period of time.
No.		Criteria for listing	Explanatory notes
And B. Spread			
6.1.	And	Likelihood of iInternational spread, of the *pathogenic agent* including (via live *aquatic animals*, their *aquatic animal products*, vectors or fomites) is likely has been proven.	International trade in aquatic animal species susceptible to the disease exists or is likely to develop and, under international trading practices, the entry and establishment of the disease is likely.
AND			
7.2.	And	At least one Several countryies or a country with a zone may or countries with zones has demonstrated country or zone freedom or impending freedom from the *disease* in populations of susceptible *aquatic animals*, may be declared free of the disease based on the general surveillance provisions principles outlined in of Chapters 1.4. and 1.5.	Free countries/zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a disease can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread diseases, or the protection of the last remaining free zones from a widespread disease.
And C. Diagnosis			
AND			
8.3.		A repeatable and robust A precise *case* definition is available and a rReliable means of detection/ and diagnosis exists and a precise *case* definition is available to clearly identify *cases* and allow them to be distinguished from other *diseases*.	A diagnostic test should be widely available and preferably has undergone a formal standardisation and validation process using routine field samples (See *Aquatic Manual.*) or a robust case definition is available to clearly identify cases and allow them to be distinguished from other pathologies.

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— Text deleted.

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

EU comment

The EU in general supports the proposed changes to this chapter, except for the proposed listing of *Batrachochytrium salamandrivorans* (Bsal).

While acknowledging the possible devastating effects of that disease on certain wildlife species and therefore being in favour in principle of listing it from a biodiversity and conservation point of view, the EU has serious doubts as to whether Bsal meets the current criteria for listing diseases in the Aquatic Code.

Indeed, as pointed out in the assessment report in Annex 29, there are major uncertainties as regards criterion No. 8, which requires the existence of "*a repeatable and robust means of detection/diagnosis*", which is clearly not the case for Bsal. As noted in the assessment report, for both the PCR and culture methods there are only limited validation studies available. It is thus not possible to assess the repeatability and robustness of these tests, which are essential test characteristics necessary to assess whether criterion No. 8 is met.

Consequently, there must also be uncertainties as regards criterion No. 7 which requires that "*several countries or countries with zones may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4.*". This can however not be assessed in the absence of reliable detection/diagnosis methods. It thus remains unclear as to whether any country or country with a zone can be declared free of Bsal with the currently avaible surveillance and diagnosis methodologies. Furthermore, the assessment report in Annex 29 itself points out these uncertainties, by stating that there is uncertainty regarding the global distribution of Bsal and that it is merely likely that several countries may be declared free of the disease.

In conclusion, the EU is of the opinion that it would be premature at this stage to list Bsal in the Aquatic Code. Further studies to validate the diagnostic tests are required in order to confirm their repeatability and robustness, after which more data on the distribution of and the freedom from the disease should be collected in several countries, Only then can a reliable assessment of Bsal against the listing criteria be made.

Moreover, the EU would like to point out that as the listing criteria (Chapter 1.2. of the Aquatic Code) are currently in the process of being revised, it would not be advisable to envisage the listing of any new disease (or the delisting of a listed disease) at this stage. Indeed, as the criteria will likely be amended (including the one concerning diagnostic tests), any assessment done at this stage against the current criteria would necessarily not be valid against the revised criteria. Therefore, the assessment of Bsal will need to be repeated once the revised listing criteria are adopted by the OIE Word Assembly.

Finally, the EU would like to stress that in line with current Aquatic Code rules (Chapter 1.1. on notification of diseases), OIE member countries are required to notify the OIE when an emerging diseases as defined in the Aquatic Code has been detected in its territory. This provision is indeed applicable for Bsal and is suited to ensure

> **transparency of the animal health situation in OIE member countries, even when that disease is not listed in the Aquatic Code.**

Preamble: The following *diseases* are listed by the OIE according to the criteria for listing an *aquatic animal disease* (see Article 1.2.2.).

In case of modifications of this list of *aquatic animal diseases* adopted by the World Assembly of Delegates, the new list comes into force on 1 January of the following year.

[…]

Article 1.3.3.

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

–   Acute hepatopancreatic necrosis disease

–   Infection with *Aphanomyces astaci* (Crayfish crayfish plague) (*Aphanomyces astaci*)

–   Infection with yellow head virus genotype 1

–   Infection with Infectious infectious hypodermal and haematopoietic necrosis virus

–   Infection with Infectious infectious myonecrosis virus

–   Infection with *Hepatobacter penaei* (Necrotising necrotising hepatopancreatitis)

–   Infection with Taura syndrome virus

–   Infection with White white spot syndrome virus disease

–   Infection with *Macrobrachium rosenbergii* nodavirus (White white tail disease).

Article 1.3.4.

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

–   Infection with *Batrachochytrium dendrobatidis*

=   Infection with *Batrachochytrium salamandrivorans*

–   Infection with *Ranavirus* ranavirus.

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—   Text deleted.

CHAPTER 9.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

> **EU comment**
>
> **The EU thanks the OIE and supports this new chapter.**

Article 9.X.1.

For the purposes of the *Aquatic Code*, acute hepatopancreatic necrosis disease (AHPND) means *infection* with strains of ~~the bacteria~~ *Vibrio parahaemolyticus* (Vp_{AHPND}) and *V. harveyi* that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB. ~~carrying one or more extrachromosal plasmid(s) that encode for a toxin (Pir^Vp) that induces AHPND histopathological changes in the hepatopancreas (Vp_{AHPND}). V. parahaemolyticus is classified as a member of the V. harveyi clade.~~

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

Article 9.X.2.

Scope

The recommendations in this chapter apply to the following *susceptible species* which meet the criteria for listing species as susceptible in Chapter 1.5.: white leg shrimp (*Penaeus vannamei*) and giant tiger prawn (*Penaeus monodon*).

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.X.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose _regardless of the AHPND status of the exporting country, zone or compartment_ ~~from a country, zone or compartment not declared free from acute hepatopancreatic necrosis disease~~

1) *Competent Authorities* should not require any conditions related to AHPND, regardless of the AHPND 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 9.X.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 100˚C for at least three minutes (or any time/temperature equivalent which has been demonstrated to inactivate Vp_{AHPND});

   c) pasteurised crustacean products that have been subjected to heat treatment at 63˚C for at least 30 minutes (or any time/temperature equivalent which has been demonstrated to inactivate Vp_{AHPND});

   d) crustacean oil;

   e) crustacean *meal*;

   f) chemically extracted chitin.]

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.X.2., other than those referred to in point 1 of Article 9.X.3., *Competent Authorities* should require the conditions prescribed in Articles 9.X.9 to 9.X.11. relevant to the AHPND 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 9.X.2. but which could reasonably be expected to pose a *risk ~~of spread~~ of* AHPND, 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.

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Article 9.X.4.
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Country free from acute hepatopancreatic necrosis disease

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from AHPND if all the areas covered by the shared water bodies are declared countries or *zones* free from AHPND (see Article 9.X.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from AHPND if:

1) none of the *susceptible species* referred to in Article 9.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.X.2. are present and the following conditions have been met:

   a) there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of AHPND;

OR

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

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

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

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.X.5.

Article 9.X.5.

Zone or compartment free from acute hepatopancreatic necrosis disease

If a *zone* or *compartment* extends over more than one country, it can only be declared an AHPND free *zone* or *compartment* if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from AHPND may be declared free by the *Competent Authority(ies)* of the country(ies) concerned if:

1)   none of the *susceptible species* referred to in Article 9.X.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.X.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a)   there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b)   *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of AHPND;

OR

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

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

   b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

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

Article 9.X.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from AHPND following the provisions of points 1 or 2 of Articles 9.X.4. or 9.X.5. (as relevant) may maintain its status as free from AHPND provided that *basic biosecurity conditions* are continuously maintained.

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A country, *zone* or *compartment* that is declared free from AHPND following the provisions of point 3 of Articles 9.X.4. or 9.X.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from AHPND provided that conditions that are conducive to clinical expression of AHPND, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of AHPND, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.X.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from acute hepatopancreatic necrosis disease

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* declared free from AHPND, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.X.4. or 9.X.5. (as applicable) and 9.X.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from AHPND.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.X.3.

Article 9.X.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from acute hepatopancreatic necrosis disease

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

    a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

    b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of Vp_{AHPND}.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

    a) identify stock of interest (cultured or wild) in its current location;

    b) evaluate stock health and disease history;

    c) take and test samples for Vp_{AHPND}, pests and general health/disease status;

    d) import of a founder (F-0) population and quarantine in a secure facility;

    e) produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for Vp_{AHPND} and perform general examinations for pests and general health/disease status;

g)   if Vp_{AHPND} is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as AHPND free or specific pathogen free (SPF) for $Vp_{AHPND;}$

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.X.3.

Article 9.X.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from acute hepatopancreatic necrosis disease

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, 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 9.X.3., or products described in point 1 of Article 9.X.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of Vp_{AHPND}; or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.X.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from acute hepatopancreatic necrosis disease

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should require that:

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

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of Vp_{AHPND};.

This Article does not apply to *commodities* referred to in point 1 of Article 9.X.3.

Annex 13 (contd)

Article 9.X.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from acute hepatopancreatic necrosis disease

1) *Competent Authorities* should not require any conditions related to AHPND, regardless of the AHPND status of the *exporting country, zone* or *compartment*, when authorising the importation or transit of [frozen peeled shrimp or decapod crustacea (shell off, head off)] which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

    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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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'Clean version'
ARTICLE X.X.8. FOR ALL DISEASE-SPECIFIC CHAPTERS (OR ARTICLE 10.4.12. FOR INFECTION WITH INFECTIOUS SALMON ANAEMIA)

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from 'disease X'

1) When importing for *aquaculture*, *aquatic animals* of species referred to in Article X.X.2. from a country, *zone* or *compartment* not declared free from 'disease *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 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, effluent and waste materials to inactive 'pathogen X' (in accordance with Chapter 4.3.) and biosecure disposal of effluent and waste.

3) 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 'disease X' .

   b) In the importing country:

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

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

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

      iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of 'disease X' (as described in the *Aquatic Manual*) and test for "disease X" in accordance with Chapter 1.4.;

      v) if 'disease X' is not detected in the F-1 population may be defined as free from 'disease X' and may be released from *quarantine*;

      vi) if 'disease X' is detected in the F-1 population they should not be released from *quarantine* and should be destroyed and disposed of in a biosecure manner.

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— Text deleted.

OIE Aquatic Animal Health Standards Commission/February 2016

'Track changes version'
Article X.X.8. for all disease-specific chapters (or Article 10.4.12.
for Infection with infectious salmon anemia)

> **EU comment**
>
> **The EU in general supports the proposed changes to this article. Comments are inserted in the text below.**

Importation of ~~live~~ aquatic animals for aquaculture from a country, zone or compartment not declared free from 'disease X'

1) When importing ~~live~~ aquatic animals of species referred to in Article 10.10.2. from a country, zone or compartment not declared free from 'disease X', ~~for aquaculture,~~ the Competent Authority of the importing country should assess the risk ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below

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

   a) the direct delivery to and lifelong holding of the *aquatic animals* in a *quarantine* facility; ~~consignment in biosecure facilities for continuous isolation from the local environment~~; and

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate 'pathogen X' (in accordance with Chapter 4.3) and biosecure disposal of effluent and waste.

~~1.~~ ~~If the intention of the introduction is the establishment of a new stock, the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.~~

3) If the intention is to establish a new stock for *aquaculture*, consider applying the following: ~~For the purposes of the Aquatic Code, the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following main points:~~

   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 'disease X'.

   b) In the importing country:

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

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

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

   iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of 'disease X' (as described in the *Aquatic Manual*) and test for "disease X" in accordance with Chapter 1.4.;

v)   if 'disease *X*' is not detected in the F-1 population may be defined as free from 'disease *X*' and may be released from *quarantine*;

EU comment

The wording in point v) above should slightly be amended for clarity reasons, as follows:

"if 'disease X' is not detected in the F-1 population, that population may be defined as free from 'disease X' and may be released from quarantine;".

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vi)   if 'disease *X*' is detected in the F-1 population they should not be released from *quarantine* and should be destroyed and disposed of in a biosecure manner.

EU comment

A similar change is suggested to point Vi) above, as follows:

" if 'disease X' is detected in the F-1 population, that population they should not be released from quarantine and should be destroyed and disposed of in a biosecure manner.".

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a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for 'pathogen *X*', pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in quarantine;

f   )culture F-1 stock and at critical times in its development (life cycle) sample and test for '*disease X*' and perform general examinations for pests and general health/disease status;

g)   if '*disease X*' is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the basic biosecurity conditions of the importing country, zone or compartment, the F-1 stock may be defined as '*disease X*' free or specific pathogen free (SPF) for '*disease X*';

h)   release SPF F-1 stock from quarantine for aquaculture or stocking purposes in the country, zone or compartment.

2.   With respect to point 3 e), quarantine conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If quarantine conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low infection level.

This Article does not apply to aquatic animals referred to in point 1 of Article 10.10.3.

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—   Text deleted.

CHAPTER 4.4.

RECOMMENDATIONS FOR SURFACE DISINFECTION OF SALMONID EGGS

EU comment

The EU in general supports the proposed changes to this chapter. One comment is inserted in the text below.

Article 4.4.1.

Introduction

The practice of disinfecting salmonid *eggs* at hatcheries is an essential part of ensuring that endemic *diseases* are not transferred between incubators and between facilities and forms a part of routine hatchery hygiene protocols. The *disinfection* process is also important for international trade in when trading salmonid *eggs* between countries*, zones or compartments* compartments, zones or countries to prevent the transfer of some *pathogenic agents*. Although generally effective for *disinfection* of the *egg* surface and reproductive fluids, the use of *disinfectants* will not prevent vertical transmission.

Salmonid *eggs* may be disinfected with a number of chemical agents. However, the most common method used is *disinfection* with the iodine-based product, povidine-iodine.

Iodophores, commonly povidone-iodine solutions, have the advantage of providing a neutral pH, being non-irritant and are relatively non-toxic. The neutral pH is important for minimising toxicity and ensuring efficacy. It is recommended to follow manufacturer's instructions to identify circumstances where pH may be a concern. If other iodine based agents are used for *disinfection* it is essential that they be adequately buffered.

Article 4.4.2.

Disinfection protocol for salmonid eggs

This *disinfection* protocol may be applied to newly fertilised or eyed salmonid *eggs*. However newly fertilised *eggs* should be allowed to commence hardening prior to undergoing the *disinfection* protocol. Although there is a considerable margin of safety for hardened *eggs*, the *disinfection* protocol is not recommended for unfertilised ova or during fertilisation. It is essential that the pH of the iodophore solution is maintained between 6 and 8.

To disinfect salmonid *eggs* the following protocol should be applied:

1) rinsed in pathogen free 0.9% to 1.1% pathogen free saline (30-60 seconds) to remove organic matter; then

2) immersed in a an iodophore solution containing 100 ppm available iodine for a minimum of 10 minutes. The iodophore solution concentration should be monitored to ensure effective levels are maintained used only once. The ratio of *eggs* to iodophore solution should be a minimum of 1:4; then

EU comment

In point 2 above, the EU would prefer keeping the recommendation to use iodophore solutions only once. Indeed, the Aquatic Code should remain strict on this as using the iodophore solution only once is more reliable and less open to user error. We therefore suggest adding the following after "[...] levels are maintsained.":

"The iodophor solution should be used only once."

3) rinsed again in pathogen free 0.9% to 1.1% pathogen free saline for 30-60 seconds; then

4) held in pathogen free water.

All rinsing and *disinfection* solutions should be prepared using pathogen free water. Iodophore solutions may be buffered using sodium bicarbonate (NaHCO₃) if the pH is low.

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— Text deleted.

CHAPTER 9.1.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

EU comment

The EU in general supports the proposed changes to this chapter. One comment is inserted in the text below.

Article 9.1.1.

For the purposes of the *Aquatic Code*, infection with *Aphanomyces astaci* crayfish plague means *infection* with *Aphanomyces A. astaci* Schikora. This organism is a member of a group commonly known as the Class Oomycota (water moulds) (the *Oomycetida*). The disease is commonly known as crayfish plague. Common synonyms are listed in the corresponding chapter of the *Aquatic Manual*.

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

Article 9.1.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5: noble crayfish (*Astacus astacus*), Danube crayfish (*A. leptodactylus*), signal crayfish (*Pacifastacus leniusculus*), Red swamp crawfish (*Procambarus clarkii*), *Austropotamobius torrentium, A. pallipes, Orconectes limosus, O. immunis, Procambarus alleni* and *Potamon potamios.* all species of crayfish in all three crayfish families (*Cambaridae, Astacidae* and *Parastacidae*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 9.1.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with *A. astaci* status of the exporting country, zone or compartment from a country, zone or compartment not declared free from crayfish plague

1) *Competent Authorities* should not require any conditions related to infection with *A. astaci* crayfish plague, regardless of the infection with *A. astaci* crayfish plague 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 9.1.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

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

    d) frozen crayfish products that have been subjected to minus 20°C or lower temperatures for at least 72 hours;

    e) crayfish oil;

    f) crayfish *meal*;

g)   chemically extracted chitin.

2)   When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.1.2., other than those referred to in point 1 of Article 9.1.3., *Competent Authorities* should require the conditions prescribed in Articles 9.1.7. to 9.1.11. relevant to the infection with *A. astaci* ~~crayfish plague~~ 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 9.1.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of infection with *A. astaci* ~~crayfish plague~~, 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.

Article 9.1.4.

Country free from infection with *A. astaci* ~~crayfish plague~~

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with *A. astaci* ~~crayfish plague~~ if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with *A. astaci* ~~crayfish plague~~ (see Article 9.1.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with *A. astaci* ~~crayfish plague~~ if:

1)   none of the *susceptible species* referred to in Article 9.1.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.1.2. are present and the following conditions have been met:

a)   there has been no observed occurrence of the *disease* for at least the last 25 years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

b)   *basic biosecurity conditions* have been continuously met for at least the last 10 years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

a)   *basic biosecurity conditions* have been continuously met for at least the last five years; and

b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last five years without detection of infection with *A. astaci* ~~crayfish plague~~;

OR

4)   it previously made a *self-declaration of freedom* from infection with *A. astaci* ~~crayfish plague~~ and subsequently lost its *disease* free status due to the detection of infection with *A. astaci* ~~crayfish plague~~ but the following conditions have been met:

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

b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last five years without detection of <u>infection with *A. astaci*</u> ~~crayfish plague~~.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.1.5.

Article 9.1.5.

Zone or compartment free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from <u>infection with *A. astaci*</u> ~~crayfish plague~~ if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~ may be declared free by the *Competent Authority(ies)* of the country~~(ies)~~ concerned if:

1) none of the *susceptible species* referred to in Article 9.1.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.1.2. are present in the *zone* or *compartment* and the following conditions have been met:

    a) there has not been any observed occurrence of the *disease* for at least the last 25 years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

    b) *basic biosecurity conditions* have been continuously met for at least the last 10 years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

    a) *basic biosecurity conditions* have been continuously met for at least the last five years; and

    b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last five years without detection of <u>infection with *A. astaci*</u> ~~crayfish plague~~;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from <u>infection with *A. astaci*</u> ~~crayfish plague~~ and subsequently lost its *disease* free status due to the detection of <u>infection with *A. astaci*</u> ~~crayfish plague~~ in the *zone* but the following conditions have been met:

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

    b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

    d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last five years without detection of <u>infection with *A. astaci*</u> ~~crayfish plague~~.

<u>Annex 16</u> (contd)

Article 9.1.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~ following the provisions of points 1 or 2 of Articles 9.1.4. or 9.1.5. (as relevant) may maintain its status as free from <u>infection with *A. astaci*</u> ~~crayfish plague~~ provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~ following the provisions of point 3 of Articles 9.1.4. or 9.1.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from crayfish plague provided that conditions that are conducive to clinical expression of <u>infection with *A. astaci*</u> ~~crayfish plague~~, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

EU comment

The EU suggests also replacing the words "free from crayfish plague" in the third line of the paragraph above by the words "free from <u>infection with *A. astaci*</u>" (consistency).

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of <u>infection with *A. astaci*</u> ~~crayfish plague~~, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.1.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.1.2. from a country, *zone* or *compartment* declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.1.4. or 9.1.5. (as applicable) and 9.1.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.1.3.

Article 9.1.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of *A. astaci*.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a) identify stock of interest (cultured or wild) in its current location;

b) evaluate stock health and disease history;

c) take and test samples for *A. astaci*, pests and general health/disease status;

d) import of a founder (F-0) population and quarantine in a secure facility;

e) produce F-1 generation from the F-0 stock in *quarantine*;

f) culture F-1 stock and at critical times in its development (life cycle) sample and test for *A. astaci* and perform general examinations for pests and general health/disease status;

g) if *A. astaci* is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as <u>free from infection with *A. astaci*</u> ~~crayfish plague free~~ or specific pathogen free (SPF) for *A. astaci*;

h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4) With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.1.3.

Article 9.1.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~, 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 9.1.3., or products described in point 1 of Article 9.1.11., or other products authorised by the *Competent Authority*; and

2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of *A. astaci* or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.1.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~, the *Competent Authority* of the *importing country* should require that:

<u>Annex 16</u> (contd)

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

2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of *A. astaci*.

This Article does not apply to *commodities* referred to in point 1 of Article 9.1.3.

Article 9.1.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~

1) *Competent Authorities* should not require any conditions related to crayfish plague, regardless of the <u>infection with *A. astaci*</u> ~~crayfish plague~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of the following *commodities* which have been prepared and packaged for retail trade and which comply with Article 5.4.2.:

   – no *commodities* listed.

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from <u>infection with *A. astaci*</u> ~~crayfish plague~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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— Text deleted.

CHAPTER 9.3.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

EU comment

The EU supports the proposed changes to this chapter.

Article 9.3.1.

For the purposes of the *Aquatic Code*, <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u> (IHHN) means *infection* with infectious hypodermal and haematopoietic necrosis virus (IHHNV). IHHNV is classified as the species *Penaeus stylirostris densovirus* in of the <u>Genus</u> genus *Brevidensovirus* in the <u>Family</u> family *Parvoviridae*.

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

Article 9.3.2.

Scope

The recommendations in this chapter apply to <u>the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5.:</u> giant tiger prawn *(Penaeus monodon)*, Pacific white <u>leg</u> shrimp *(P. vannamei),* and blue shrimp *(P. stylirostris),* <u>spiny king crab *(P. californiensis),* white shrimp *(P. setiferus)* and giant river prawn *(Macrobrachium rosenbergii)*.</u> These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.3.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose <u>regardless of the infection with IHHNV status of the exporting country, zone or compartment</u> from a country, zone or compartment not declared free from infection with infectious hypodermal and haematopoietic necrosis virus

1) *Competent Authorities* should not require any conditions related to <u>infection with IHHNV</u> IHHN, regardless of the <u>infection with IHHNV</u> IHHN 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 9.3.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 90°C for at least 20 minutes (or any time/temperature equivalent which has been demonstrated to inactivate IHHNV);

   c) crustacean oil;

   d) crustacean *meal*.

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.3.2., other than those referred to in point 1 of Article 9.3.3., *Competent Authorities* should require the conditions prescribed in Articles 9.3.7. to 9.3.11. relevant to the <u>infection with IHHNV</u> IHHN 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 9.3.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of <u>infection with IHHNV</u> ~~IHHN~~, 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.

Article 9.3.4.

Country free from <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u>

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from <u>infection with IHHNV</u> ~~IHHN~~ if all the areas covered by the shared water bodies are declared countries or *zones* free from <u>infection with IHHNV</u> ~~IHHN~~ (see Article 9.3.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from <u>infection with IHHNV</u> ~~IHHN~~ if:

1)   none of the *susceptible species* referred to in Article 9.3.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.3.2. are present and the following conditions have been met:

a)   there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with IHHNV</u> ~~IHHN~~;

OR

4)   it previously made a *self-declaration of freedom* from <u>infection with IHHNV</u> ~~IHHN~~ and subsequently lost its *disease* free status due to the detection of <u>infection with IHHNV</u> ~~IHHN~~ but the following conditions have been met:

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

b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with IHHNV</u> ~~IHHN~~.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.3.5.

Article 9.3.5.

Zone or compartment free from <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u>

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from <u>infection with IHHNV</u> ~~IHHN~~ if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from infection with <u>IHHNV</u> ~~IHHN~~ may be declared free by the *Competent Authority(ies)* of the country~~(ies)~~ concerned if:

1) none of the *susceptible species* referred to in Article 9.3.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.3.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a) there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of <u>infection with IHHNV</u> ~~IHHN~~;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from <u>infection with IHHNV</u> ~~IHHN~~ and subsequently lost its *disease* free status due to the detection of <u>infection with IHHNV</u> ~~IHHN~~ in the *zone* but the following conditions have been met:

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with IHHNV</u> ~~IHHN~~.

Annex 17 (contd)

Article 9.3.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with IHHNV IHHN following the provisions of points 1 or 2 of Articles 9.3.4. or 9.3.5. (as relevant) may maintain its status as free from infection with IHHNV IHHN provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with IHHNV IHHN following the provisions of point 3 of Articles 9.3.4. or 9.3.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from infection with IHHNV IHHN provided that conditions that are conducive to clinical expression of infection with IHHNV IHHN, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with IHHNV IHHN, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.3.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from infection with infectious hypodermal and haematopoietic necrosis virus

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* declared free from infection with IHHNV IHHN, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.3.4. or 9.3.5. (as applicable) and 9.3.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with IHHNV IHHN.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.3.3.

Article 9.3.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with infectious hypodermal and haematopoietic necrosis virus

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from infection with IHHNV IHHN, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of IHHNV.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for IHHNV, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for IHHNV and perform general examinations for pests and general health/disease status;

g)   if IHHNV is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as <u>free from</u> <u>infection with IHHNV</u> I̶H̶H̶N̶ f̶r̶e̶e̶ or specific pathogen free (SPF) for IHHNV;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

3)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.3.3.

Article 9.3.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u>

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from <u>infection with IHHNV</u> I̶H̶H̶N̶, 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 9.3.3., or products described in point 1 of Article 9.3.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of IHHNV or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.3.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u>

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from <u>infection with IHHNV</u> I̶H̶H̶N̶, the *Competent Authority* of the *importing country* should require that:

Annex 17 (contd)

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

2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of IHHNV.

This Article does not apply to *commodities* referred to in point 1 of Article 9.3.3.

Article 9.3.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with</u> infectious hypodermal and haematopoietic necrosis <u>virus</u>

1) *Competent Authorities* should not require any conditions related to <u>infection with IHHNV</u> ~~IHHN~~, regardless of the IHHN status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from <u>infection with IHHNV</u> ~~IHHN~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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C H A P T E R   9 . 4 .

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

EU comment

The EU supports the proposed changes to this chapter.

Article 9.4.1.

For the purposes of the *Aquatic Code*, infection with infectious myonecrosis virus ~~(IMN)~~ means infection with infectious myonecrosis virus (IMNV)~~. This virus~~ which is similar to members of the ~~family~~ Family *Totiviridae*.

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

Article 9.4.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5.: brown tiger prawn *(Penaeus esculentus),* banana prawn *(Penaeus merguiensis),* ~~Pacific white shrimp~~ and white leg shrimp *(Penaeus vannamei).* ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.4.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with IMNV status of the *exporting country, zone or compartment* ~~from a country, zone or compartment not declared free from infectious myonecrosis~~

1) *Competent Authorities* should not require any conditions related to infection with IMNunderline{V}, regardless of the infection with IMNunderline{V} 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 9.4.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

    b) cooked crustacean products that have been subjected to heat treatment at 60˚C for at least three minutes (or any time/temperature equivalent which has been demonstrated to inactivate IMNV);

    c) crustacean oil;

    d) crustacean *meal*;

    e) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.4.2., other than those referred to in point 1 of Article 9.4.3., *Competent Authorities* should require the conditions prescribed in Articles 9.4.7. to 9.4.11. relevant to the infection with IMNV 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 9.4.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of infection with

IMN<u>V</u>, 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.

Article 9.4.4.

Country free from <u>infection with</u> infectious myonecrosis <u>virus</u>

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from <u>infection with</u> IMN<u>V</u> if all the areas covered by the shared water bodies are declared countries or *zones* free from <u>infection with</u> IMN<u>V</u> (see Article 9.4.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from <u>infection with</u> IMN<u>V</u> if:

1)   none of the *susceptible species* referred to in Article 9.4.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.4.2. are present and the following conditions have been met:

    a)   there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

    b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

    a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

    b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with</u> IMN<u>V</u>;

OR

4)   it previously made a *self-declaration of freedom* from <u>infection with</u> IMN<u>V</u> and subsequently lost its *disease* free status due to the detection of <u>infection with</u> IMN<u>V</u> but the following conditions have been met:

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

    b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

    d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with</u> IMN<u>V</u>.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.4.5.

Article 9.4.5.

Zone or compartment free from <u>infection with</u> infectious myonecrosis <u>virus</u>

If a *zone* or *compartment* extends over more than one country, it can only be declared <u>a</u> ~~an IMN free~~ *zone* or *compartment* <u>free from infection with IMNV</u> if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from <u>infection with</u> IMN<u>V</u> may be declared free by the *Competent Authority~~(ies)~~* of the country~~(ies)~~ concerned if:

1)   none of the *susceptible species* referred to in Article 9.4.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.4.2. are present in the *zone* or *compartment* and the following conditions have been met:

a)   there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

b)   *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of <u>infection with</u> IMN<u>V</u>;

OR

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

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

b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with</u> IMN<u>V</u>.

<u>Annex 18</u> (contd)

Article 9.4.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from <u>infection with</u> IMN<u>V</u> following the provisions of points 1 or 2 of Articles 9.4.4. or 9.4.5. (as relevant) may maintain its status as free from <u>infection with</u> IMN<u>V</u> provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from <u>infection with</u> IMN<u>V</u> following the provisions of point 3 of Articles 9.4.4. or 9.4.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from <u>infection with</u> IMN<u>V</u> provided that conditions that are conducive to clinical expression of <u>infection with</u> IMN<u>V</u> as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of <u>infection with</u> IMN<u>V</u>, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.4.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from <u>infection with</u> infectious myonecrosis <u>virus</u>

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.4.2. from a country, *zone* or *compartment* declared free from <u>infection with</u> IMN<u>V</u>, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.4.4. or 9.4.5. (as applicable) and 9.4.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from <u>infection with</u> IMN<u>V</u>.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.4.3.

Article 9.4.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from <u>infection with</u> infectious myonecrosis <u>virus</u>

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from <u>infection with</u> IMN<u>V</u>, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of IMNV.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for IMNV, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for IMNV and perform general examinations for pests and general health/disease status;

g)   if IMNV is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as <u>free from infection with</u> IMN<u>V</u> ~~free~~ or specific pathogen free (SPF) for IMNV;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.4.3.

Article 9.4.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from <u>infection with</u> infectious myonecrosis <u>virus</u>

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from <u>infection with</u> IMN<u>V</u>, 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 9.4.3., or products described in point 1 of Article 9.4.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of IMNV or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.4.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from <u>infection with</u> infectious myonecrosis <u>virus</u>

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from <u>infection with</u> IMN<u>V</u>, the *Competent Authority* of the *importing country* should require that:

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

Annex 18 (contd)

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of IMNV.

This Article does not apply to *commodities* referred to in point 1 of Article 9.4.3.

Article 9.4.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with</u> infectious myonecrosis <u>virus</u>

1)   *Competent Authorities* should not require any conditions related to <u>infection with</u> IMN<u>V</u>, regardless of the <u>infection with</u> IMN<u>V</u> status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from <u>infection with</u> IMN<u>V</u>, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

―   Text deleted.

CHAPTER 9.5.

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

EU comment

The EU supports the proposed changes to this chapter.

Article 9.5.1.

For the purposes of the *Aquatic Code*, infection with *Hepatobacter penaei* necrotising hepatopancreatitis (NHP) means infection with Candidatus *Hepatobacter penaei*. This an obligate intracellular bacterium is a member of the order Order α-Proteobacteria. The disease is commonly known as necrotising hepatopancreatitis.

Article 9.5.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5.: Pacific white white leg shrimp *(Penaeus vannamei)*, blue shrimp *(P. stylirostris)*, northern white shrimp *(P. setiferus)* and northern brown shrimp *(P. aztecus)*. These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.5.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with *H. penaei* status of the *exporting country, zone or compartment* from a country, zone or compartment not declared free from necrotising hepatopancreatitis

1) *Competent Authorities* should not require any conditions related to infection with *H. penaei* NHP, regardless of the infection with *H. penaei* NHP 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 9.5.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

    b) cooked crustacean products that have been subjected to heat treatment at 100°C for at least three minutes (or any time/temperature equivalent which has been demonstrated to inactivate Candidatus H. *penaei*);

    c) pasteurised crustacean products that have been subjected to heat treatment at 63°C for at least 30 minutes (or any time/temperature equivalent which has been demonstrated to inactivate Candidatus H. *penaei*);

    d) crustacean oil;

    e) crustacean *meal*;

    f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.5.2., other than those referred to in point 1 of Article 9.5.3., *Competent Authorities*

should require the conditions prescribed in Articles 9.5.7. to 9.5.11. relevant to the <u>infection with *H. penaei*</u> ~~NHP~~ 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 9.5.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of <u>infection with *H. penaei*</u> ~~NHP~~, 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.

Article 9.5.4.

Country free from <u>infection with *H. penaei*</u> ~~necrotising hepatopancreatitis~~

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from <u>infection with *H. penaei*</u> ~~NHP~~ if all the areas covered by the shared water bodies are declared countries or *zones* free from <u>infection with *H. penaei*</u> ~~NHP~~ (see Article 9.5.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from <u>infection with *H. penaei*</u> ~~NHP~~ if:

1) none of the *susceptible species* referred to in Article 9.5.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.5.2. are present and the following conditions have been met:

   a) there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with *H. penaei*</u> ~~NHP~~;

OR

4) it previously made a *self-declaration of freedom* from <u>infection with *H. penaei*</u> ~~NHP~~ and subsequently lost its *disease* free status due to the detection of <u>infection with *H. penaei*</u> ~~NHP~~ but the following conditions have been met:

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with *H. penaei*</u> ~~NHP~~.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.5.5.

Article 9.5.5.

Zone or compartment free from <u>infection with *H. penaei*</u> ~~necrotising hepatopancreatitis~~

If a *zone* or *compartment* extends over more than one country, it can only be declared <u>a</u> ~~an NHP free~~ *zone* or *compartment* <u>free from infection with *H. penaei*</u> ~~NHP~~ if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from <u>infection with *H. penaei*</u> ~~NHP~~ may be declared free by the *Competent Authority~~(ies)~~* of the country~~(ies)~~ concerned if:

1)   none of the *susceptible species* referred to in Article 9.5.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.5.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a)   there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b)   *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of <u>infection with *H. penaei*</u> ~~NHP~~;

OR

4)   it previously made a *self-declaration of freedom* in the *zone* from <u>infection with *H. penaei*</u> ~~NHP~~ and subsequently lost its *disease* free status due to the detection of <u>infection with *H. penaei*</u> ~~NHP~~ in the *zone* but the following conditions have been met:

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

   b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with *H. penaei*</u> ~~NHP~~.

Annex 19 (contd)

Article 9.5.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with *H. penaei* NHP following the provisions of points 1 or 2 of Articles 9.5.4. or 9.5.5. (as relevant) may maintain its status as free from infection with *H. penaei* NHP provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with *H. penaei* NHP following the provisions of point 3 of Articles 9.5.4. or 9.5.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from infection with *H. penaei* NHP provided that conditions that are conducive to clinical expression of infection with *H. penaei* NHP, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with *H. penaei* NHP, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.5.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from infection with _H. penaei_ necrotising hepatopancreatitis

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* declared free from infection with *H. penaei* NHP, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.5.4. or 9.5.5. (as applicable) and 9.5.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with *H. penaei* NHP.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.5.3.

Article 9.5.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with _H. penaei_ necrotising hepatopancreatitis

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from infection with *H. penaei* NHP, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of Candidatus H. *penaei.*

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for Candidatus *H. penaei*, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for Candidatus *H. penaei* and perform general examinations for pests and general health/disease status;

g)   if Candidatus *H. penaei* is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as <u>free from</u> <u>infection with *H. penaei*</u> ~~NHP~~ free or specific pathogen free (SPF) for Candidatus *H. penaei*;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.5.3.

Article 9.5.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from <u>infection with *H. penaei*</u> ~~necrotising hepatopancreatitis~~

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from <u>infection with *H. penaei*</u> ~~NHP~~, 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 9.5.3., or products described in point 1 of Article 9.5.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of Candidatus *H.* or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.5.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from <u>infection with *H. penaei*</u> ~~necrotising hepatopancreatitis~~

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from <u>infection with *H. penaei*</u> ~~NHP~~, the *Competent Authority* of the *importing country* should require that:

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

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2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of Candidatus *H. penaei.*

This Article does not apply to *commodities* referred to in point 1 of Article 9.5.3.

Article 9.5.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with *H. penaei*</u> ~~necrotising hepatopancreatitis~~

1)   *Competent Authorities* should not require any conditions related to <u>infection with *H. penaei*</u> ~~NHP~~, regardless of the <u>infection with *H. penaei*</u> ~~NHP~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

      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 *commodities*   Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from <u>infection with *H. penaei*</u> ~~NHP~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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C H A P T E R   9 . 6 .

INFECTION WITH TAURA SYNDROME VIRUS

> **EU comment**
>
> **The EU supports the proposed changes to this chapter.**

Article 9.6.1.

For the purposes of the *Aquatic Code*, infection with Taura syndrome virus (TS) means infection with Taura syndrome virus (TSV). Taura syndrome virus is classified as a species of the Genus *Aparavirus,* in the family Family *Dicistroviridae*. Common synonyms are listed in the corresponding chapter of the *Aquatic Manual.*

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

Article 9.6.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5.: Pacific white shrimp or white leg shrimp *(Penaeus vannamei)*, blue shrimp *(P. stylirostris)*, northern white shrimp *(P. setiferus)*, southern white shrimp *(P. schmitti)*, greasyback shrimp prawn *(P. ensis),* and giant tiger prawn *(P. monodon)* and northern brown shrimp (*P. aztecus*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.6.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with TSV TS status of the *exporting country, zone* or *compartment* from a country, zone or compartment not declared free from Taura syndrome

1) *Competent Authorities* should not require any conditions related to infection with TSV TS, regardless of the infection with TSV TS 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 9.6.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

    b) cooked crustacean products that have been subjected to heat treatment at 70°C for at least 30 minutes (or any time/ temperature equivalent which has been demonstrated to inactivate TSV);

    c) pasteurised crustacean products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time / temperature equivalent which has been demonstrated to inactivate TSV);

    d) crustacean oil;

    e) crustacean *meal*;

    f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.6.2., other than those referred to in point 1 of Article 9.6.3., *Competent Authorities*

should require the conditions prescribed in Articles 9.6.7. to 9.6.11. relevant to the infection with TSV TS 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 9.6.2. but which could reasonably be expected to pose a *risk* of spread of infection with TSV TS, 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.

Article 9.6.4.

Country free from infection with Taura syndrome virus

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with TSV TS if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with TSV TS (see Article 9.6.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with TSV TS if:

1)   none of the *susceptible species* referred to in Article 9.6.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.6.2. are present and the following conditions have been met:

   a)   there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with TSV TS;

OR

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

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

   b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the *risk* likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with TSV TS.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.6.5.

Annex 20 (contd)

Article 9.6.5.

Zone or compartment free from <u>infection with</u> Taura syndrome <u>virus</u>

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from <u>infection with TSV</u> ~~TS~~ if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from <u>infection with TSV</u> ~~TS~~ may be declared free by the *Competent Authority~~(ies)~~* of the country~~(ies)~~ concerned if:

1) none of the *susceptible species* referred to in Article 9.6.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.6.2. are present in the *zone* or *compartment* and the following conditions have been made:

   a) there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been made:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of TS;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from <u>infection with TSV</u> ~~TS~~ and subsequently lost its *disease* free status due to the detection of <u>infection with TSV</u> ~~TS~~ in the *zone* but the following conditions have been met:

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~risk~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with TSV</u> ~~TS~~.

Annex 20 (contd)

Article 9.6.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TSV TS following the provisions of points 1 or 2 of Articles 9.6.4. or 9.6.5. (as relevant) may maintain its status as free from infection with TSV TS provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with TSV TS following the provisions of point 3 of Articles 9.6.4. or 9.6.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from infection with TSV TS provided that conditions that are conducive to clinical expression of infection with TSV TS, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with TSV TS, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.6.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from infection with Taura syndrome virus

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* declared free from infection with TSV TS, 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* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.6.4. or 9.6.5. (as applicable) and 9.6.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with TSV TS.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.6.3.

Article 9.6.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with Taura syndrome virus

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from infection with TSV TS, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

    a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

    b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of TSV.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for TSV, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for TSV and perform general examinations for pests and general health/disease status;

g)   if TSV is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as <u>free from</u> <u>infection with TSV</u> ~~TS~~ free or specific pathogen free (SPF) for TSV;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.6.3.

Article 9.6.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from <u>infection with</u> Taura syndrome <u>virus</u>

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from <u>infection with TSV</u> ~~TS~~, 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 9.6.3., or products described in point 1 of Article 9.6.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of TSV or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.6.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from <u>infection with</u> Taura syndrome <u>virus</u>

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from <u>infection with TSV</u> ~~TS~~, the *Competent Authority* of the *importing country* should require that:

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

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2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of TSV.

This Article does not apply to *commodities* referred to in point 1 of Article 9.6.3.

Article 9.6.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with</u> Taura syndrome <u>virus</u>

1)   *Competent Authorities* should not require any conditions related to <u>infection with TSV</u> ~~TS~~, regardless of the <u>infection with TSV</u> ~~TS~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp or decapod crustacea (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from <u>infection with TSV</u> ~~TS~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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—   Text deleted.

CHAPTER 9.8.

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

EU comment

The EU supports the proposed changes to this chapter.

Article 9.8.1.

For the purposes of the *Aquatic Code*, infection with *Macrobrachium rosenbergii* nodavirus means infection with *Macrobrachium rosenbergii* nodavirus (MrNV) of the Family *Nodaviridae*. The disease is commonly known as white tail disease. white tail disease (WTD) means *infection* with macrobrachium nodavirus (MrNV). This virus has yet to be formally classified.

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

Article 9.8.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5.: the giant fresh water prawn *(Macrobrachium rosenbergii)*. Other common names are listed in the *Aquatic Manual.* These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.

Article 9.8.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose regardless of the infection with MrNV status of the *exporting country, zone or compartment* from a country, zone or compartment not declared free from white tail disease

1) *Competent Authorities* should not require any conditions related to infection with MrNV WTD, regardless of the infection with MrNV the WTD 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 9.8.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

    b) cooked crustacean products that have been subjected to heat treatment at 60°C for at least 60 minutes (or any time/temperature equivalent which has been demonstrated to inactivate MrNV);

    c) pasteurised crustacean products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been shown to inactivate MrNV);

    d) crustacean oil;

    e) crustacean *meal*;

    f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.8.2., other than those referred to in point 1 of Article 9.8.3., *Competent Authorities*

should require the conditions prescribed in Articles 9.8.7. to 9.8.11. relevant to the infection with MrNV WTD 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 9.8.2. but which could reasonably be expected to pose a *risk* of spread of infection with MrNV WTD, 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.

Article 9.8.4.

Country free from infection with MrNV white tail disease

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with MrNV WTD if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with MrNV WTD (see Article 9.8.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with MrNV WTD if:

1)   none of the *susceptible species* referred to in Article 9.8.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2)   any of the *susceptible species* referred to in Article 9.8.2. are present and the following conditions have been met:

    a)   there has been no observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

    b)   *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3)   the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

    a)   *basic biosecurity conditions* have been continuously met for at least the last two years; and

    b)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with MrNV WTD;

OR

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

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

    b)   infected populations have been destroyed or removed from the *infected zone* by means that minimise the *risk* likelihood of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

    d)   *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with MrNV WTD.

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.8.5.

Article 9.8.5.

Zone or compartment free from <u>infection with MrNV</u> ~~white tail disease~~

If a *zone* or *compartment* extends over more than one country, it can only be declared a ~~WTD~~ free *zone* or *compartment* <u>free from infection with MrNV</u> if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from <u>infection with MrNV</u> ~~WTD~~ may be declared free by the *Competent Authority~~(ies)~~* of the country~~(ies)~~ concerned if:

1) none of the *susceptible species* referred to in Article 9.8.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.8.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a) there has not been any observed occurrence of the *disease* for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:

   a) *basic biosecurity conditions* have been continuously met for at least the last two years; and

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of <u>infection with MrNV</u> ~~WTD~~;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from <u>infection with MrNV</u> ~~WTD~~ and subsequently lost its *disease* free status due to the detection of <u>infection with MrNV</u> ~~WTD~~ in the *zone* but the following conditions have been met:

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

   b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the ~~*risk*~~ <u>likelihood</u> of further spread of the *disease*, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

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

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of <u>infection with MrNV</u> ~~WTD~~.

Annex 21 (contd)

Article 9.8.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with MrNV WTD following the provisions of points 1 or 2 of Articles 9.8.4. or 9.8.5. (as relevant) may maintain its status as free from infection with MrNV WTD provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with MrNV WTD following the provisions of point 3 of Articles 9.8.4. or 9.8.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as free from infection with MrNV WTD provided that conditions that are conducive to clinical expression of infection with MrNV WTD, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with MrNV WTD, *targeted surveillance* needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.8.7.

Importation of aquatic animals and aquatic animal products from a country, zone or compartment declared free from infection with MrNV white tail disease

When importing *aquatic animals* and *aquatic animal products* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* declared free from infection with MrNV WTD, 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 e*xporting country* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles 9.8.4. or 9.8.5. (as applicable) and 9.8.6., the place of production of the *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with MrNV WTD.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.8.3.

Article 9.8.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with MrNV white tail disease

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV WTD, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

   a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and

   b) the treatment of water used in transport and of all effluent and waste materials in a manner that ensures inactivation of MrNV WTDV.

2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.

3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx) may be summarised to the following points:

a)   identify stock of interest (cultured or wild) in its current location;

b)   evaluate stock health and disease history;

c)   take and test samples for MrNV WTDV, pests and general health/disease status;

d)   import of a founder (F-0) population and quarantine in a secure facility;

e)   produce F-1 generation from the F-0 stock in *quarantine*;

f)   culture F-1 stock and at critical times in its development (life cycle) sample and test for MrNV WTD and perform general examinations for pests and general health/disease status;

g)   if MrNV WTDV is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as free from infection with MrNV WTD free or specific pathogen free (SPF) for MrNV WTDV;

h)   release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.

4)   With respect to point 3 e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* listed in point 1 of Article 9.8.3.

Article 9.8.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with MrNV white tail disease

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV WTD, 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 9.8.3., or products described in point 1 of Article 9.8.11., or other products authorised by the *Competent Authority*; and

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of WTDV or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.8.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with MrNV white tail disease

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV WTD, the *Competent Authority* of the *importing country* should require that:

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

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<u>Annex 21</u> (contd)

2)   water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of <u>MrNV</u> ~~WTDV~~.

This Article does not apply to *commodities* referred to in point 1 of Article 9.8.3.

Article 9.8.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from <u>infection with MrNV</u> ~~white tail disease~~

1)   *Competent Authorities* should not require any conditions related to <u>infection with MrNV</u> ~~WTD~~, regardless of the <u>infection with MrNV</u> ~~WTD~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of frozen peeled shrimp (shell off, head off) which have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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 *commodities* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *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, of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from <u>infection with MrNV</u> ~~WTD~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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CHAPTER 2.2.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

EU comment

The EU supports this draft new chapter.

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1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (Vp_{AHPND}) and *V. harveyi* that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent isolates

AHPND has a bacterial aetiology (Kondo *et al.*, 2015; Kwai *et al.,* 2014; Tran *et al.,* 2013a; 2013b; Liu *et al.,* 2014). It is caused by specific virulent strains of *Vibrio* species, including *V. parahaemolyticus* (Vp_{AHPND}) and *V. harveyi*, 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.,* 2015; Kondo *et al.,* 2014; Lee *et al.,* 2015; Yang *et al.,* 2014). The plasmid within AHPND-causing *V. parahaemolyticus* (Vp_{AHPND}) has been designated pVA1, and its size may vary slightly. Removal (or "curing") of pVA1 abolishes the AHPND-causing ability of the virulent strain of *V. parahaemolyticus.* A pVA1-cured strain fails to induce the massive sloughing of cells in the hepatopancreatic tubules that is a primary histopathological characteristic of AHPND (Lee *et al.,* 2015).

Within a population of AHPND-causing bacteria, natural deletion of the Pir^{vp} region may occur in a few individuals (Tinwongger *et al.,* 2014). This deletion is due to the instability caused by the repeat sequences or transposase that flank the Pir toxin operon, and although different strains exhibit different levels of stability, when the deletion occurs, it means that a virulent strain of *V. parahaemolyticus* will lose its ability to induce AHPND. However, if the Pir toxin sequence is used as a target for detection, then a colony that has this deletion will produce a negative result even though the colony was derived from an isolate of AHPND-causing bacteria.

The plasmid pVA1 also carries a cluster of genes related to conjugative transfer, which means that this plasmid is potentially able to transfer to other bacteria. The pVA1 plasmid also carries the *pnd*A gene, which is associated with a post-segregational killing (psk) system. For a bacterium that harbours a plasmid with the psk system (PSK^+), only progeny that inherit the PSK^+ plasmid will be viable. Progeny that do not inherit the PSK^+ plasmid will die because the stable *pnd*A mRNA will be translated to PndA toxin that will kill the bacterium. The presence of a psk system on a plasmid thus ensures that the plasmid is inherited during bacterial replication. The pVA1 plasmid will therefore be passed on to subsequent generations of Vp_{AHPND} producing $PirA^{vp}$ and $PirB^{vp}$.

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

AHPND-causing strains of *V. parahaemolyticus* (Vp_{AHPND}) would be expected to possess similar properties to other strains of *V. parahaemolyticus* found in seafood which have been shown to survive up to 9 and 18 days in filtered estuarine water and filtered seawater, respectively (Karunasagar *et al.*, 1987).

2.1.3. Stability of the agent

Experimental studies have shown that AHPND could not be transmitted via frozen infected shrimp (Tran *et al.*, 2013a). In addition, other strains of *V. parahaemolyticus* are known to be sensitive to

freezing, refrigeration, heating and common disinfectants (Muntada-Garriga *et al.*, 1995; Thompson & Thacker, 1973; Andrews *et al.*, 2000; Su & Liu, 2007).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species (common and Latin names)

Species that fulfil the criteria for listing a species as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Code* include: white leg shrimp (*Penaeus vannamei*); giant tiger prawn (*P. monodon*).

2.2.2. Species with incomplete evidence for susceptibility

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

2.2.3. Susceptible stages of the host

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; Leaño & Mohan, 2013; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b). Interestingly, there is a report (de la Pena *et al.*, 2015) of disease outbreaks in the Philippines occurring as late as 46–96 days after pond-stocking.

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

Not applicable.

2.2.4. Target organs and infected tissue

Gut-associated tissues and organs.

2.2.5. Persistent infection

No data/not known.

2.2.6. Vectors

None are known, although since *Vibrio* spp. are ubiquitous in the marine environment, the presence of vectors would not be unexpected.

2.3. Disease pattern

2.3.1. Transmission mechanisms

AHPND has been transmitted experimentally by immersion, in feed and reverse gavage (Joshi *et al.*, 2014b; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b), simulating natural horizontal transmission via oral routes and co-habitation.

2.3.2. Prevalence

Vibrio spp. are ubiquitous in the marine environment. In regions where AHPND is enzootic in farmed shrimp evidence indicates a near 100% prevalence (Tran *et al.*, 2014a).

2.3.3. Geographical distribution

The disease was reported initially in 2010 from China (People's Rep. of), and subsequently from Vietnam (2010), Malaysia (2011), Thailand (2012) (Flegel, 2012; Lightner *et al.*, 2012), Mexico (2013) (Nunan *et al.*, 2014) and the Philippines (2014) (de la Pena *et al.*, 2015; Dabu *et al.*, 2015).

2.3.4. Mortality and morbidity

AHPND is characterised by sudden, mass mortalities (up to 100%) within 30-35 days of stocking grow-out ponds with PLs or juveniles (FAO, 2013; NACA, 2012) and can be reproduced experimentally (Joshi *et al.,* 2014a; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; and Tran *et al.,* 2013b).

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

Water sources with low salinity (<20 ppt) seem to reduce the incidence of the disease. Although AHPND can be found year round in South-East Asia, the hot and dry season from April to July seems to be the peak. Overfeeding, poor seed quality, poor water quality, poor feed quality, algal blooms or crashes are also factors that may lead to AHPND in endemic areas (FAO, 2013; NACA, 2012).

2.4. Control and prevention

2.4.1. Vaccination

Not applicable.

2.4.2. Chemotherapy

Not applicable.

2.4.3. Immunostimulation

Not applicable.

2.4.4. Resistance breeding

Not applicable.

2.4.5. Restocking with resistant species

None available.

2.4.6. Blocking agents

None available.

2.4.7. Disinfection of eggs and larvae

None known.

2.4.8. General husbandry practices

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, reduced over-crowding etc. are all well-established practices that reduce the impact of disease, including AHPND (NACA, 2012).

3. Sampling

3.1. Selection of individual specimens

Samples of moribund shrimp or shrimp that show clinical signs (see Section 4.1.1) should be selected for AHPND diagnosis. It is assumed that adults (broodstock) can carry Pir toxin-bearing strains of *V. parahaemolyticus* or other *Vibrio* spp. (Han *et al.,* 2015; Lee *et al.*, 2015; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b). Therefore, broodstock without clinical signs may also be selected for diagnostic testing.

3.2. 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 polymerase chain reaction (PCR) detection and (iii) preserved in Davison's AFA fixative for histology (Joshi *et al.,* 2014a; 2014b; Leaño & Mohan, 2013; Lee *et al.,* 2015; Nunan *et al.,* 2104; Sirikharin *et al.,* 2015; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b).

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3.3. Pooling of samples

Samples, especially PL or specimens up to 0.5 g can be pooled for molecular testing. Larger shrimp should be processed individually.

3.4. Best organs or tissues

Samples of gut-associated tissues and organs, such as hepatopancreas, stomach, the midgut and the hindgut are suitable. In addition, faecal (non-lethal) samples may be collected from valuable broodstock.

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

Samples other than gut-associated tissues and organs are not appropriate (FAO, 2013; NACA, 2012; 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Gross signs

The onset of clinical signs and mortality can start as early as 10 days post-stocking and can be used for presumptive diagnosis. Clinical signs include a pale-to-white hepatopancreas (HP), significant atrophy of the HP, soft shells, guts with discontinuous, or no, contents, 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, 2012; 2014).

4.1.2. Behavioural changes

Not applicable.

4.2. Clinical methods

4.2.1. Clinical chemistry

None are known.

4.2.2. Microscopic pathology

The disease has two distinct phases:

i) The acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (FAO, 2013; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; 2013b; 2014a; 2014b).

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 (FAO, 2013; Leaño & Mohan, 2013; NACA, 2012; 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; 2013b; 2014a; 2014b).

4.2.3. Wet mounts

Not applicable.

4.2.4. Smears

Not applicable.

4.2.5. Fixed sections (for ISH)

ISH is not currently available (October 2015).

4.2.6. Electron microscopy or cytopathology

Not applicable.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Not applicable.

4.3.1.1.2. Smears

Not applicable.

4.3.1.1.3. Fixed sections

See Section 4.2.2.

4.3.1.2. Agent isolation and identification

Pir toxin-producing strains of *V. parahaemolyticus* (and other bacterial species) can be isolated on standard media used for isolation of bacteria from diseased shrimp (Lee *et al.,* 2015; Soto-Rodriguez *et al.,* 2015). Bacterial identification may be carried out using 16S rRNA PCR and sequencing (Weisburg *et al.,* 1991), and their probable ability to cause AHPND using AHPND-specific PCR methods described in section 4.3.1.2.3.

4.3.1.2.1. Cell culture or artificial media

See sections 4.3.1.2.3.1.1 and 4.3.1.2.3.1.2.

4.3.1.2.2. Antibody-based antigen detection methods

None is available to date (October 2015).

4.3.1.2.3. Molecular techniques

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

PCR methods have been developed that target the AHPND toxin genes. The AP3 method is a single-step PCR that targets the 12.7 kDa *pir*A^{vp} gene (Sirikharin *et al.,* 2015). It was validated for 100% positive and negative predictive value by testing 104 isolates of AHPND-causing and non-pathogenic bacteria (including other *Vibrio* and non-*Vibrio* species) that had previously been tested by bioassay (Kwai *et al.,* 2014; Sirikharin *et al.,* 2015). Subsequently, Soto-Rodriguez *et al.* (2015), using 9 AHPND-causing 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. *Vp*PirA-284, *Vp*PirB-392 (Han *et al.*, 2015a) and TUMSAT-Vp3 (Tinwongger *et al.*, 2014), have relatively low sensitivity when used for detection of AHPND-causing bacteria 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 4.3.1.2.3.1.1) is recommended.

Alternatively, a nested PCR method, AP4, has been developed with a 100% positive predictive value for AHPND-causing bacteria 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 AHPND-causing bacteria), allowing it to be used directly with tissue and environmental samples without an enrichment step.

In addition, real-time PCR (qPCR) methods, for example the AHPND-specific TaqMan qPCR developed by Han *et al.* (2015b), and an isothermal loop-mediated amplification protocol (LAMP) method developed by Koiwai *et al.* (2015) also have high sensitivity and can be used directly with tissue and environmental samples without an enrichment step.

4.3.1.2.3.1.1 Enrichment of samples prior to DNA extraction

Preliminary enrichment culture for detection of AHPND-causing bacteria 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.

4.3.1.2.3.1.2 Agent purification

The causative agent of 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.*, 2013a; 2013b). Confirmation of identification as an AHPND-causing bacteria may be undertaken by PCR analysis and bioassay.

4.3.1.2.3.1.3 DNA extraction

A general DNA extraction method may be used to extract DNA from the stomach or hepatopancreatic tissue of putatively infected shrimp, from cultures of purified bacterial isolates or from bacterial pellets from enrichment cultures (see above). The amount of template DNA in a 25 µl PCR reaction volume should be in the range of 0.01–1 ng of DNA when extracted from bacterial isolates (i.e. directly from a purified culture) and in the range of 10–100 ng of total DNA when extracted from shrimp tissues or from a bacterial pellet derived from an enrichment culture.

4.3.1.2.3.1.4 PCR primers for one-step PCR detection of AHPND-causing bacteria

Four one-step PCR methods (AP3, TUMSAT-Vp3, VpPirA-284 and VpPirB-392) are described here for detection of Pir toxin genes. The primers, target gene and the size of the expected amplicons are listed in Table 4.1.

Table 4.1. *PCR primers for one-step PCR detection of AHPND-causing bacteria*

Method name	Primers	Target gene	Expected amplicon size	Reference
AP3	AP3-F: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP3-R: 5'-GTG-GTA-ATA-GAT-TGT-ACA-GAA-3'	*pirA*^{vp}	333bp	Sirikharin *et al.*, 2014, 2015
TUMSAT-Vp3	TUMSAT-Vp3 F: 5'-GTG-TTG-CAT-AAT-TTT-GTG-CA-3' TUMSAT-Vp3 R: 5'-TTG-TAC-AGA-AAC-CAC-GAC-TA-3'	*pirA*^{vp}	360bp	Tinwongger *et al.*, 2014
VpPirA-284	VpPirA-284F: 5'-TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG-3' VpPirA-284R: 5'-CAC-GAC-TAG-CGC-CAT-TGT-TA-3'	*pirA*^{vp}	284bp	Han *et al.,* 2015a
VpPirB-392	VpPirB-392F: 5'-TGA-TGA-AGT-GAT-GGG-TGC-TC-3' VpPirB-392R: 5'-TGT-AAG-CGC-CGT-TTA-ACT-CA-3'	*pirB*^{vp}	392bp	Han *et al.,* 2015a

4.3.1.2.3.1.5 AP4 nested PCR primers for detection of AHPND bacteria

The nested PCR primers, designed using the China (People's Rep. of) isolate of AHPND bacteria (Yang *et al.,* 2014), are shown in Table 4.2. 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 4.2. *Primers for the AP4, nested PCR method for detection of AHPND-causing bacteria*

Method name	Primers	Expected amplicon size	Reference
AP4 Step 1	AP4-F1: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP4-R1: 5'-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'	1269	Dangtip *et al.,* 2015
AP4 Step 2	AP4-F2: 5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3' AP4-R2: 5'- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'	230	

4.3.1.2.3.1.6 Primers and Probe for AHPND-specific qPCR

The primers and probe and target gene for the AHPND-specific qPCR are listed in Table 4.3.

Table 4.3. *Primers and probe for the qPCR method for detection of AHPND-causing bacteria*

Primer/ probe name	Sequence	Target gene	Reference
VpPirA-F	5'-TTG-GAC-TGT-CGA-ACC-AAA-CG-3'		
VpPirA-R	5'-GCA-CCC-CAT-TGG-TAT-TGA-ATG-3'	pirA	Han *et al.,* 2015
VpPirA Probe	5'-6FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA-3'		

4.3.1.2.3.1.7 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.

4.3.1.2.3.1.8 Protocol for the VpPirA-284 and VpPirB-392 PCR methods

This protocol follows the method described by Han *et al.* (2015) 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.

4.3.1.2.3.1.9 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.

4.3.1.2.3.1.10 Protocol for the AP4 nested PCR method

This protocol follows the method described by Sritnyalucksana *et al.* (2015). The first PCR reaction mixture consists of 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.

4.3.1.2.3.1.11 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 6x 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 4.1, 4.2 and 4.3) indicate a positive result. Positive results must be confirmed by sequence analysis.

4.3.1.2.3.1.12 Protocol for the AHPND-specific qPCR method

This protocol is based on the method described by Han *et al.* (2015). The TaqMan Fast Universal PCR Master Mix (Life Technologies) is used and extracted DNA is added to the qPCR mixture containing 0.3 µM of each primer and 0.1 µM probe to a final volume of 10 µM. qPCR 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 qPCR 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.

4.3.1.2.3.1.13 Controls for all PCR methods

The following controls should be included in all 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 AHPND-affected shrimp tissue or DNA from an 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 the 16S rRNA PCR for bacteria (Weisburg *et al.*, 1991).

While details of each PCR protocol are provided here, as with any diagnostic test individual laboratories should validate the tests for the specific reagents and platform used within their own laboratories.

4.3.2. Serological methods

Not applicable.

4.3.3. Bioassay

AHPND has been transmitted experimentally by immersion and reverse gavage (Joshi *et al.*, 2014b; Nunan *et al.*, 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b), 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 are processed for AHPND PCR and sequence analysis. Moribund shrimp are processed for histology, bacterial re-isolation and AHPND PCR and sequence analysis.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of AHPND are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PL	Juveniles	Adults		
Gross signs	d	d	c	c	C	d
Bioassay	d	d	d	d	D	a
Histopathology	d	c	a	c	A	b
qPCR	d	a	a	a	A	b
Nested PCR and sequence	d	b	b	b	A	a
1-step PCR and Sequence	d	d	d	d	A	a

PL = postlarvae; PCR = polymerase chain reaction; qPCR = real-time PCR.

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

As indicated in Table 5.1, qPCR is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

i)   Mortality associated with clinical signs of AHPND

ii)   Histopathology indicative of AHPND

iii)   Detection of Pir toxin genes by PCR or qPCR.

7.2. Definition of confirmed case

AHPND is considered to be confirmed if two or more of the following criteria are met:

i)   Histopathology indicative of AHPND

ii)   Detection of Pir toxin gene and pVA1 plasmid by PCR and sequence analysis

iii)   Positive results by bioassay (clinical signs, mortality, histopathology, PCR and sequence).

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CHAPTER 2.2.1.

INFECTION WITH ~~CRAYFISH PLAGUE~~ ~~(~~*APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

EU comment

The EU in general supports the proposed changes to this chapter. Comments are inserted in the text below.

1. Scope

Infection with *Aphanomyces astaci* means infection with *A. astaci* Schikora, a member of the Class Oomycota (water moulds). The disease is commonly known as crayfish plague. ~~For the purpose of this chapter, crayfish plague is considered to be infection of crayfish with *Aphanomyces astaci* Schikora.~~

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

~~The aetiological agent of crayfish plague is *Aphanomyces astaci*.~~ *Aphanomyces astaci* is a member of a group of organisms commonly known as the water moulds. Although long regarded to be fungi, this group, the Oomycetida, are now considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

Four groups (A–D) 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): Group A (the so called *Astacus* strains) comprises a number of strains that were isolated from *Astacus astacus* and *Astacus leptodactylus*; these strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus strains* I) includes isolates from both *A. astacus* in Sweden and *Pacifastacus leniusculus* from Lake Tahoe, USA. Imported *P. leniusculus* have probably introduced *A. astaci* and infected the native *A. astacus* in Europe. 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* strain). This strain shows temperature/growth curves with higher optimum temperatures compared with isolates from northern Europe (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 more recently with crayfish imports from North America since the 1960s.

EU comment

The EU would like to point out that five, not four groups of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR).

Reference:

Kozubíková, E., Viljamaa-Dirks S., Heinikainen, S. and Petrusek, A. (2011) Spiny-cheek crayfish Orconectes limosus carry a novel genotype of the crayfish plague pathogen Aphanomyces astaci. Journal of Invertebrate Pathology 108:214-216. DOI: 10.1016/j.jip.2011.08.002

2.1.2. Survival outside the host

Although *A. astaci* is not an obligate parasite and will grow well under laboratory conditions on artificial media (Alderman & Polglase, 1986; Cerenius *et al.*, 1988), in the natural environment it does not survive well for long periods in the absence of a suitable host.

Aphanomyces astaci zoospores remain motile for up to 3 days and cysts survive for 2 weeks in distilled water (Svensson & Unestam, 1975; Unestam, 1966). As *A. astaci* can go through three cycles of zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension kept at 2°C for 2 months (Unestam, 1966).

2.1.3. Stability of the agent (effective inactivation methods)

Aphanomyces astaci, both in culture and in infected crayfish, is killed by a short exposure to temperatures of 60°C or to temperatures of −20°C (or below) for 48 hours (or more) (Alderman, 2000; Oidtmann *et al.*, 2002). Sodium hypochlorite and iodophors are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection, since organic matter was found to decrease the effectiveness of iodophors (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation.

2.1.4. Life cycle

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. Free-swimming zoospores appear to be chemotactically attracted to crayfish cuticle (Cerenius & Söderhäll, 1984a) and often settle on the cuticle near a wound (Nyhlen & Unestam, 1980). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infective viability (Cerenius & Söderhäll, 1984b). Growth and sporulation capacity is strain-and temperature-dependent (Dieguez-Uribeondo *et al.*, 1995).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with *Aphanomyces astaci* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: noble crayfish (*Astacus astacus*), Danube crayfish (*A. leptodactylus*), signal crayfish (*Pacifastacus leniusculus*), Red swamp crawfish (*Procambarus clarkii*), *Austropotamobius torrentium*, *A. pallipes*, *Orconectes limosus, O. immunis, Procambarus alleni* and *Potamon potamios.*

EU comment

According to this Manual chapter, confirmation of *A. astaci* can be done by PCR and sequencing. *A. astaci* has indeed been confirmed in *Procambarus fallax forma virginalis* (marbled crayfish) by PCR and sequencing.

Reference:

Mrugala et al. (2015) Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome. Biological Invasions 17:1313-1326   DOI: 10.1007/s10530-014-0795-x

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To date, all species of freshwater crayfish have to be considered as susceptible to infection with *A. astaci*. The outcome of an infection varies depending on species. All stages of European crayfish species, 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 (*Astacus leptodactylus*) of eastern Europe and Asia Minor are highly susceptible (Alderman, 1996; Alderman *et al.*, 1984; Rahe & Soylu, 1989; Unestam, 1969b; 1976; Unestam & Weiss, 1970). Laboratory challenges have demonstrated that Australian species of crayfish are also highly susceptible (Unestam, 1976). North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Orconectes* 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 (Oidtmann *et al.*, 2006; Unestam, 1969b; Unestam &

~~Weiss, 1970) 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*) but this was reported only under laboratory conditions (Benisch, 1940).~~

2.2.2.   Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of Aquatic Code include: *Astacopsis fluviatilis, A. gouldi,* red claw crayfish (*Cherax quadricarinatus*), yabby crayfish (*C. destructor*), *C. papuanus, Euastacus crassus, E. claydensis, E. kershawi, Geocherax gracilis,* and Chinese mitten crab (*Eriochier sinensis*).

EU comment

The EU understands that the species mentioned in the paragraph above concern mainly Asia. We suggest including information on North American species as well.

In addition, pathogen-specific positive PCR results have been reported in the following organisms but an active infection has not been demonstrated: *Orconectes cf. virlis, Procambarus fallax virginalis* and *Macrobrachium dayanum.*

EU comment

Please replace the word "virlis" by the word "virilis" in the paragraph above (typographical error).

Given the wide range of hosts found to meet some of all the criteria for susceptibility it is likely that other species, as yet untested or naturally exposed to *A. astaci,* may meet some or all criteria for susceptibility'

2.2.3~~2~~.   Susceptible stages of the host

All live stages need to be considered as susceptible to infection.

2.2.4~~3~~.   Species   or   subpopulation   predilection   (probability   of   detection)

The host species susceptible to infection with *A. astaci* fall largely into 2 categories: those highly susceptible to infection with development of clinical disease and mortalities, and those which are infected without associated clinical disease or mortalities.

Highly susceptible species: ~~in natural~~ clinical disease outbreaks ~~of crayfish plague,~~ 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.

North American crayfish species: the prevalence of infection tends to be lower in animals that have gone through a recent moult (B. Oidtmann, unpublished data). However, large scale systematic studies have not been undertaken to corroborate these observations. Juvenile crayfish go through several moults per year, whereas adult crayfish usually moult at least once per year in temperate climates. Therefore, animals in which the last moult was some time ago may show higher prevalence compared with animals that have recently moulted.

2.2.5~~4~~.   Target organs and infected tissue

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

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

2.2.6~~5~~.   Persistent infection ~~with lifelong carriers~~

A number of North American crayfish species have been ~~investigated for their susceptibility to infection~~ <u>shown to be infected</u> with *A. astaci* ~~and disease~~ (Oidtmann *et al.*, 2006; Unestam, 1969a; Unestam & Söderhäll, 1977). ~~So far, infection has been consistently shown in all North American crayfish species tested to date. Animals investigated were usually clinically healthy.~~ <u>Infection in naturalised or aquaculture reared North American crayfish populations is usually without development of clinical disease or mortalities.</u>

~~This is supported by a recent study where the chances of detecting an *A. astaci* positive signal crayfish were shown to increase significantly with increasing crayfish length. Furthermore, large female crayfish expressed significantly higher levels of *A. astaci* than large males (Vrålstad *et al.,* 2011). The results probably reflect the decreased moult frequency of larger mature individuals compared with smaller immature crayfish (Reynolds, 2002), where mature females tend to moult even less frequently than mature males (Skurdal & Qvenild, 1986).~~

Based on the observations made in North American crayfish species, it seems reasonable to assume that all crayfish species native to the North American continent can be infected with *A. astaci* without development of clinical disease and they may therefore act as ~~lifelong~~ carriers of the pathogen.

<u>A recent report from Finland also suggests that low density Noble crayfish populations in cold water environments may be infected at low levels in a chronic infection (Viljamaa-Dirks *et al.*, 2011).</u>

2.2.<u>7</u>.~~6~~. Vectors

There is good field and experimental evidence that movements of fish from areas in which <u>there is a clinical outbreak of disease due to infection with *A. astaci*</u> ~~crayfish plague is active~~ can transmit infection from one watershed to another (Alderman *et al.*, 1987; Oidtmann *et al.*, 2006).

Fomites: A. astaci can also be spread by contaminated equipment (nets, boots, clothing etc.).

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

~~A number of studies have shown that crayfish species native to North America can act as carriers of *A. astaci* (e.g. signal crayfish, spiny cheek crayfish, red swamp crayfish (Alderman *et al.*, 1990; Oidtmann *et al.*, 2006). Since all North American species tested to date have been shown to be potential carriers of the disease, it is also assumed that North American species not tested to date are likely to act potentially as carriers of *A. astaci*. North American species are wide spread in several regions of Europe.~~

2.3. Disease pattern

2.3.1. Transmission mechanisms

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 fish movements, or 3) through colonisation of habitats by North American crayfish species.

Transmission from crayfish to crayfish occurs, in short, through the release of zoospores from an infected animal and attachment of such zoospores to a naïve crayfish. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (Cerenius & Söderhäll, 1984a).

The main route of spread of ~~crayfish plague~~ <u>*A. astaci*</u> in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms (Alderman, 1996; Dehus *et al.*, 1999). Nowadays, spread mainly occurs through expanding populations of North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (Edsman, 2004; Oidtmann *et al.*, 2005).

Colonisation of habitats, initially occupied by highly susceptible species, by North American crayfish species carrying *A. astaci* is likely to result in an epidemic among the highly susceptible animals. The velocity of spread will depend, among other factors, on the prevalence of infection in the population of North American crayfish.

Fish transports may facilitate the spread of *A. astaci* in a number of ways, such as through the presence of spores in the transport water, *A. astaci* surviving on fish skin, co-transport of infected crayfish specimens, or a combination of all three (Alderman *et al.*, 1987; Oidtmann *et al.*, 2002). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.).

2.3.2. Prevalence

In the highly susceptible European crayfish species, exposure to *A. astaci* spores is considered to lead to infection and eventually to death. The minimal infectious dose has still not been established, but it may be as low as a single spore per animal (B. Oidtmann, unpublished data). Prevalence of infection within a population in the early stage of an outbreak may be low (only one or a 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 velocity of spread from initially affected animals depends on several factors, one being water temperature (Oidtmann *et al.*, 2005). 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 outbreak of crayfish plague in 2001 that was followed in subsequent years suggest that in sparse Noble crayfish populations, spread throughout the host population may be prolonged over a time span of several years.

Prevalence levels in North American crayfish appear to vary greatly. Limited studies suggest prevalence levels ranging from anywhere between 0 and 100% are possible (Oidtmann *et al.*, 2006).

2.3.3. Geographical distribution

First reports of large crayfish mortalities go back to 1860 in Italy (Ninni, 1865; Seligo, 1895). These were followed by further reports of crayfish mortalities, where no other aquatic species were affected, in the Franco-German border region in the third quarter of the 19th century. From there a steady spread of infection occurred, principally in two directions: down the Danube into the Balkans and towards the Black Sea, and across the North German plain into Russia and from there south to the Black Sea and north-west to Finland and, in 1907, to Sweden. In the 1960s, the first outbreaks in Spain were reported, and in the 1980s further extensions of infection to the British Isles, Turkey, Greece and Norway followed (Alderman, 1996). The reservoir of the original infections in the 19th century was never established; *Orconectes* spp. were not known to have been introduced until the 1890s, but the post-1960s extensions are largely linked to movements of North American crayfish introduced more recently for purposes of crayfish farming (Alderman, 1996). Escapes of such introduced species were almost impossible to prevent and *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

Since North American crayfish serve as a reservoir of *A. astaci*, any areas where North American crayfish species are found have to be considered as areas where *A. astaci* is present (unless shown otherwise).

Australia and New Zealand have never experienced any outbreaks of crayfish plague to date and are currently considered free of the infection with *A. astaci* (OIE WAHID website, accessed June 2011).

2.3.4. Mortality and morbidity

When the infection first reaches a naïve population of highly susceptible crayfish species, high levels of mortality are usually observed within a short space of time, so that in areas with high crayfish densities the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread quickly from the initial outbreak site downstream, whereas upstream spread is slower. Where population densities of susceptible crayfish are low fewer zoospores will be produced, the spread of infection will be slower and evidence of mortality less dramatic. Water temperature has some effect on the speed of spread and this is most evident in low-density crayfish populations where animal-to-animal spread takes longer and challenge intensity will be lower. Lower water temperatures and reduced numbers of zoospores are associated with slower mortalities and a greater range of clinical signs in affected animals (Alderman *et al.*, 1987). Observations from Finland suggest that at low water temperatures, noble crayfish can be infected for several months without the development of noticeable mortalities (S. Viljamaa-Dirks, unpublished data).

On rare occasions, single specimens of the highly susceptible species have been found after a wave of crayfish plague 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/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 crayfish plague wave). However, low-virulent strains of ~~crayfish plague~~ 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 (Alderman, 1996; Souty-Grosset *et al.*, 2006). Populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with infection, new outbreaks of crayfish plague in the form of large-scale mortalities will occur.

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows slightly varies depending on the strain. In a study, which compared a number of *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 crayfish plague outbreaks occur at a wide temperature range, and at least in the temperature range from 4–20°C. The velocity 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 epidemic is enhanced by higher water temperatures. At low water temperatures, the epidemic curve can increase very slowly and the period during which mortalities are observed can be several months (B. Oidtmann, unpublished data).

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 in a pH range from 6.–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 & Söderhäll, 1984b).

2.4. Control and prevention

Once *A. astaci* has been introduced into a population of highly susceptible crayfish species in the wild, the spread within the affected population cannot be controlled. Therefore, prevention of introduction is essential. To avoid the main pathways of introduction, the following measures are necessary:

1. 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.

2. When fish transfers are being planned, it should be considered whether the source water may harbour infected crayfish (including North American carrier crayfish).

3. Any fish movements from the site of a current epidemic of crayfish plague carries a high risk of spread and should generally be avoided.

4. If fish movements from a source containing North American crayfish are being planned, fish harvest methods at the source site need to ensure that: a) crayfish are not accidentally co-transported; b) the transport water does not carry *A. astaci* spores, and, c) equipment is disinfected between use; d) the consignment does not become contaminated during transport.

5. The release of North American crayfish into the wild in areas where any of the highly susceptible species are present should be prevented. Once released, North American crayfish tend to spread, sometimes over long distances. Therefore prior to any planned release, careful consideration needs to be given to the long-term potential consequences of such a release. Highly susceptible crayfish populations at a distance from the release site may eventually be affected.

6. Aquaculture facilities for the cultivation of crayfish are very rarely suitable for preventing the spread of crayfish from such sites. Therefore, careful consideration needs to be given, as to whether such facilities should be established.

Certain pathways of introduction, such as the release of North American crayfish by private individuals are difficult to control.

2.4.1. Vaccination

Currently, there is no evidence that vaccines offer long-term protection in crustaceans and even if this were not to be the case, vaccination of natural populations of crayfish is impossible.

2.4.2. Chemotherapy

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. Resistance breeding

In the 125 years since crayfish plague first occurred in Europe, there is little evidence of resistant populations of European crayfish. However, the fact that North American crayfish are not very susceptible to developing clinical disease suggests that selection for resistance may be possible and laboratory studies using *A. astaci* strains attenuated for virulence might be successful. However, there are currently no published data referring to such studies.

2.4.5. Restocking with resistant species

North American crayfish have been used in various European countries to replace the lost stocks of native crayfish. However, since North American crayfish are potential hosts for *A. astaci*, restocking with North American crayfish would further the spread of *A. astaci*. Given the high reproduction rates and the tendency of several North American crayfish species to colonise new habitats, restocking with North American crayfish species would largely prevent the re-establishment of the native crayfish species.

2.4.6. Blocking agents

No data available.

2.4.7. Disinfection of eggs and larvae

Limited information is available on the susceptibility of crayfish eggs to infection with *A. astaci*. Unestam & Söderhäll mention that they experimentally exposed *Astacus astacus* and *P. leniusculus* eggs to zoospore suspensions and were unable to induce infection (Unestam & Söderhäll, 1977). However, the details of these studies have not been published.

Although published data are lacking, disinfection of larvae, once infected, is unlikely to be successful, since *A. astaci* would be protected from disinfection by the crayfish cuticle, in which it would be present.

2.4.8. General husbandry practices

If a crayfish farm for highly susceptible species is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or whether North American crayfish populations may be present upstream (for sites that are "online" on a stream or abstracting water from a stream), even if at a great distance upstream. If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

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

1. 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 must be prevented.

2. If fish transfers are being planned, these must not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of crayfish plague or North American carrier crayfish).

3. North American crayfish must not be brought onto the site.

4. Fish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of crayfish plague may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment that will kill *A. astaci* (see Section 2.1.3).

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

6. General biosecurity measures should be in place (e.g. controlled access to premises; disinfection of boots when site is entered; investigation of mortalities if they occur; introduction of live animals (crayfish, fish) only from sources known to be free of ~~crayfish plague~~ infection with *A. astaci*).

3. Sampling

3.1. Selection of individual specimens

In the case of a suspected outbreak of crayfish plague in a population of highly susceptible crayfish species, the batch of crayfish selected for investigation for the presence of *A. astaci* should ideally consist of: a) live crayfish showing signs of disease, b) live crayfish appearing to be still healthy, and, c) dead crayfish that may also be suitable, although this will depend on their condition.

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

The container should provide insulation against major temperature differences outside the container. In periods of hot weather, freezer packs should be used to avoid temperatures deleterious to the animals. These can be attached at the inside bottom of the transport container. The crayfish must however be protected from direct contact with freezer packs. This can be achieved using, for instance, cardboard or a several layers of newspaper.

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 from lack of oxygen.

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, these might still be suitable for diagnosis. Depending on the condition they are in, they 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 Section 3.2).

Animals showing advanced decay are unlikely to give a reliable result, however, if no other animals are available, these might still be tested.

3.2. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If, for practical reasons, transport of recently dead or moribund crayfish cannot be arranged quickly, crayfish may be 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).

3.3. Pooling of samples

Not recommended.

3.4. Best organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended.

3.5. Samples/tissues that are not suitable

Autolytic material is not suitable for analysis.

4. Diagnostic methods

Large numbers of dead crayfish of the highly susceptible species with the remaining aquatic fauna being unharmed gives rise to a suspicion that the population may be affected by crayfish plague. Clinical signs of crayfish plague include behavioural changes and a range of visible external lesions. However, clinical signs are of limited diagnostic value. The main available diagnostic methods are PCR and isolation of the pathogen in culture media followed by confirmation of its identity. Isolation can be difficult and requires that samples are in good condition when they arrive at the investigating laboratory (Oidtmann *et al.*, 1999). Molecular methods are now available that are less dependent on speed of delivery and can deal with a greater range of samples compared with methods relying on agent isolation (Oidtmann *et al.*, 2006; Vrålstad *et al.*, 2009).

4.1. Field diagnostic methods

4.1.1.   Clinical signs

Highly susceptible species

Gross clinical signs are extremely variable and depend on challenge severity and water temperatures. The first sign of a crayfish plague mortality may be the presence of numbers of crayfish at large during daylight (crayfish are normally nocturnal), some of which may show evident loss of co-ordination in their movements, and easily fall over on their backs and remain unable to right themselves. Often, however, unless waters are carefully observed, the first sign that there is a problem will be the presence of large numbers of dead crayfish in a river or lake (Alderman *et al.*, 1987).

In susceptible species, where sufficient numbers of crayfish are present to allow infection to spread rapidly, particularly at summer water temperatures, infection will spread quickly and stretches of over 50 km may lose all their crayfish in less than 21 days from the first observed mortality (D. Alderman, pers. comm.). Crayfish plague has unparalleled severity of effect, since infected susceptible crayfish generally do not survive. It must be emphasised, however, that the presence of large numbers of dead crayfish, even in crayfish plague-affected watersheds, is not on its own sufficient for diagnosis. The general condition of other aquatic fauna must be assessed. Mortality or disappearance of other aquatic invertebrates, as well as crayfish, even though fish survive, may indicate pollution (e.g. insecticides such as cypermethrin have been associated with initial misdiagnoses).

North American crayfish species

Melanised cuticle has sometimes been suggested as a sign of infection with *A. astaci*. However, melanisation can have a wide variety of causes and is not a specific sign of *A. astaci* infection. Conversely, animals without signs of melanisation are often infected.

4.1.2.   Behavioural changes

Highly susceptible species

Infected crayfish of the highly susceptible crayfish species may leave their hides during daytime (which is not normally seen in crayfish), have a reduced escape reflex, and progressive paralysis. Dying crayfish are sometimes found lying on their backs. The animals are often no longer able to upright themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

North American crayfish species

Infected North American crayfish do not show any behavioural changes (B. Oidtmann, unpublished data).

4.2. Clinical methods

4.2.1.   Gross pathology

Highly susceptible species

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular 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 and finally the gills.

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example the soft abdominal cuticle. However, it must be stressed that these melanisations can be caused by mechanical injuries or infections with other water moulds and are very unspecific. Conversely, visible melanisation is not always associated with carrier status. Infected animals can appear completely devoid of visible melanisations.

4.2.2.   Clinical chemistry

No suitable methods available.

4.2.3.   Microscopic pathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. Additionally, such material does not prove that any hyphae observed are those of the primary pathogen. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used.

See also Section 4.2.4.

4.2.4. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 4.2.1) and examined under a compound microscope using low to medium power will confirm the presence of aseptate fungal 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).

4.2.5. Smears

Not suitable.

4.2.6. Fixed sections

See section 4.2.3.

4.2.7. Electron microscopy/cytopathology

Not suitable.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

As indicated above (Section 4.2.4.), presumptive identification of *A. astaci* may be made from the presence of hyphae pervading the cuticle and sporangia of the correct morphological types (see below) on the surface of crayfish exoskeletons.

4.3.1.1.2. Smears

Not suitable.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Highly susceptible species

Care should be taken that animals to be used for isolation of *A. astaci* via cultivation are not exposed to desiccation.

Isolation methods have been described by Benisch (1940); Nyhlen & Unestam (1980); Alderman & Polglase (1986); Cerenius *et al.* (1988); Oidtmann *et al.* (1999) and Viljamaa-Dirks (2006).

Isolation medium (IM) according to Alderman & Polglase (1986): 12.0 g agar; 1.0 g yeast extract; 5.0 g glucose; 10 mg oxolinic acid; 1000 ml river water; and 1.0 g penicillin G (sterile) added after

autoclaving and cooling to 40°C. River water is defined as any natural river or lake water, as opposed to demineralised water.

Any superficial contamination should first be removed from the soft intersternal abdominal cuticle or any other areas from which cuticle will be excised by thoroughly wiping the cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. Simple aseptic excision of infected tissues, which are then placed as small pieces (3–5 mm^2) on the surface of isolation medium plates , will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 hours) animals. Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low-power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

Provided that care is taken in excising infected tissues for isolation, contaminants need not present significant problems. Small pieces of cuticle and muscle may be transferred to a Petri dish of sterile water and there further cut into small pieces with sterile instruments for transfer to isolation medium (IM). Suitable instruments for such work are scalpels, fine forceps and scissors.

To reduce potential contamination problems, disinfection of the cuticle with ethanol and melting a sterile glass ring 1–2 mm deep into the isolation medium can improve isolation success (Nyhlen & Unestam, 1980; Oidtmann *et al.,* 1999). The addition of potassium tellurite into the area inside the glass ring has been described (Nyhlen & Unestam, 1980).

Inoculated agar can be incubated at temperatures between 16°C and 24°C. The Petri dishes should be sealed with a sealing film (e.g. Parafilm^2) to avoid desiccation.

On IM agar, growth of new isolates of *A. astaci* is almost entirely within the agar except at temperatures below 7°C, when some superficial growth occurs. Colonies are colourless. Dimensions and appearance of hyphae are much the same in crayfish tissue and in agar culture. Vegetative hyphae are aseptate and (5)7–9(10) µm in width (i.e. normal range 7–9 µm, but observations have ranged between 5 and 10 µm). Young, actively growing hyphae are densely packed with coarsely granular cytoplasm with numerous highly refractile globules (Alderman & Polglase, 1986). Older hyphae are largely vacuolate with the cytoplasm largely restricted to the periphery, leaving only thin strands of protoplasm bridging the large central vacuole. The oldest hyphae are apparently devoid of contents. Hyphae branch profusely, with vegetative branches often tending to be somewhat narrower than the main hyphae for the first 20–30 µm of growth.

When actively growing thalli or portions of thalli from broth or agar culture are transferred to river water (natural water with available cations encourages sporulation better than distilled water), sporangia form readily in 20–30 hours at 16°C and 12–15 hours at 20°C. Thalli transferred from broth culture may be washed with sterile river water in a sterile stainless steel sieve, before transfer into fresh sterile river water for induction of sporulation. Thalli in agar should be transferred by cutting out a thin surface sliver of agar containing the fungus so that a minimum amount of nutrient-containing agar is transferred. Always use a large volume of sterile river water relative to the amount of fungus being transferred (100:1). Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. The sporangial form is variable: terminal sporangia are simple, developing from new extramatrical hyphae, while intercalary sporangia can be quite complex in form. Intercalary sporangia develop by the growth of a new lateral extramatrical branch, which forms the discharge tube of the sporangium. The cytoplasm of such developing discharge tubes is noticeably dense, and these branches are slightly wider (10–12 µm) than ordinary vegetative hyphae. Sporangia are delimited by a single basal septum in the case of terminal sporangia and by septa at either end of the sporangial segment in intercalary sporangia. Such septa are markedly thicker than the hyphal wall and have a high refractive index. Successive sections of vegetative hypha may develop into sporangia, and most of the vegetative thallus is capable of developing into sporangia.

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

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Within developing sporangia, the cytoplasm cleaves into a series of elongate units (10–25 × 8 μm) that are initially linked by strands of protoplasm. Although the ends of these cytoplasmic units become rounded, they remain elongate until and during discharge. Spore discharge is achlyoid, that is, the first spore stage is an aplanospore that encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been found for the existence of a flagellated primary spore, thus, in this description, the terms 'sporangium' not 'zoosporangium' and 'primary spore' not 'primary zoospore' have been used. Discharge is fairly rapid (<5 minutes) and the individual primary spores (=cytoplasmic units) pass through the tip of the sporangium and accumulate around the sporangial orifice. The speed of cytoplasmic cleavage and discharge is temperature dependent. At release, each primary spore retains its elongate irregularly amoeboid shape briefly before encystment occurs.

Encystment is marked by a gradual rounding up followed by the development of a cyst wall, which is evidenced by a change in the refractive index of the cell. The duration from release to encystment is 2–5 minutes. Some spores may drift away from the spore mass at the sporangial tip and encyst separately. Formation of the primary cyst wall is rapid, and once encystment has taken place the spores remain together as a coherent group and adhere well to the sporangial tip so that marked physical disturbance is required to break up the spore mass.

Encysted primary spores are spherical, (8)9–11(15) μm in diameter, and are relatively few in number, (8)15–30(40) per sporangium in comparison with other *Aphanomyces* spp. Spores remain encysted for 8–12 hours. Optimum temperatures for sporangial formation and discharge for the majority of European isolates of *A. astaci* are between 16 and 24°C (Alderman & Polglase, 1986). For some isolates, particularly from Spanish waters, slightly higher optimal temperatures may prevail (Dehus *et al.*, 1999). The discharge of secondary zoospores from the primary cysts peaks at 20°C and does not occur at 24°C. In new isolates of *A. astaci*, it is normal for the majority of primary spore cysts to discharge as secondary zoospores, although this varies with staling in long-term laboratory culture. Sporangial formation and discharge occurs down to 4°C. *A. astaci* does not survive at –5°C and below for more than 24 hours in culture, although –20°C for >48 hours may be required in infected crayfish tissues, nor does it remain viable in crayfish tissues that have been subject to normal cooking procedures (Alderman, 2000; Oidtmann *et al.*, 2002).

In many cases, some of the primary spores are not discharged from the sporangium and many sporangia do not discharge at all. Instead, the primary spores appear to encyst *in situ* within the sporangium, often develop a spherical rather than elongate form and certainly undergo the same changes in refractive index that mark the encystment of spores outside the sporangium. This within-sporangial encystment has been observed on crayfish. Spores encysted in this situation appear to be capable of germinating to produce further hyphal growth.

Release of secondary zoospores is papillate, the papilla developing shortly before discharge. The spore cytoplasm emerges slowly in an amoeboid fashion through a narrow pore at the tip of a papilla, rounds up and begins a gentle rocking motion as a flagellar extrusion begins and the spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral (Scott, 1961); zoospores are typical saprolegniaceous secondary zoospores measuring 8 × 12 μm. Active motility takes some 5–20 minutes to develop (dependent on temperature) and, at first, zoospores are slow and uncoordinated. At temperatures between 16 and 20°C, zoospores may continue to swim for at least 48 hours (Alderman & Polglase, 1986).

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.

North American crayfish species

Isolation of *A. astaci* by culture following the methods described for the highly susceptible species usually fails. Currently, the recommended method for detecting infection in such species is by PCR.

4.3.1.2.2. Antibody-based antigen detection methods

None available.

4.3.1.2.3. Molecular techniques

Animals

In the case of a suspected outbreak of the disease in highly susceptible crayfish species, moribund or recently dead (<24 hours) crayfish are preferably selected for DNA extraction. Live crayfish can be killed using chloroform. If the only animals available are animals that have died a few days prior to DNA extraction, they can be tested, but a negative PCR result must be interpreted with caution as DNA degradation may have occurred. Endogenous controls can be used to assess whether degradation may have occurred. These should preferably use host tissues richer in host cells compared to the cuticle; cuticle itself contains very few host cell nuclei. If circumstances prevent delivery of crayfish to the specialist laboratory within 24 hours, fixation in 70% ethanol (≥3:1 ethanol to crayfish tissue) is possible, but may result in a reduction of the DNA yield.

DNA extraction

Where animals of the highly susceptible species are analysed, 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 a wet (using autoclaved H_2O) clean disposable paper towel. The soft abdominal cuticle is then excised and 30–50 mg ground in liquid nitrogen to a fine powder using a pestle and mortar (alternative grinding techniques may be used, but should be compared with the liquid nitrogen method before routine use). For carrier identification, 30–50 mg tissue from each soft abdominal cuticle, and telson and uropods are sampled and processed separately. DNA is extracted from the ground cuticle using a proteinase K-based DNA extraction method (e.g. DNeasy tissue kit; Qiagen, Hilden, Germany; protocol for insect tissue) following the manufacturer's instructions (Oidtmann *et al.*, 2006) or using a CTAB (cetyltrimethylammonium bromide-based)-based assay (Vrålstad *et al.,* 2009). Negative controls should be run alongside the samples. Shrimp tissues may be used as negative controls.

4.3.1.2.3.1. PCR

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here that have proven highly sensitive and specific. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome. As should be standard for any PCR-based diagnostic tests, negative controls should be run alongside the samples to control for potential contamination. Environmental controls (using for example shrimp tissue as described above) and extraction blank controls from the DNA extraction should be included along with 'no template' PCR controls (template DNA replaced with molecular grade water). The no template PCR controls should include an environmental PCR control left open during pipetting of sample DNA.

Method 1:

This conventional PCR assay uses species-specific primer sites located in the ITS1 and ITS2 regions. Forward primer (BO 42) 5'-GCT-TGT-GCT-GAG-GAT-GTT-CT-3' and reverse primer (BO 640) 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3'. The PCR is carried out in a 50 µl reaction volume containing 1 × PCR buffer 75 mM Tris/HCl, pH 8.8, 20 mM $[NH_4]_2SO_4$, 0.01% (v/v) Tween 20), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 0.5 µM of each primer, and 1.25 units of DNA polymerase (e.g. Thermoprime Plus DNA Polymerase; AB Gene, Epsom, UK) or equivalent Taq polymerase and 2 µl DNA template. The mixture is denatured at 96°C for 5 minutes, followed by 40 amplification cycles of: 1 minute at 96°C, 1 minute at 59°C and 1 minute at 72°C followed by a final extension step of 7 minutes at 72°C. Amplified DNA is analysed by agarose gel electrophoresis. The target product is a 569 bp fragment. Confirmation of the identity of the PCR product by sequencing is recommended. 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).

Method 2:

This assay is a TaqMan minor groove binder (MGB) real-time PCR assay that targets a 59 bp unique sequence motif of *A. astaci* in the ITS1 region. Forward primer AphAstITS-39F (5'-AAG-GCT-TGT-GCT-GGG-ATG-TT-3'), reverse primer AphAstITS-97R (5'-CTT-CTT-GCG-AAA-CCT-TCT-GCT-A-3') and TaqMan MGB probe AphAstITS-60T (5'-6-FAM-TTC-GGG-ACG-ACC-CMG-BNF-Q-3') labelled with the fluorescent reporter dye FAM at the 5'-end and a non-fluorescent quencher MGBNFQ at the 3'-end. Real-time PCR amplifications are performed in a total volume of 25 µl containing 12.5 µl PCR Master Mix (e.g. Universal PCR Master Mix or Environmental PCR Master Mix, Applied Biosystems), 0.5 µM of the forward (AphAstITS-39F) and reverse (AphAstITS-97R) primers, 0.2 µM 200 nM of the MGB probe (AphAstITS-60T), 1.5 µl molecular grade water and 5 µl template DNA (undiluted and tenfold diluted). Amplification and detection is performed in Optical Reaction Plates sealed with optical adhesive film or similar on a real-time thermal cycler. The PCR programme consists of an initial decontamination step of 2 minutes at 50°C to allow optimal UNG enzymatic activity, followed by 10 minutes at 95°C to activate the DNA polymerase, deactivate the UNG and denature the template DNA, and successively 50 cycles of 15 seconds at 95°C and 60 seconds at 58°C. A dilution series with reference DNA of known DNA content needs to be run alongside with the samples.

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

The diagnostic test sensitivity of either assay largely depends on the quality of the samples taken. Where a crayfish plague outbreak is investigated, the test sensitivity in animals that had died of crayfish plague 12 hours or less prior to sampling or in live crayfish showing clear clinical signs of disease is expected to be high. Studies to investigate the effect of sensitivity loss caused by deteriorating sample quality (for instance because of delayed sampling, processing or unsuitable storage of samples) have not been carried out. It is recommended that multiple (5–10) crayfish be tested, to compensate for variations in sample quality and invasion site of the pathogen.

Analytical test specificity has been investigated (Oidtmann *et al.*, 2006; Tuffs & Oidtmann, 2011; Vrålstad *et al.*, 2009) and no cross reaction was observed. However, owing to the repeated discovery of new *Aphanomyces* strains, sequencing is recommended to confirm diagnosis. In the case of the real-time PCR assay, this requires separate amplification of a PCR product, either using the primers as described in method 1, or using primers ITS 1 and ITS4 (see section 'sequencing' below).

4.3.1.2.3.2. *Sequencing*

A PCR product of 569 bp can be amplified using primers BO42 and BO640. The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel (e.g. using the Freeze n' Squeeze DNA purification system, Anachem, Luton, UK). Both DNA strands must be sequenced using the primers used in the initial amplification. The consensus sequence is generated using sequence analysis software and compared with published sequences using an alignment search tool such as BLAST. If 100% identity between the submitted sequence and the published sequences is found, then the amplified product is *A. astaci*. If the sequence is not 100% identical, further sequencing should be performed using primers ITS-1 (5'-TCC-GTA-GGT-GAA-CCT-GCG-G-3') and ITS-4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White *et al.,* 1990), which generate an amplicon of 757 bp that provides sequence data in the same region, but expanded at both ends relative to the sequence generated by primers BO42 and BO640. This expanded sequence should confirm the identity of the pathogen to the species level.

Highly susceptible species

PCR (conventional or real-time) is a suitable method to investigate suspected crayfish plague outbreaks (see Section 7.1). Where the conditions of a suspect case are fulfilled, amplification of a PCR product of the expected size using conventional PCR or real-time PCR can be considered sufficient as a confirmatory diagnosis, if a high level of template DNA is detected. Where low levels of template DNA are detected (weak amplification) or the samples are investigated from a site not meeting the conditions of a suspect case, it is recommended to sequence PCR products generated as described under the section sequencing to confirm the diagnosis.

4.3.1.2.4. Agent purification

None available.

4.3.2. Serological methods

None available.

5. Rating of tests against purpose of use

The methods currently available for diagnosis of clinical diseases of crayfish plague (*Aphanomyces astaci*) in highly susceptible species are listed in Table 5.1. Methods for targeted surveillance to demonstrate freedom from infection with *A. astaci* in highly susceptible species are displayed in Table 5.2.

Clinical disease is extremely rare in North American crayfish. Therefore a rating of methods for diagnosing clinical disease in these species is not provided. However, methods for targeted surveillance to demonstrate freedom from infection in North American crayfish are listed in Table 5.3.

The designations used in the tables indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. ~~Crayfish plague~~ Diagnostic methods for infection with A. astaci in highly susceptible crayfish species

Method	Presumptive diagnosis	Confirmatory diagnosis
Gross and microscopic signs	b	d
Isolation and culture	b	d
Histopathology	d	d
PCR	a	b or a[1]
qPCR	a	b or a[1]
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
In-situ DNA probes	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative (real-time) PCR; EM = electron microscopy;
n/a = not applicable or not available;
1 = see definitions of confirmed case in Section 7.1.

Table 5.2. Methods for targeted surveillance in highly susceptible crayfish species
to declare freedom from ~~crayfish plague~~ infection with A. astaci

Method	Screening method	Confirmatory method
Inspection for gross signs and mortality	a	c
Microscopic signs (wet mounts)	c	c
Isolation and culture	c	b

Method	Screening method	Confirmatory method
Histopathology	d	d
PCR	a	b, possibly a[1]
qPCR	a	b, possibly a[1]
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
In-situ DNA probes	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative (real-time) PCR; EM = electron microscopy;
n/a = not applicable or not available;
1 = see definitions of confirmed case in Section 7.1.

Table 5.3. Methods for targeted surveillance in North American crayfish species
to declare freedom from ~~crayfish plague~~ infection with A. astaci

Method	Screening method	Confirmatory method
Gross and microscopic signs	d	d
Isolation and culture	c	c
Histopathology	d	d
PCR	a	b
qPCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	d	d
Antibody-based assays	n/a	n/a
In-situ DNA probes	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative (real-time) PCR; EM = electron microscopy;
n/a = not applicable or not available

6. Test(s) recommended for targeted surveillance to declare freedom from ~~crayfish plague~~ infection with (*Aphanomyces astaci*)

6.1. Highly susceptible species

Crayfish farms keeping susceptible crayfish would need to be inspected at a frequency outlined in Chapter 2.2.0 *General information* (on diseases of crustaceans). A history of no mortalities (this does not include losses due to predation) occurring within the population over a period of at least 12 months combined with absence of clinical signs, as well as gross and microscopic pathology at the time of inspection are suitable methods for this purpose. Surveillance of wild crayfish stocks presents greater problems, especially where the species concerned is endangered. As movements of fish stocks from infected waters present a risk of pathogen transmission, monitoring the status of crayfish populations to confirm that they remain healthy, is necessary.

In a farm setting, an infection with ~~crayfish plague~~ A. astaci should be noticed relatively quickly, due to a relatively quick onset of mortalities in the farmed crayfish population.

To undertake targeted surveillance, regular inspections are recommended, where samples are collected if there is any suspicion of mortality or disease. If moribund or dead animals are found, it is recommended that samples are analysed by PCR and if PCR returns a positive result, that PCR products generated using primers 42 and 640, or ITS-1 and -4 are sequenced and the sequences analysed.

6.2. North American crayfish species

In North American crayfish species, animals would need to be sampled and analysed using one of the PCR assays described above. For reasons of higher sensitivity, the real-time PCR assay is the preferred method. This applies to both farmed and naturalised stocks, and surveillance programmes need to take into account the risks of indirect transmission by movements of fish.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Highly susceptible crayfish species

Any extensive mortality solely of the highly susceptible species of freshwater crayfish where all other aspects of the flora and fauna, particularly other aquatic crustaceans, are normal and healthy.

North American crayfish species

Any population of North American crayfish is generally to be regarded as potentially infected with *A. astaci*.

7.2. Definition of confirmed case

Highly susceptible crayfish species

Confirmation of presence of *A. astaci* by PCR or qPCR and sequencing.

Where (1) a crayfish mortality meets the definition of a suspect case and (2) PCR results indicate the presence of high levels of template DNA (in case of real-time PCR, this corresponds to Ct values ≤ 30), and (3) the investigated suspect case is not the first case of detection of *A. astaci* in the country or region, the PCR result alone may be considered sufficient as a confirmation.

North American crayfish species

Confirmation of presence of *A. astaci* by PCR or qPCR and sequencing

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EU comment

The EU requests a clarification as to what exactly is meant by the OIE recommendation as regards the confirmation of presence of *A. astaci* in the sentence above. Indeed, use of "by" followed by "or" and "and" makes this recommendation ambiguous.

Since this is crucial for the definition of a confirmed case, that clarification would be very important.

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CHAPTER 2.2.2.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

> **EU comment**
>
> **The EU supports the proposed changes to this chapter.**

1. Scope

Infection with infectious hypodermal and haematopoietic necrosis virus (IHHN) disease means is caused by infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) of the genus *Brevidensovirus* in the family *Parvoviridae* (Bonami & Lightner, 1991; Bonami *et al.,* 1990; Lightner, 1996a; 2011; Lightner *et al.,* 1983a, 1983b; Lotz *et al.,* 1995; Tang & Lightner, 2002).

Synonyms: the International Committee on Taxonomy of Viruses has assigned IHHNV (a parvovirus) as a tentative species in to the genus *Brevidensovirus*, *Penstyldensovirus* family *Parvoviridae* with the species name of PstDNV (for *Penaeus stylirostris* densovirus) (Fauquet *Decapod penstyldensovirus* 1 (King *et al.,* 2005 2012). For the purpose of this *Aquatic Manual*, most references to the viral agent of IHHN will be as IHHNV.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

IHHNV is the smallest of the known penaeid shrimp viruses. The IHHN virion is a 20–22 nm, non-enveloped icosahedron, with a density of 1.40 g ml^{-1} in CsCl, contains linear single-stranded DNA with an estimated a size of 3.9 kb, 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 AF218266).

At least two distinct genotypes of IHHNV have been identified (Tang & Lightner, 2002; Tang *et al.,* 2003b): Type 1 is from the Americas and East Asia (principally the Philippines). Type 2 is from South-East Asia. These genotypes are infectious to *Penaeus vannamei* and *P. monodon*. Two putative related sequences are found embedded in the genome of penaeids 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). There is evidence that these sequences are not infectious to *P. vannamei* and *P. monodon* (Tang & Lightner, 2002; Tang *et al.,* 2003b; 2007). IHHNV type 3A and type 3B related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.,* 2007). The putative IHHNV sequences in the *P. monodon* genome are not infectious to the representative host species *P. vannamei* and *P. monodon* (Lightner *et al.,* 2009; Tang & Lightner, 2006; Tang *et al.,* 2007). Primer sets 309F/309R can distinguish the infectious forms of IHHNV from non-infectious forms. Primer sets MG831F/MG831R will distinguish the non-infectious forms of IHHNV.

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective inactivation methods)

IHHNV is believed to be the most a stable virus of the known penaeid shrimp viruses. Infected virus; infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996a; Lightner *et al.,* 1987; 2009).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with IHHNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: giant tiger prawn (*Penaeus monodon*), white leg shrimp (*P. vannamei*), blue shrimp (*P. stylirostris*), yellow leg shrimp (*P. californiensis*), northern white shrimp (*P. setiferus*) and giant river prawn (*Macrobrachium rosenbergii*).

~~Most penaeid species can be infected with IHHNV, including the principal cultured species, *P. monodon* (black tiger shrimp/prawn), *P. vannamei* (Pacific white shrimp), and *P. stylirostris* (Pacific blue shrimp).~~

~~IHHNV infections are most severe in the Pacific blue shrimp, *P. stylirostris*, where the virus can cause acute epizootics and mass mortality (> 90%). In *P. stylirostris,* the juvenile and subadult life stages are the most severely affected (Bell & Lightner, 1984; 1987; Brock & Lightner 1990; Brock *et al.,* 1983; Lightner, 1996a; Lightner & Redman, 1998a; Lightner *et al.,* 1983a).~~

~~IHHNV causes the chronic disease runt-deformity syndrome (RDS) in *P. vannamei* in which reduced, irregular growth and cuticular deformities, rather than mortalities, are the principal effects (Bray *et al.,* 1994; Browdy *et al.,* 1993; Castille *et al.,* 1993; Kalagayan *et al.,* 1991; Lightner, 1996a; 1996b; Motte *et al.,* 2003). IHHNV infection in *P. monodon* is usually subclinical, but RDS, reduced growth rates and reduced culture performance have been reported in IHHNV infected stocks (Chayaburakul *et al.,* 2004; Primavera & Quinitio, 2000).~~

2.2.2. Species with incomplete evidence for susceptibility

Species or which there is incomplete evidence to fulfil the criteria for listing a species as susceptible to infection with IHHNV according to Chapter 1.5. of the *Aquatic Code* include: northern brown shrimp (*P. 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: northern pink shrimp (*P. duorarum*), western white shrimp (*P. occidentalis*), kuruma prawn (*P. japonicas*), *P. semisulcatus, Hemigrapsus penicillatus, Artemesia longinarus, Callinectes arcuatus, Archirus mazatlanus, Gerres cinerus, Oreochromis* sp., *Lile stolifera* and *Centopomus medium.*

2.2.3~~2~~. Susceptible stages of the host

Infection with IHHNV has been detected ~~demonstrated~~ in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei.* Eggs, produced by IHHNV-infected females with high virus loads, were found to generally fail to develop and hatch. Those nauplii produced from infected broodstock that do hatch have *a* high prevalence of infection with IHHNV ~~infection~~ (Motte *et al.,* 2003).

2.2.3~~4~~. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1 and 2.2.2.

2.2.4~~5~~. Target organs and infected tissue

IHHNV infects and has been shown to replicate (using *in-situ* hybridisation [ISH] with specific DNA probes) in tissues of ectodermal and mesodermal origin from the embryo. Thus, the principal target organs include: the gills, cuticular epithelium (or hypodermis), all connective tissues, the haematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, and striated muscle show no histological signs of infection with ~~by~~ IHHNV and are usually negative ~~for IHHNV~~ by ISH (Lightner, 1993; 1996a; 2011; Lightner *et al.,* 1992b).

<u>Annex 24</u> (contd)

2.2.~~5~~6. Persistent infection ~~with lifelong carriers~~

Some members of *P. stylirostris* and *P. vannamei* populations that survive <u>infection with</u> IHHNV ~~infections~~ or epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (Bell & Lightner 1984; Lightner, 1996a; 1996b; Morales-Covarrubias & Chavez-Sanchez, 1999; Motte *et al.,* 2003).

2.2.~~6~~7. Vectors

No vectors are known in natural infections.

~~2.2.8. Known or suspected wild aquatic animal carriers~~

~~IHHNV is common in wild penaeid shrimp in South East Asia (*P. monodon*) and in the Americas (*P. vannamei, P. stylirostris* and other Pacific side wild penaeid species) (Fegan & Clifford, 2001; Lightner, 1996a; Lightner *et al.,* 2009; Morales Covarrubias *et al.,* 1999).~~

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of IHHNV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water (Lightner, 1996a; Lightner *et al.,* 1983a; 1983b; 1985), and vertical transmission via infected eggs (Motte *et al.,* 2003) have been demonstrated.

2.3.2. Prevalence

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); and from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte *et al.,* 2003). Other penaeids collected during some of these surveys and found to be IHHNV positive included the brown shrimp, *P. californiensis* and the Western white shrimp *P. occidentalis.* In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence, approaching 100%, is typical (Chayaburakul *et al.,* 2004; Lightner, 1988; 1996a; 1996b; Lightner *et al.,* 1992a; 1983a; Martinez-Cordova, 1992).

2.3.3. Geographical distribution

IHHNV appears to have a world-wide distribution in both wild and cultured penaeid shrimp (Brock & Lightner, 1990; Lightner, 1996a; 1996b; Owens *et al.,* 1992). In the Western Hemisphere, <u>infection with</u> IHHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although <u>infection with</u> IHHNV has been reported from cultured *P. vannamei* and *P. stylirostris* in most of the shrimp-culturing regions of the Western Hemisphere and in wild penaeids throughout their range along the Pacific coast of the Americas (Peru to northern Mexico), the virus has not been reported in wild penaeid shrimp on the Atlantic coast of the Americas (Bondad-Reantaso *et al.,* 2001; Brock & Main, 1994; Lightner, 1996a, 1996b; Lightner *et al.,* 1992a; Lightner & Redman, 1998a). IHHNV has also been reported in cultured penaeid shrimp from Pacific islands including the Hawaiian Islands, French Polynesia, Guam, and New Caledonia. In the Indo-Pacific region, the virus has been reported from cultured and wild penaeid shrimp in East Asia, South-East Asia, and the Middle East (Bondad-Reantaso *et al.,* 2001; Lightner, 1996a).

~~Infectious~~ <u>The</u> IHHNV was detected for the first time in farmed prawns in Australia in 2008. Additionally an IHHN-like virus sequence has been reported from Australia (Krabsetsve *et al.*, 2004; Owens *et al.*, 1992), and the presence of IHHN in farmed prawns in Australia was reported to the OIE in 2008. As discussed in Section 2.1.1, IHHNV-related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.*, 2007).

2.3.4. Mortality and morbidity

~~Depending on the host~~ The effects of infection with IHHNV varies among shrimp species and ~~the genotype of the virus, IHHN may take three distinct forms:~~ populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with ~~by~~ IHHNV results in acute, usually catastrophic, disease with mortalities approaching 100%. In contrast, in populations of *P. vannamei*, some selected lines of *P. stylirostris*, and some populations of *P. monodon* ~~under some conditions~~, infection with ~~by~~ IHHNV results in a more subtle, chronic disease, RDS, in which high mortalities are unusual, but ~~significant~~ where growth suppression and cuticular deformities are common. ~~In the third situation, a large portion of the IHHNV genome has been found to be inserted in the genome of some genetic lines of *P. monodon*. There is evidence that in *P. monodon*, this inserted IHHNV sequence is not infectious to other penaeids (Tang & Lightner, 2002; 2006~~ (Kalagayan *et al.*, 1991).

Infection with IHHNV interferes with normal egg, larval, and postlarval development~~: poor~~. When broodstock are used from wild or farmed stocks where IHHNV is enzootic, hatching success of eggs is reduced, and ~~poor~~ survival and culture performance of the larval and postlarval stages is ~~observed when broodstock are used from wild or farmed stocks where IHHNV is enzootic~~ lowered (Motte *et al.,* 2003).

~~Farmed~~ Stocks of *P. stylirostris*, juveniles, subadults, and adults ~~may~~ show persistently high mortality rates. In *P. vannamei~~, P. stylirostris,~~* and possibly *P. monodon~~,~~* ~~IHHNV infected~~ stocks infected with IHHNV ~~may~~ show poor and highly disparate growth~~, poor overall culture performance~~, and cuticular deformities, including especially bent rostrums and deformed sixth abdominal segments.

Demonstration of eosinophilic to pale basophilic intranuclear inclusion bodies in the typical target tissues for IHHNV, as IHHNV intranuclear inclusion bodies are nearly identical in appearance to those occurring in the early stages of ~~WSSV~~ IHHNV infections, their presence in tissue sections should be considered as a presumptive diagnosis of IHHNV until confirmed with a second test method, such as dot-blot or ISH with IHHNV-specific DNA probes or positive PCR test results for IHHNV.

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 102 lower viral load than shrimp held at 24°C~~. However, even at the higher temperature, significant (up to 105 virus copies 50 ng -1 of shrimp DNA) IHHNV replication still occurred in shrimp held at 32°C~~ (Montgomery-Brock *et al.*, 2007).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods for IHHNV have been developed.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Resistance breeding

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 (Clifford, 1998; Lightner, 1996a; 1996b; Weppe 1992; Zarian-Herzberg & Ascencio-Valle, 2001). Some selected lines of *P. stylirostris* bred for IHHNV resistance were found to be refractory to infection (Tang *et al.,* 2000). However, such stocks do not have increased resistance to diseases such as white spot syndrome virus (WSSV), and, hence, their use has been limited. In some stocks a genetic basis for IHHN susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.,* 1997).

2.4.5. Restocking with resistant species

There has been some limited application and success with IHHNV-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarin-Herzberg & Ascencio 2001). The relative resistance of *P. vannamei* to clinical IHHN disease, despite infection by IHHNV, is considered to be among the principal factors that led to *P.vannamei* being the principal shrimp species farmed in the Western Hemisphere and, since 2004, globally (Lightner, 2005; Lightner *et al.,* 2009; Rosenberry, 2004).

2.4.6. Blocking agents

~~There are reports of shrimp with high viral loads of IHHNV being resistant to infection by WSSV (Bonnichon *et al.,* 2006; Tang *et al.,* 2003a). However, there~~ There are no reports to date for IHHNV blocking agents.

2.4.7. Disinfection of eggs and larvae

Infection with IHHNV is transmitted vertically by the transovarian route (Motte *et al.,* 2003). Hence, while disinfection of eggs and larvae is good management practice (Chen *et al.,* 1992) and is recommended for its potential to reduce IHHNV contamination of spawned eggs and larvae produced from them (and contamination by other pathogenic agents), the method is not effective for preventing transmission of IHHNV (Motte *et al.,* 2003).

2.4.8. General husbandry practices

Some husbandry practices have been successfully ~~applied to~~ in ~~the~~ prevent~~ion~~ing the spread ~~on~~ of infection with IHHNV ~~infections~~ and clinical disease. Among these has been the application of PCR pre-screening of wild or pond-reared broodstock and/or their spawned eggs/nauplii, and discarding those that test positive for the virus (Fegan & Clifford, 2001; Motte *et al.,* 2003), as well as the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Lotz *et al.,* 1995; Pruder *et al.,* 1995; Wyban 1992). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (Jaenike *et al.,* 1992; Lightner, 2005; Pruder *et al.,* 1995). Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status (Lightner *et al.,* 2009). The development of SPF *P. vannamei* that were free not only of IHHNV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Lightner *et al.,* 2009; Rosenberry, 2004).

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection ~~by~~ with IHHNV are all life stages (eggs, larvae, postlarvae, juveniles and adults) (Motte *et al.,* 2003). While infection with IHHNV may ~~infect~~ affect 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 ~~IHHNV~~ detection or certification of ~~for IHHN~~ freedom from infection with IHHNV.

3.2. 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.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and postlarvae, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 postlarvae depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs and 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, 1996a; Lightner & Redman, 1998a). Hence, whole shrimp (e.g. larvae or postlarvae) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

Haemolymph or excised pleopods may be collected and used for testing (usually for PCR, or dot-blot hybridisation with specific probes) when non-lethal testing of valuable broodstock is necessary (Lightner, 1996a; Lightner & Redman, 1998a).

3.5. Samples/tissues that are not suitable

IHHNV 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 by IHHNV (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

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 pathognomonic for infection with ~~by~~ IHHNV (see Section 4.2.1.2). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV. As *P. vannamei*, *P. stylirostris,* and *P. monodon* can be infected by IHHNV and not present obvious signs of infection (e.g. they may show markedly reduced growth rates or 'runting'), molecular tests are recommended when evidence of freedom from infection with IHHNV is required.

4.1.2. Behavioural changes

In acute clinical ~~IHHN~~ disease, *P. stylirostris* may present behavioural changes (see Section 4.2.1.1) but with RDS, no consistent behavioural changes have been reported for affected shrimp.

4.2. Clinical methods

4.2.1. Gross pathology

4.2.1.1. Infection with IHHNV in Penaeus stylirostris

Infection with IHHNV often causes an acute clinical disease with very high mortalities in juveniles of this species. 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 and/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; Bondad-Reantaso *et al.*, 2001; Brock *et al.*, 1983; Brock & Main, 1994; Lightner, 1983; 1988; 1993; 1996a; 2011; Lightner *et al.*, 1983a, 1983b). Gross signs are not of infection with IHHNV are not 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 with infection 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* as such individuals become more bluish. In *P. stylirostris* and *P. monodon* with terminal-phase IHHNV infections, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Bondad-Reantaso *et al.*, 2001; ~~Lightner, 1983; 1988; 1993; 1996a; 2011;~~ Lightner *et al.*, 1983a; 1983b).

4.2.1.2. Infection with IHHNV ~~disease~~ in Penaeus vannamei

RDS, a chronic form of <u>infection with</u> IHHNV ~~disease~~, occurs in *P. vannamei*. ~~as a result of IHHNV infection~~. 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 <u>postlarval</u> ~~PL~~ 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 ~~IHHNV free (and thus RDS-free)~~ populations of juvenile *P. vannamei* and *P. stylirostris* <u>free of infection with IHHNV</u> (and thus RDS-free) usually show CVs of 10–30% (Bray *et al.,* 1994; Brock & Lightner, 1990; Brock *et al.,* 1983; Brock & Main, 1994; Browdy *et al.,* 1993; Carr *et al.,* 1996; Lightner, 1996a; Primavera & Quinitio, 2000; Pruder *et al.,* 1995).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute ~~IHHNV~~ infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained histological methods (see Section 4.2.6). Chronic <u>infection with IHHNV</u> ~~IHHNV infections~~ and RDS are much more difficult to diagnose using routine H&E histological methods. For diagnosis of chronic infections, the use of molecular methods are recommended for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or ISH of histological sections).

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of <u>infection with IHHNV</u> ~~IHHNV infection~~. These characteristic IHHN<u>V</u> 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, 1996a), 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 <u>infection with</u> IHHNV may be easily confused with developing intranuclear inclusion bodies caused by WSSV infection. ISH assay (see Section 4.3.1.2.3 of this chapter) of such sections with a DNA probe specific to IHHNV provides a definitive diagnosis of IHHNV infection (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4.2.4. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

Histopathology: histology may be used to provide a definitive diagnosis of <u>infection with IHHNV</u> ~~IHHNV infection~~. Because 10% buffered formalin and other fixatives provide, at best, only fair fixation of the shrimp, 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 less the abdomen) is immersed in fixative for from 24 to no more than 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported (via post or courier to the diagnostic laboratory) by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags (see Section 4.2.3).

In-situ hybridisation (see Section 4.3.1.2.3 below).

4.2.7.   Electron microscopy/cytopathology

Electron microscopy is not recommended for routine diagnosis of IHHNV.

4.3. Agent detection and identification methods

4.3.1.   Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See section 4.2.6.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

IHHNV **has not been grown** *in vitro*. **No crustacean cell lines exist (Lightner, 1996a; Lightner &** **Redman, 1998a: 1998b).**

4.3.1.2.2. Antibody-based antigen detection methods

None has been successfully developed.

4.3.1.2.3. Molecular techniques

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. PCR tests for IHHNV have been developed and a number of methods and commercial ~~products using these methods~~ PCR detection kits are readily available.

DNA probes for dot-blot and ISH applications: gene probe and PCR methods provide greater diagnostic specificity and sensitivity than ~~do more~~ traditional techniques ~~for IHHN diagnosis~~ 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 ~~direct~~ dot-blot hybridisation test.

~~*Dot-blot hybridisation procedure for IHHNV:* the probe is labelled with a non-radioactive label, digoxigenin 11 dUTP (DIG 11 dUTP). The system using DIG to label nucleic acid probes was developed by Boehringer Mannheim Biochemicals (this company is now owned by Roche Diagnostic Corporation), which is described in the Roche *DIG Nonradioactive Labeling and Detection Product Selection Guide* and *DIG Application Manual for Filter Hybridization^TM System User's Guide for Membrane Hybridization* and from Boehringer Mannheim's *Nonradioactive In Situ Hybridization Application Manual* (Roche Applied Science, 2006a; 2006b). The protocols given below use a DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (Lightner, 1996a; Mari *et al.*, 1993). An alternative method for producing DIG labelled probes uses specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit^TM.~~

Dot-blot hybridisation procedure for IHHNV: the dot-blot hybridisation method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al.* (1993) and Lightner (1996a). Formulas for the required reagents are given after the protocols.

i)   Prepare a positively charged nylon membrane ~~(Roche Diagnostics Cat. No. 1 209 299 or equivalent~~), cut ~~pieces~~ to a size to fit samples and controls and mark with a soft-lead pencil making 1 cm squares for each sample. Include a positive and a negative control on each filter. Lay out on to a piece of filter paper (Whatman 3MM).

ii)   If necessary, ~~dilute~~ samples ~~to~~ can be ~~assayed~~ diluted in TE ~~(Tris/EDTA [ethylene diamine tetra acetic acid])~~ buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) plus 50 µg ml⁻¹ salmon sperm DNA~~, using 1 µl sample in 9 µl buffer in 1.5 ml microcentrifuge tubes~~. Samples for dot-~~blots~~ blot hybridisation can be haemolymph, tissues homogenised in TN ~~(Tris/NaCl: 0.4 M NaCl and~~ buffer (20 mM Tris-HCl, pH 7.4, 0.4 M NaCl), or extracted DNA in 10 mM Tris-HCl.

iii)   Boil samples for ~~10~~ 5 minutes and quench on ice for ~~5~~ 1–2 minutes. ~~Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein.~~ Keep on ice until samples are dotted on to the membrane.

iv)   Dot 1–3 µl of each sample on to an appropriate place on the filters. Allow to air-dry and then fix samples on to the membrane by baking at 80°C for 30 minutes or by UV cross-linking using a DNA transilluminator for 3 minutes.

~~v)   Adjust a water bath to 68°C and prepare the prehybridisation solution. For a 10 × 15 cm membrane, prepare 8 ml per membrane. Set a stirring hot plate to 'low' and stir while warming the solution for 30 minutes until the blocking agent has dissolved and the solution is cloudy. Also, prepare some heat seal bags that are slightly larger in size than the membrane: five to six bags will be needed per membrane.~~

~~vi~~v)   Remove membranes from the oven or transilluminator and put into a heat-seal bag with 4 ml per membrane of prehybridisation solution. Seal the bags and put into a 68°C water bath for ~~30 minutes~~ 1 hour.

~~vii~~vi)   Boil the DIG-labelled probe for ~~10~~ 3–5 minutes, keep on ice ~~and then microfuge in the cold to bring all the liquid down in the microcentrifuge tube. Keep on ice~~. Remove the prehybridisation solution from the bags. Add 2 ml of fresh prehybridisation solution to each bag and then add the ~~correct,~~ predetermined amount of DIG-labelled probe to each, mixing well as it is being added. Seal the bags, place back in the 68°C water bath and incubate for 8–12 hours.

~~viii~~vii)   Wash membranes well with:

2 × ~~standard saline citrate (~~SSC/0.1% ~~sodium dodecyl sulphate (~~SDS	2 ×	5 minutes at room temperature
0.1 × SSC/0.1% SDS	3 ×	15 minutes at 68°C
~~(use 4 ml/filter and seal in bags)~~		
Buffer I	1 ×	5 minutes at room temperature
~~Buffer II~~	~~1 ×~~	~~30 minutes at room temperature~~
~~Buffer I~~	~~1 ×~~	~~5 minutes at room temperature~~
~~(Buffers are prepared ahead of time).~~		

~~ix~~viii)   React the membrane in bags with anti-DIG ~~AP~~ alkaline phosphatase conjugate (Roche Diagnostics[3] ~~1 093 274~~) diluted 1/5000 in Buffer I. ~~Use 3 ml per membrane;~~ incubate for 30–45 minutes at room temperature on a shaker platform.

~~x~~ix)   Wash membrane well with:

Buffer I	2 ×	15 minutes at room temperature
Buffer III	1 ×	5 minutes at room temperature

~~xi~~x)   Develop the membranes in bags using ~~3 ml per membrane of~~ a development solution (nitroblue tetrazolium salt [NBT]/X-phosphate in Buffer III) made up just prior to use. React in the dark at room temperature for 1–2 hours. Stop the reactions in Buffer IV and dry the membranes on 3MM filter paper.

~~xii~~xi)   Photograph the results (colour fades over time).

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

Annex 24 (contd)

<p style="text-align:right">xiiixii)</p>

xiiixii)   Store dry membranes in heat-seal bags.

In-situ hybridisation (ISH) procedure: the ISH method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al.* (1993) and Lightner (1996a). ~~Formulas for the required reagents are given after the protocols.~~

i)   Embed tissue in paraffin and cut sections at 4–6 µm thickness. Place sections on to positively charged microscope slides (do not put gelatine in water to float sections; just use water).

ii)   Put slides in a slide rack, such as a Tissue-Tek rack. Heat the slides in an oven for 45 minutes at 60°C. In the staining centre, rehydrate the tissue as follows:

Xylene (or suitable substitute)	3 ×	5 minutes each
Absolute alcohol	2 ×	1 minute each
95% alcohol	2 ×	10 dips each
80% alcohol	2 ×	10 dips each
50% alcohol	1 ×	10 dips
Distilled water		six rinses (do not let slides dry out)

iii)   Wash the slides for 5 minutes in ~~phosphate buffered saline (~~PBS (or Tris/NaCl/EDTA [TNE] buffer). Prepare fresh proteinase K at 100 µg ml^{-1} in PBS (or TNE). Place slides flat in a humid chamber, pipette on 500 µl of the proteinase K solution and incubate for 10–15 minutes at 37°C. Drain fluid onto blotting paper.

iv)   Return slides to slide rack. Fix sections in 0.4% cold formaldehyde for 5 minutes at room temperature.

v)   Incubate slides in 2 × SSC for 5 minutes at room temperature.

vi)   With slides flat, add 0.5–1 ml prehybridisation buffer and incubate in a humid chamber for 15–30 minutes at 37°C.

vii)   Boil the DIG-labelled probe for ~~10~~ 3–5 minutes and quench on ice; spin briefly in the cold and keep on ice. Dilute the probe to 25 ng ml^{-1} in prehybridisation solution and cover the tissue with 250 µl of the solution. Incubate the slides for 2–4 hours at 42°C or overnight at 37°C in a humid chamber. ~~Drain fluid onto blotting paper. During this incubation, pre-warm the wash buffers at 37°C.~~

viii)   Place slides in slide rack. Wash the slides as follows:

2 × SSC	2 ×	5–30 minutes at 37°C
1 × SSC	2 ×	5 minutes at 37°C
0.5 × SSC	2 ×	5 minutes at 37°C

ix)   Wash the slides for ~~5~~ 1–3 minutes in Buffer I at room temperature. Put the slides flat in a humid chamber and block with 0.5 ml per slide of Buffer II. Incubate for 15 minutes at 37°C. ~~Drain the fluid on to blotting paper.~~

x)   Dilute the anti-DIG alkaline phosphotase conjugate ~~(Roche Applied Science cat. 10686322)~~ at a ratio of 1/1000 in Buffer II ~~(1 µl anti-DIG-AP per 1 ml buffer)~~. Cover tissue with 500 µl of diluted conjugate and incubate in a humid chamber for 30 minutes at 37°C.

xi)   Place the slides in a slide rack. Wash in Buffer I twice for 5–10 minutes each time at room temperature. Wash once with Buffer III for ~~5–10~~ 1–2 minutes.

xii)   Prepare the development solution by first adding 4.5 µl NBT per 1 ml buffer III. Mix well. Then add 3.5 µl X-phosphate per ml of solution and mix well. Pipette on 500 µl per slide and incubate in a humid chamber in the dark for 2–3 hours at room temperature.

xiii)   Stop the reaction by returning the slides to a slide rack and washing in Buffer IV for 15 minutes at room temperature.

xiv)   Counterstain the slides by dipping for 5 minutes in 0.5% aqueous Bismarck brown Y.

xv)   Dehydrate the slides in the staining centre as follows:

95% alcohol	3 ×	10 dips each
Absolute alcohol	3 ×	10 dips each
Xylene (or suitable substitute)	4 ×	10 dips each
Do not allow the slides to dry out – leave them in the last xylene (or xylene substitute) container until ready for cover-slips.		

xvi)   Mount with cover-slips and mounting medium (Permount).

xvii) Examine the slides under bright-field for a dark-blue or black precipitate that marks sites where IHHNV DNA is present. Pathodiagnostic intranuclear Cowdry type A inclusions are well marked with the probe. Also often marked are host cell nuclei without obvious inclusions, cytoplasmic inclusions, and accumulation of free virus in the tissue spaces and haemolymph.

~~NOTE: Always run a known positive and negative control.~~

Reagent formulas for ISH method:

~~i)~~ ~~10 × phosphate buffered saline~~

~~NaCl~~	~~160 g~~
~~KH$_2$PO$_4$~~	~~4 g~~
~~Na$_2$HPO$_4$~~	~~23 g~~
~~KCl~~	~~4 g~~
~~DD H$_2$O~~	~~1950 ml (qs to 2 litres)~~

~~pH to 8.2 with NaOH; autoclave to sterilise; store at room temperature. To make 1 × PBS, dilute 100 ml 10 × PBS in 900 ml DD H$_2$O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.~~

~~ii)~~ i) 10 × Tris/NaCl/EDTA (TNE) buffer

Tris base	60.57 g
NaCl	5.84 g
EDTA	3.72 g
H$_2$O	900 ml (qs to 1 litre)

pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 × TNE in 900 ml ~~DD~~ H$_2$O; ~~Filter 1 × solution through a 0.45 µm filter; store at 4°C.~~

~~iii~~ii) Proteinase K, 100 µg ml^{-1} (prepare just prior to use)

PBS	10 ml 1 × PBS
Proteinase K	1 mg

~~iv)~~ ~~0.4% formaldehyde~~

~~37% formaldehyde~~	~~5.4 ml~~
~~DD H$_2$O~~	~~500 ml~~

~~Store at 4°C; can be reused up to four times before discarding.~~

~~v~~iii) Prehybridisation buffer (50 ml final volume)

4 × SSC	10 ml 20 × SSC
50% formamide	25 ml 100% formamide
1 × Denhardt's	2.5 ml 20 × Denhardt's
5% dextran sulphate	10 ml 25% dextran sulphate
Warm to 60°C	

Boil 2.5 ml of 10 mg ml^{-1} salmon sperm DNA and add to buffer for final concentration of 0.5 mg ml^{-1} salmon sperm DNA~~; store at 4°C.~~

~~vi)~~ ~~20 × SSC buffer~~

~~3M NaCl~~	~~175.32 g NaCl~~
~~0.3 M Na$_3$C$_6$H$_5$O$_7$.2H$_2$O~~	~~88.23 g Na citrate.2H$_2$O~~
~~DD H$_2$O~~	~~1000 ml (qs)~~

~~pH to 7.0; autoclave; store at 4°C.~~

~~To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H$_2$O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H$_2$O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H$_2$O. Filter solutions through a 0.45 µm filter; store at 4°C.~~

~~vii)~~ ~~20 × Denhardt's solution~~

~~BSA (Fraction V)~~	~~0.4 g bovine serum albumin~~
~~Ficoll 400~~	~~0.4 g Ficoll~~
~~PVP 360~~	~~0.4 g polyvinylpyrollidine~~
~~DD H$_2$O~~	~~100 ml~~

~~Filter solutions through a 0.45 µm filter; store at 4°C. Aliquot 2.5 ml into small tubes and store frozen.~~

Annex 24 (contd)

viii) 25% dextran sulphate
    Dextran sulphate   25 g
    DD H$_2$O   100 ml
    Mix to dissolve; store frozen in 10 ml aliquots.

ix) Salmon sperm DNA (10 mg ml^{-1})
    Salmon sperm DNA   0.25 g
    DD H$_2$O   25 ml
    To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18 gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.

x iv) 10 × Buffer I
    1 M Tris/HCl   121.1 g Tris base
    1.5 M NaCl   87.7 g NaCl
    DD H$_2$O   1000 ml (qs)

    pH to 7.5 with HCl. Autoclave; store at 4°C.
     To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H$_2$O. Filter through a 0.45 µm filter; store at 4°C.

x v) Buffer II (blocking buffer)
    Blocking reagent   0.25 g Blocking reagent (Roche Diagnostics 1-096-176)

    Buffer I   50 ml 1 × Buffer I
    Store at 4°C for up to 2 weeks.

xii vi) Buffer III

    100 mM Tris/HCl   1.21 g Tris base
    100 mM NaCl   0.58 g NaCl
    DD H$_2$O   100 ml (qs)

    pH to 9.5 with HCl
    Then add:
    50 mM MgCl$_2$ 1.02 g MgCl$_2$.6H$_2$O
    Filter through a 0.45 µm filter; store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)

    Polyvinyl alcohol   10 g
    DD H$_2$O   100 ml

    To prepare, slowly add PVA to water while stirring on low heat. (It takes 2–3 hours for PVA to go into solution.) Dispense 10 ml per tube and store frozen at –20°C.

    xiv vii) Development solution

     Mix 90 ml Buffer III with 10 ml of 10% PVA. Store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

    4.5 µl NBT   75 mg NBT ml^{-1} in 70% dimethylformamide
                                            (Roche Diagnostics 1-383-213)
    3.5 µl X-phosphate   5-bromo-4-chloro-3-indoyl phosphate, toluidine salt
                                            (50 mg ml^{-1} in dimethylformamide)
                                            (Roche Diagnostics 1-383-221)

    xv xiii) Buffer IV

    10 mM Tris/HCl   1.21 g Tris base
    1 mM EDTA   0.37 g EDTA.2H$_2$O (disodium salt)
    DD H$_2$O   1000 ml

    pH to 8.0 with HCl. Filter through a 0.45 µm filter; store at 4°C.
    xvi ix) 0.5% Bismarck Brown Y

    Bismarck Brown Y   2.5 g
    DD H$_2$O   500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

Polymerase chain reaction for IHHNV: several one-step PCR methods (Krabsetsve *et al.,* 2004; Nunan *at al.,* 2000; Shike *et al.,* 2000; Tang *et al.,* 2000; 2007; ~~Tang & Lightner 2001~~), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available from commercial sources.

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods for IHHNV. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (~~Krabsetsve *et al.,* 2004; Tang & Lightner, 2002~~ Tang *et al.,* 2007). However these tests also detect IHHNV-related sequences called types 3A and 3B, which are inserted into the genome of certain ~~geographic~~ stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (~~Duda & Palumbi, 1999;~~ Saksmerprome *et al.,* 2011; Tang & Lightner, 2006; Tang *et al.,* 2007). PCR primers have been developed that can detect the IHHN viral sequence but do not react with IHHNV-related sequences 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 infection with IHHNV), but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Primer set MG831F/R reacts only with types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang *et al.,* 2007). Hence, confirmation of unexpected positive and/or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. ~~PCR using primers from another region of the genome, real time PCR~~ histology, bioassay, ISH) is highly recommended.

Table 4.1. Recommended primer sets for one-step PCR detection of IHHNV

Primer	Product	Sequence	G+C%/Temp.	GenBank & References
389F	389 bp	5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3'	50%/72°C	AF218266
389R		5'-GGC-AAA-GAC-CAA-AAT-ACG-AA-3'	45%/71°C	(Tang *et al.,* 2000)
77012F	356 bp	5'-ATC-GGT-GCA-CTA-CTC-GGA-3'	50%/68°C	AF218266
77353R		5'-TCG-TAC-TGG-CTG-TTC-ATC-3'	55%/63°C	(Nunan *et al.,* 2000)
392F	392 bp	5'-GGG-CGA-ACC-AGA-ATC-ACT-TA-3'	50%/68°C	AF218266
392R		5'-ATC-CGG-AGG-AAT-CTG-ATG-TG-3'	50%/71°C	(Tang *et al.,* 2000; 2007)
309F	309 bp	5'-TCC-AAC-ACT-TAG-TCA-AAA-CCA-A-3'	36%/68°C	AF218266
309R		5'-TGT-CTG-CTA-CGA-TGA-TTA-TCC-A-3'	40%/69°C	(Tang *et al.,* 2007)
MG831F	831 bp	5'-TTG-GGG-ATG-CAG-CAA-TAT-CT-3'	45%/58°C	DQ228358
MG831R		5'-GTC-CAT-CCA-CTG-ATC-GGA-CT-3'	55%/62°C	(Tang *et al.,* 2007)

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region ~~(ORF 1)~~ of the IHHNV genome. Primers 77012F/77353R are from a region in between the nonstructural and ~~the structural (coat protein)~~ 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). Cumulative experience with the technique has led to modifications with respect to template ~~(DNA extraction of clinical specimens),~~ methods, choice of primers (Table 4.1), and volume of reaction.

Annex 24 (contd)

i) Use as a template, the DNA extracted from ~~ground tissue homogenate (TN buffer, 0.4 M NaCl, 20 mM Tris, pH 7.4) or haemolymph (collected with a small amount of 10% sodium citrate) or from~~ tissue<u>s</u> or haemolymph that was ~~fixed~~ <u>preserved</u> in 95% ethanol and then dried. A control consisting of tissue<u>s</u> or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods, but excellent results have been obtained using kits from Roche Diagnostics ~~(Cat. No. 1 796 828) or Qiagen (Cat. No. 51304)~~. Other 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. Use 1–5 µl of extracted DNA <u>as a template</u> per ~~50~~ <u>25</u> 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 ~~100 µl^{-1}~~ <u>10 µM</u> in distilled water. ~~Keep frozen at –70°C.~~

iv) ~~Use a 'hot start' method for the polymerase: if Applied Biosystem's AmpliTaq Gold is used, this~~ <u>If PuReTaqTM Ready-To-Go PCR Beads (GE Healthcare) are used, the PCR profile</u> involves a <u>3–</u>5 minutes at 95°C to denature DNA ~~prior to the primers binding and activation of the enzyme. This programme is then linked to the cycling programme (~~ <u>followed by</u> 35 cycles~~)~~ ~~and an~~ <u>of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final</u> extension ~~programme. The programme is set as follows:~~ <u>at 72oC for 5 minutes.</u>

~~Hot start~~	~~Programme 1~~	~~5 minutes 95°C~~	
~~Linked to~~	~~Programme 2~~	~~30 seconds 95°C~~	
		~~30 seconds 55°C~~	~~35 cycles~~
		~~1 minute 72°C~~	
~~Linked to~~	~~Programme 3~~	~~7 minutes 72°C~~	
~~Linked to~~	~~Programme 4~~	~~4°C until off~~	

<u>v) Prepare a 'Master Mix' consisting of water and primers.</u>

vi) For a ~~50~~ <u>25</u> µl reaction mix, add ~~49~~ <u>24</u> µl Master Mix to each tube and then add 1 µl of the ~~sample~~ <u>DNA template</u> to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. ~~If the thermal cycler does not have a heated lid to prevent condensation, then carefully overlay the top of each sample with 25–50 µl mineral oil and re-cap the tubes.~~ Insert tubes into the thermal cycler and start ~~programme 1 ('hot start'), which is linked to cycling, extension and soak cycles~~ <u>the PCR program</u>.

~~viii) If mineral oil was used, recover samples from under the mineral oil using a pipette set at 50 µl and transfer to a fresh tube. Using the long-tipped pipette tips (designed for loading gels) results in less oil being carried over with the sample.~~

ix) ~~Run~~ <u>After PCR, run</u> 6–10 µl of the sample in a 1.5% agarose gel (containing 0.5 µg ml^{-1} ethidium bromide 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 Southern transfer of the gel or a dot blot can be run for more sensitive detection. The DNA can also be precipitated (0.3 M sodium acetate and 2.5 volumes 100% ethanol, –70°C, for 1–3 hours, centrifuge for 20 minutes) and resuspended in 1/10th volume (i.e. 4 µl) TE (10 mM Tris, 1 mM EDTA, pH 7.5) or water and either re-run in the gel or tested in a dot blot.~~ <u>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.</u>

Real-time PCR (qPCR) method for IHHNV: qPCR methods have been developed for the detection of IHHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHHNV genome (Dhar *et al.*, 2001; Tang & Lightner, 2001). ~~Using primers 309F/309R, it is possible to distinguish infectious forms of IHHNV from non-infectious forms. Using MG831F/MG831R it is possible to distinguish the non-infectious forms.~~

The qPCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (2001).

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 primers and TaqMan probe are designed by the Primer Express software (~~Applied Biosystems~~ Life Technologies). 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'), ~~which corresponds to the region from nucleotide 1632 to 1644,~~ is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end ~~(Applied Biosystems, part no. 450025)~~.

ii) Preparation of DNA template: the extraction ~~and purification~~ of DNA template is the same as that described ~~in the section of traditional PCR~~ above.

iii) The qPCR reaction mixture contains: TaqMan ~~Universal PCR~~ Fast virus 1-step Master Mix (~~Applied Biosystems, part no. 4324018~~ 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 ~~25~~ 20 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the ~~GeneAmp 5700 Sequence Detection~~ StepOnePlus PCR System (~~Applied Biosystems; ABI PRISM 7000, 7300, or 7500~~ Life Technologies; or equivalent ~~can also be used~~ PCR systems). The cycling profile is: ~~activation~~ initial denaturation ~~of AmpliTaq Gold for 10 minutes~~ 20 seconds at 95°C, followed by 40 cycles of denaturation at 95°C for ~~15 seconds~~ 1 second and annealing/extension at 60°C for ~~1 minute. The levels of fluorescence are measured at the end of the annealing and extension step~~ 20 seconds.

v)   At the end of the reaction, ~~real-time fluorescence measurements will be taken with a built-in charge coupled device (CCD) camera~~ fluorescence intensity is measured. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. A cut-off Ct value is set through the analyses of several independent runs of negative and positive controls. Samples with a Ct value lower than ~~40~~ cut-off cycles are considered to be positive.

vi) 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 ~~or in the heat block of the thermal cycler~~. A positive control should also be included, and it can be a plasmid containing the target sequence, or purified virions, or DNA extracted from IHHNV-infected tissue.

Sequencing: 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 insertion of non-infectious IHHNV genome in host DNA (Tang & Lighter, ~~2002;~~ 2006).

Through PCR, IHHNV was detected in *P. monodon* from South-East Asia. ~~Most~~ Some of these IHHNV PCR ~~assays~~ primers also ~~detected~~ reacted to IHHNV-related sequences in *P. monodon* populations in Africa, Australia and Thailand (Tang & Lightner, 2006; Saksmerprome *et al.*, 2011). To discriminate the IHHNV-related sequences from the actual virus, PCR assays using primers that detect the IHHN viral sequence and do not react with IHHNV-related sequences present in the *P. monodon* stocks from Africa or Australia (Tang *et al.*, 2007), or Thailand (e.g. Saksmerprome *et al.*, 2011) have been developed.

Annex 24 (contd)

PCR commercial kits are available for IHHNV diagnosis and can be acceptable provided they have been validated as fit for such purpose. The OIE validation procedure is described in Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases*.

4.3.2. Serological methods

Shrimp are invertebrate animals and do not produce antibodies. Therefore, serological methods for IHHN are not available.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of infection with IHHNV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable

Table 5.1. Infection with IHHNV surveillance, detection and diagnostic methods

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	d	d
Bioassay	d	d	d	d	c	c
Direct LM	d	d	d	d	d	d
Histopathology	d	d	c	c	a	b
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	d	c	d	d
In-situ ~~DNA probes~~ hybridisation	d	d	b	b	a	a
PCR, qPCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; qPCR = real-time polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with infectious hypodermal and haematopoietic necrosis

As indicated in Table 5.1, PCR is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic IHHNV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with IHHNV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with IHHNV shall be suspected if at least one of the following criteria is met:

i)   Clinical signs indicative of Infection with IHHNV and a positive result by *in-situ* hybridisation

or

ii)   Histopathology indicative of Infection with IHHNV and a positive result by *in-situ* hybridisation.

7.2. Definition of confirmed case

Infection with IHHNV is considered to be confirmed if two of the following criteria are met:

i)   Positive result by *in-situ* hybridisation

ii)   Positive result by PCR (always genotype specific)

iii)   Sequence analysis to confirm IHHNV nucleic acid sequence.

The two methods must target different areas of the genome.

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CHAPTER 2.2.3.

INFECTION WITH
INFECTIOUS MYONECROSIS VIRUS

> **EU comment**
>
> **The EU supports the proposed changes to this chapter.**

1. Scope

Infection with infectious myonecrosis <u>virus</u> means infection with infectious myonecrosis virus (IM<u>N</u>V), which is similar to members of the Family *Totiviridae*. is a viral disease of penaeid shrimp caused by infection with infectious myonecrosis virus (IMNV) (Lightner *et al.,* 2004; Nibert 2007; Poulos *et al.,* 2006).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

IMNV is a totivirus. Phylogenetic analysis of its RNA-dependent RNA polymerase (RdRp) gene coding sequence indicates that IMNV is most closely related to *Giardia lamblia virus*, a member of the family *Totiviridae* (Fauquet *et al.,* 2005; Lightner, 2011; Nibert, 2007; Poulos *et al.,* 2006).

IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml^{-1} in caesium chloride. The genome consists of a single, double-stranded (ds) RNA molecule of 7560 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 59 first ORF (ORF1, nt 136–4953 470–5596) 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 39 second ORF (ORF2, nt 5241–7451 5884–8133) encodes a putative RdRp (Poulos *et al.,* 2006).

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 *P. vannamei* stocks from Brazil) indicate that the virus was introduced from Brazil to Indonesia in 2006.

<u>Infection with IMNV</u> IMN disease is not the same disease as white tail disease of Penaeid shrimp and white tail disease of *Macrobrachium rosenbergii*. These two diseases exhibit gross and histological signs that mimic <u>infection with IMNV</u> IMN, but which are caused by two different types of virus: a nodavirus named *Paneaus vannamei* novavirus – PvNV (Tang *et al.,* 2007) and a nodavirus named *Macrobrachium rosenbergii* nodavirus – *Mr*NV (see Chapter 2.2.7 White tail disease <u>Infection with *Macrobrachium rosenbergii* nodavirus</u>).

2.1.2. Survival outside the host

Only anecdotal information is available. IMNV is apparently more difficult to inactivate with typical pond disinfection procedures (e.g. sun drying, chlorination, etc.) than are other penaeid shrimp viruses like white spot syndrome virus (WSSV), yellow head virus <u>genotype 1</u> (YHV<u>1</u>), Taura syndrome virus (TSV) and infectious hypodermal and haematopoietic virus (IHHNV). Reservoir hosts are suspected, but none have been documented consistently.

2.1.3. Stability of the agent (effective inactivation methods)

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No data.

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species (common and Latin names)

Species that fulfil the criteria for listing a species as susceptible to infection with IMNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: brown tiger prawn (*Penaeus esculentus*), banana prawn (*P. merguiensis*), white leg shrimp (*Penaeus vannamei*).

~~The principal host species in which IMNV is known to cause significant disease outbreaks and mortalities in farmed populations is *Penaeus vannamei* (commonly called the Pacific white shrimp or white leg shrimp) (Lightner *et al.,* 2004; Nunes *et al.,* 2004). The Pacific blue shrimp, *P. stylirostris*, and the black tiger shrimp, *P. monodon* have been infected experimentally with IMNV, but mortalities did not occur as a consequence of experimental infection in this laboratory trial (Tang *et al.,* 2005).~~

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: 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~~2~~. Susceptible stages of the host

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 ~~IMN disease~~ (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006).

2.2.4~~3~~. Species or subpopulation predilection (probability of detection)

No data.

2.2.5~~4~~. Target organs and infected tissue

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).

2.2.6~~5~~. Persistent infection ~~with lifelong carriers~~

Some members of populations of *P. vannamei* that survive IMNV infections and/or epizootics may carry the virus for life ~~and, although this has not been demonstrated scientifically, are believed to transmit virus vertically to progeny.~~

2.2.7.6. Vectors

There are no specific data on vectors. However, because of its non-enveloped particle structure, it is possibly that IMNV, like TSV, will remain infectious in the gut and faeces of seabirds that feed on dead or moribund shrimp at farms with on-going infection with IMNV ~~IMN~~ epizootics, and be spread within and among farms by faeces or regurgitated shrimp carcasses (Vanpatten *et al.,* 2004).

~~2.2.7. Known or suspected wild aquatic animals carriers~~

~~Native wild penaeid shrimp in north-eastern Brazil have been anecdotally reported as hosts.~~

2.3. Disease pattern

In early juvenile, juvenile, or adult *P. vannamei* in regions where IMNV is enzootic, outbreaks of infection with IMNV ~~IMN disease~~ associated with sudden high mortalities may follow stressful events such as capture by cast-netting, feeding, sudden changes in water salinity or temperature, etc. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. Shrimp in the acute phase of infection with IMNV ~~IMN disease~~ will 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 shrimp. Severely affected shrimp become moribund and mortalities can be high immediately following a "stress" event and continue for several days (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006).

2.3.1. Transmission mechanisms

IMNV has been demonstrated to be transmitted horizontally by cannibalism (Lightner, 2011; Poulos *et al.,* 2006). Transmission via water and vertical transmission from broodstock to progeny 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.

2.3.2. Prevalence

In regions where IMNV is enzootic in farmed stocks of *P. vannamei*, its prevalence may reach 100% (Andrade *et al.,* 2007; Nunes *et al.,* 2004).

2.3.3. Geographical distribution

IMNV has been reported to occur in north-eastern Brazil (Andrade *et al.,* 2007; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006) and in the East Java Island (Senapin *et al.,* 2007) as well as west Java, Sumatra, Bangka, west Borneo, south Sulawesi, Bali, Lombok and Sumbawa in South-East Asia (Sutanto, 2011). There are unofficial and anecdotal reports of IMNV occurring in other South-East Asian countries (Senapin *et al.*, 2011).

2.3.4. Mortality and morbidity

Mortalities from infection with IMNV ~~IMN disease~~ can range from 40% to 70% in cultivated *P. vannamei*, and feed conversion ratios (FCR) of affected populations can increase from a normal value of ~ 1.5 up to 4.0 or higher (Andrade *et al.,* 2007).

2.3.5. Environmental factors

Temperature and salinity effects are considered to be likely predisposing factors to disease outbreaks, but no experimental data are available (Nunes *et al.,* 2004).

2.4. Control and prevention

2.4.1. Vaccination

No effective "vaccines" for infection with IMNV ~~IMN~~ are available.

2.4.2. Chemotherapy

No effective therapeutic agents have been reported for infection with IMNV ~~IMN~~.

2.4.3. Immunostimulation

No data.

2.4.4. Resistance breeding

There are anecdotal reports of some selected lines of *P. vannamei* having better survival and culture performance in farms where infection with IMNV ~~IMN~~ 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).

2.4.5. Restocking with resistant species

While there are no published reports, some shrimp farms in Indonesia are believed have stocked *P. monodon* and *P. stylirostris* because of data from a preliminary study showing these species to be more resistant to infection with IMNV IMN than *P. vannamei* (Tang *et al.,* 2005).

2.4.6. Blocking agents

No data.

2.4.7. 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 transmission potential of a number of penaeid shrimp pathogens from female spawners to their eggs or larvae, and the practice may reduce IMNV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry practices have been applied successfully to prevent IMNV infections and development of clinical disease IMN disease at shrimp farms. Foremost among these has been the application of reverse-transcription-PCR (RT-PCR) for screening pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test PCR-positive (Andrade *et al.,* 2007). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P. vannamei*, and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* most suited to local culture conditions has proven to be the most successful husbandry practice for preventing and controlling other viral pathogens of shrimp, and should be applicable to control and prevent infection with IMNV IMN disease (Lee & O'Bryen, 2003; Lightner, 2005; Lightner *et al.,* 2009; Moss & Moss, 2009).

3. Sampling

3.1. Selection of individual specimens

Specimens suitable for testing for IMNV infection using molecular methods (e.g. RT-PCR, nested RT-PCR, qRT-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 detecting IMNV or certification for freedom of infection with IMNV IMN disease.

3.2. 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 *General information* (on diseases of crustaceans).

3.3. Pooling of samples

Tissue taken for molecular tests may be pooled. Pool sizes of 5 or less are recommended for tissue sampled from juveniles, subadults and adults. Eggs, larvae and PL can be pooled in larger numbers (e.g. up to 150 eggs or larvae and 5–50 PL depending on their size/age) may be necessary to extract sufficient RNA for RT-PCR testing. See also chapter 2.2.0.

3.4. Best organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of IMNV infection 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.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.5. Samples/tissues that are not suitable

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.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only the acute-phase of IMN disease can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase infection with IMNV IMN disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase of IMN disease present behavioural changes. Typically, severely affected shrimp become lethargic during or soon after stressful events such as capture by cast-netting, feeding, sudden changes in water temperature, sudden reductions in water salinity, etc.). Severely affected shrimp may have been feeding just before the onset of stress and often have a full gut.

4.2. Clinical methods

4.2.1. Gross pathology

Shrimp in the acute phase of IMN 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. These signs may have a sudden onset following stresses (e.g. capture by cast-netting, feeding, and sudden changes in temperature or salinity). Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days.

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).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Infection with IMNV IMN disease 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 IMN White tail disease of penaeid shrimp caused by the nodavirus PvNV can mimic infection with IMNV IMN (Tang *et al.,* 2007). Hence, diagnostic information from other sources (e.g. history, gross signs, morbidity, mortality, or RT-PCR findings) may be required to confirm a diagnosis of infection with IMNV IMN.

By histology using routine haematoxylin–eosin (H&E) stained paraffin sections (Bell & Lightner, 1988), tissue sections from shrimp with acute-phase infection with IMNV IMN 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. In these shrimp, the affected muscle fibres appear to progress from presenting coagulative necrosis to presenting 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).

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Significant hypertrophy of the LO caused by accumulations of LO spheroids (LOS) is a highly consistent lesion in shrimp with acute or chronic-phase <u>infection with IMNV</u> ~~IMN~~ 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).

4.2.4.   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 (LOS) amongst normal LO tubules.

4.2.5.   Smears

Not applicable.

4.2.6.   Fixed sections

See Section 4.2.1.

4.2.7.   Electron microscopy/cytopathology

Not applicable for diagnostic purposes.

4.3. Agent detection and identification methods

4.3.1.   Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Sections 4.2.3 and 4.2.6.

4.3.1.2.   Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

None reported to date.

4.3.1.2.2. Antibody-based antigen detection methods

Monoclonal antibodies (MAbs) have been developed to the capsid protein of IMNV (Kunanopparat *et al.,* 2011). Three MAbs were developed and when used in combination, they provided better sensitivity than any one of the MAbs used in isolation. However, the sensitivity was approximately tenfold lower than that of a one-step RT-PCR assay using the same sample.

4.3.1.2.3. Molecular techniques

Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and quantitative real-time (q)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. All PCR tests have proved to be specific to IMNV.

As the sensitivity of the nested and real-time RT-PCR is greater than any other diagnostic method available currently, approaching a detection limit of 10 viral genome copies, these tests are the gold standard for IMNV (Andrade *et al.,* 2007; Poulos *et al.,* 2006).

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).

RT-PCR for detection of IMNV

A nested RT-PCR method was developed to detect IMNV that uses two PCR primer sets that produce a 328 bp one-step amplicon and 139 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).

Viral RNA can be isolated using any commercially available RNA isolation kit. The amount of tissue required will depend on the kit selected (i.e. Qiagen RNA extraction kit, Promega and Roche RNA purification kit recommend using 25–50 mg of tissue[4]). Depending on the kit used, the elution volume for Roche and Qiagen and low elution volume RNA isolation Promega extraction kit is 100 µl. The high elution volume RNA isolation Promega extraction kit is 500 µl. Extracted RNA should be maintained at –20°C before testing, however, for long-term storage the RNA should be kept at –70°C.

Following RNA extraction, the method is summarised below:

RNA templates:

1. Frozen or ethanol-fixed tissue (pleopods, cephalothorax, muscle)
2. Haemolymph (less sensitive than when other tissues are used)

RT-PCR reaction mixture (Applied Biosystems rTth Enzyme and 5 × EZ Buffer #N808 0178 SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Life Technologies):

Reagent	Volume 25 µl reaction	Final concentration
DD dH$_2$O	6.5 5.5 µl	–
5 × EZ Buffer 2 × reaction mix	5.0 12.5 µl	1 ×
dNTP mix Forward/reverse primer (10mM each)	3.0 1.0 µl	300 µM each 0.4 µm
Primer F (100 ng µl^{-1}) RT/Taq enzyme mix	1.0 µl	0.62 µM
Primer R (100 ng µl^{-1})	1.0 µl	0.62 µM
Mn(Oac)$_2$ (25 mM)	2.5 µl	2.5 mM
rTth Enzyme (2.5 U µl^{-1})	6.5 1.0 µl	0.1 U µl^{-1}
RNA template[1]	1–5 5.0 µl	1–50 ng total RNA

[1]Template must be boiled for 3 minutes and chilled on ice just prior to adding to reaction mix.

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

Annex 25 (contd)

RT-PCR thermal cycling conditions:

PCR Primers	Temperature (°C)	Time	No. cycles	Amplicon length
4587F/4914R	60, 95	30 minutes, 2 minutes	1	328 bp
	95, ~~60~~ 62	45 seconds, 45 seconds	39	
	60	7 minutes	1	

Nested PCR reaction (~~Amersham Biosciences pure Taq~~ illustra™ PuReTaq™ Ready-To-Go PCR Beads ~~#27 9558 01~~, GE Healthcare):

Reagent	25 µl reaction	Final concentration
DD H$_2$O	~~22.5~~ 23 µl	–
Primer NF (~~100 ng µl^{-1}~~ 10 µM)	~~1.0~~ 0.5 µl	~~0.465~~ 0.2 µM
Primer NR (~~100 ng µl^{-1}~~ 10 µM)	~~1.0~~ 0.5 µl	~~0.465~~ 0.2 µM
Template[2]	~~0.5~~ 1.0 µl	–

[2]Template for the nested reaction is the product from the first step reaction

Nested PCR thermal cycling conditions:

Primers	Temperature (°C)	Time	No. cycles	Amplicon length
4725 NF/ 4863 NR	95	2 minutes	1	139 bp
	95, 65, 72	30 seconds, 30 seconds, 30 seconds	39	
	72	2 minutes	1	

Primer sequences:

Primer	Sequence (5' to 3')	Amplicon Length	Ref.
4587F	CGA-CGC-TGC-TAA-CCA-TAC-AA	328 bp	Poulos & Lightner, 2006
4914R	ACT-CGG-CTG-TTC-GAT-CAA-GT		
4725 NF	GGC-ACA-TGC-TCA-GAG-ACA	139 bp	
4863 NR	AGC-GCT-GAG-TCC-AGT-CTT-G		

Quantitative (real-time) RT-PCR for detection of IMNV

A real-time qRT-PCR method was developed to detect and quantify IMNV in shrimp tissue. The method can detect as few as 10 IMNV RNA copies per µl total RNA (Andrade *et al.,* 2007). The method as published is summarised below.

The Primer Express software (Applied Biosystems) was used to aid the design of the PCR primers and TaqMan probe targeted to the ORF1 region of the IMNV genome (GenBank accession no. AY570982 (Andrade *et al.,* 2007; Poulos *et al.,* 2006). Primers IMNV412F (5'-GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA-3') and IMNV545R (5'-AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT-3') amplify a 134 bp DNA. The TaqMan probe, IMNVp1 (5'-6FAM-CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG-TAMRA-3'), which corresponds to the nucleotides 467–500, is labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) at its 5'-end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at its 3'-end. The IMNV genome fragment is amplified using ~~an ABI GeneAmp 5700 sequence detection system~~ StepOnePlus PCR System and the TaqMan ~~One~~ Fast virus 1-Step ~~RT-PCR master mix (Applied Biosystems)~~ Master Mix (Life Technologies). Prior to the ~~real-time~~ qRT-PCR, extracted RNA is boiled at ~~95~~ 100°C for ~~5~~ 3 minutes to denature the dsRNA and chilled immediately in wet ice. The reaction mixture contains 1 µl RNA sample, ~~12.5 µl~~ TaqMan Master mix (2×), ~~0.625 µl Multiscribe mix (40×),~~ 300 nM each primer IMNV412F and IMNV545R, 200 nM

IMNVp1TaqMan probe in a ~~25~~ 10–20 µl final volume. The ~~RT~~ qRT-PCR thermal cycling conditions used are ~~48~~ 50°C for ~~30~~ 3 minutes, 95°C for ~~10 minutes~~ 20 seconds followed by 40 cycles of 95°C for ~~15~~ 3 seconds and 60°C for ~~1 minute. The IMNV RNA copy number~~ 30 seconds. At the end of the ~~samples~~ reaction, fluorescence intensity is ~~determined using serial dilutions~~ measured, a threshold will be set to be above the baseline. Samples with a Ct value lower than 40 cycles are considered to be positive. It is necessary to include a 'no template' control in each reaction run. This is to rule out the presence of ~~a synthetic~~ fluorescence contaminants in the reaction mixture. A positive control should also be included, and it can be RNA extracted from IMNV-infected tissue, or in vitro transcribed IMNV RNA ~~standard~~ containing the target sequence (see below). ~~and the Gene Amp 5700 sequence detection software.~~

To synthesise an RNA standard for the real-time qRT-PCR, the PCR primers IMNV218F and IMNV682R (5'-GCT-GGA-CTG-TAT-TGG-TTG-AG-3' and 5'-AAC-CAA-GTT-CTT-CTT-CTC-CAG-TT-3', respectively) are used to amplify a 464 bp DNA product from the IMNV genome. The PCR product purified using a QIAuick PCR Purification kit (QIAGEN) was cloned into pGEM-T Easy Vector. A recombinant plasmid, pIMNV-1, confirmed to contain the 464 bp insert by sequence analysis, is linearised by digestion with *Pst*I and used as the template for an *in-vitro* RNA transcription using T7 RNA polymerase and associated reagents (Promega). RNA is synthesised at 37°C for 2 hours in a 50 µl reaction containing 1 µg plasmid DNA, followed by DNase I digestion at 37°C for 30 minutes for remove DNA. The length and integrity of the synthesic ssRNA is confirmed by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The RNA is purified using a Qiaquick PCR Purification kit, quantified by a spectrophotometer, and stored at –70°C

4.3.1.2.4. Agent purification

While IMNV has been purified from infected shrimp tissue by sucrose density gradient ultracentrifugation (Poulos *et al.,* 2006), this is not recommended for diagnostic purposes.

4.3.2. Serological methods

Not applicable because shrimp are invertebrates which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to IMNV.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of Infectious myonecrosis are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Bioassay	d	d	c	c	c	c
Direct LM	d	d	c	c	c	c
Histopathology	d	d	b	b	a	c
Transmission EM	d	d	d	d	d	d
Antibody-based assays	d	d	d	d	c	d
DNA probes (ISH)	d	d	a	a	a	a
Nested RT-PCR	a	a	a	a	a	a
qRT-PCR	d	c	a	a	a	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; ISH = *in-situ* hybridisation (ISH); RT-PCR = reverse-transcription polymerase chain reaction; qRT-PCR = quantitative (real-time) RT-PCR.

6. Test(s) recommended for targeted surveillance to declare freedom from infectious myonecrosis

As indicated in Table 5.1, nested RT-PCR (Section 4.3.1.2.3) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, histological demonstration of characteristic IMNV-induced lesions in the striated muscles and the extreme hypertrophy of the LO caused by LOS formation (with or without confirmation by ISH with IMNV-specific DNA probes) is a suitable method (Table 5.1). The occurrence of significant mortality distinguishes <u>infection with IMNV</u> IMN from penaeid white tail disease caused by PvNV, in which the gross signs and histopathology mimics <u>infection with IMNV</u> IMN disease (Tang *et al.,* 2007).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Sudden high mortalities, usually following stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult P. vannamei in regions where IMNV is enzootic or where introduction of P. vannamei from infected regions or countries has occurred. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut, and shrimp in the acute phase of infection with IMNV will 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. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Exposing the paired LO by simple dissection will show that they are hypertrophied to 3–4 times their normal size.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- Histological demonstration of diagnostic acute, transition or chronic-phase IMNV lesions in the striated muscles and/or the LO.

- ISH positive (with a IMNV-specific cDNA probe) signal to IMNV-type lesions in striated necrotic muscle fibres or to distinctive LOS in the lymphoid organs of shrimp with transition or chronic-phase IMNV infections in histological sections.

- One step or nested RT-PCR, or real time qRT-PCR with positive results for IMNV.

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C H A P T E R   2 . 2 . 4 .

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

> **EU comment**
>
> **The EU supports the proposed changes to this chapter.**

1. Scope

Infection with *Hepatobacter penaei* means infection with Candidatus *Hepatobacter penaei,* an obliagate intracellular bacterium of the Order α-Proteobacteria. The disease is commonly known as necrotising hepatopancreatitis disease is caused by infection with a Gram-negative, pleomorphic intracellular alpha-proteobacterium (Frelier *et al.,* 1992; Lightner & Redman, 1994; Lightner *et al.,* 1992; Loy *et al.,* 1996a; 1996b) preliminarily called Candidatus *Hepatobacter penaei.* The principal host species in which necrotising hepatobacterium (NHPB) can cause significant disease outbreaks and mortalities are *Penaeus vannamei* and *P. stylirostris* (Del Río Rodríguez *et al.,* 2006; Frelier *et al.,* 1993; Ibarra Gámez *et al.,* 2007; Lightner & Redman, 1994; Morales Covarrubias *et al.,* 2011).

NHP has four distinct phases: initial, acute, transition and chronic. In acute and transition phase disease, pathognomonic lesions are typically present in histological sections of the hepatopancreas, while in the initial and chronic phases of the disease, there are no pathognomonic lesions, and molecular and antibody-based methods for NHPB detection are necessary for diagnosis (Morales Covarrubias, 2010; Morales Covarrubias *et al.,* 2010; 2012; Vincent & Lotz, 2005).

Synonyms: necrotising hepatobacterium (NHPB) or NHP bacterium (NHPB); rickettsial like organism (RLO).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

NHPB Candidatus *H. penaei* is a pleomorphic, Gram-negative, intracytoplasmic bacterium preliminarily called Candidatus *Hepatobacter penaei* (Nunan *et al.,* 2013). It is a member of the α subclass of proteobacteria (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy *et al.,* 1996a; 1996b). 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 *et al.,* 1996a; 1996b). Genetic analysis of the NHPB Candidatus *H. penaei* associated with North and South American outbreaks of NHP suggest that the isolates are either identical or very closely related subspecies (Loy *et al.,* 1996a; 1996b).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent

NHPB Candidatus *H. penaei*-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. NHPB Candidatus *H. penaei* frozen at –20°C –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).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with *H.epatobacter penaei* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: white leg shrimp (*Penaeus vannamei*) Most penaeid species can be infected with NHPB, including the principal cultured species in Latin American, *P. vannamei* (Pacific white shrimp) and *P. stylirostris* (Pacific blue shrimp).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: northern white shrimp (*Penaeus setiferus*), northern pink shrimp (*P. duorarum*), blue shrimp (*P. stylirostris*), banana prawn (*P. merguiensis*), Aloha prawn (*P. marginatus*), northern brown shrimp (*P. aztecus*) and giant tiger prawn (*P. monodon*).

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: American lobster (*Homarus americanus*).

NHPB infections are most severe in *P. vannamei* where the intracellular bacterium can cause acute epizootics and mass mortality (>90%). In *P. vannamei,* the juvenile, subadult and broodstock life stages are the most severely affected (Johnson, 1990; Jory, 1997; Lightner, 1996; Morales Covarrubias, 2010).

NHPB causes chronic disease in *P. vannamei,* the main effects of which are slow growth, a soft cuticle and a flaccid body (Morales-Covarrubias, 2010; Morales-Covarrubias *et al.,* 2012).

Outbreaks of NHP disease have been reported in *P. aztecus* (Johnson, 1990; Jory, 1997; Lightner, 1996; Morales Covarrubias, 2010). NHP has also been seen in *P.californiensis* and *P. setiferus* (Frelier *et al.,* 1995; Lightner, 1996). *Penaeus setiferus* is reportedly less susceptible to disease than *P. vannamei* (Frelier *et al.,* 1995).

In an NHP survey of the Gulf of Mexico, *P.setiferus* and *P.duorarum* in the vicinity of coastal prawn farms along the Yucatan and Campeche coast revealed no histological evidence of NHP (Del Río Rodríguez *et al.,* 2006).

2.2.3. Susceptible stages of the host

NHPB Infection with *H. penaei* has been demonstrated in juveniles, adults and broodstock of *P. vannamei.*

2.2.4. Species or sub-population predilection

See Sections 2.2.1 and 2.2.2.

2.2.5. Target organs and infected tissue

The target tissue is the hepatopancreas, with NHPB infection with *H. penaei* reported in all hepatopancreatic cell types.

2.2.6. Persistent infection with lifelong carriers

Some members of *P. vannamei* populations that survive NHPB infection with *H. penaei* or epizootics may carry the intracellular bacteria for life and pass it on to other populations by horizontal transmission (Aranguren *et al.,* 2006; Lightner, 2005; Morales-Covarrubias, 2008; 2010; Vincent & Lotz, 2005).

Natural transmission of NHPB Candidatus *H. penaei* is thought to occur *per os* by cannibalism (Frelier *et al.,* 1993; 1995; Johnson, 1990; Lightner, 2005; Morales-Covarrubias, 2010), although cohabitation and dissemination of NHPB Candidatus *H. penaei* via the water column may also play a role (Frelier *et al.,* 1993; 1995). NHPB Candidatus *H. penaei* in faeces shed into pond water has also been suggested as a possible means of transmission (Aranguren *et al.,* 2006; Briñez *et al.,* 2003; Morales-Covarrubias *et al.,* 2006). Outbreaks of clinical disease are often preceded by prolonged periods of high water temperature (approximately 30°C) and salinity (up to 40 parts per thousand [ppt]) (Frelier *et al.,* 1995; Lightner & Redman, 1994; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.,* 2010; 2011; Vincent & Lotz, 2005).

2.2.7. Vectors

No vectors are known in natural infections.

2.2.7. Known or suspected wild aquatic animal carriers

NHPB is common in wild penaeid shrimp in Peru (*P. vannamei*) and Laguna Madre of Tamaulipas, Mexico (*P. aztecus, P. duorarum* and *P. setiferus*) (Aguirre Guzman *et al.,* 2010; Lightner & Redman, 1994).

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of ~~NHPB~~ Candidatus *H. penaei* can be horizontal by cannibalism; transmission by contaminated water has been demonstrated (Aranguren *et al.,* 2006; 2010; Frelier *et al.,* 1993; Gracia-Valenzuela *et al.,* 2011; Morales-Covarrubias *et al.,* 2012; Vincent *et al.,* 2004).

2.3.2. Prevalence

Some reported mean values for ~~NHPB~~ Candidatus *H. penaei* prevalence in wild stocks are between 5.6 and 15% in *P. duorarum,* and between 5 and 17% in *P. aztecus* collected from Carrizal and Carbonera, Laguna Madre of Tamaulipas, Mexico (Aguirre-Guzman *et al.,* 2010); 0.77% in *P. vannamei*, and 0.43% in *P. stylirostris* collected from Tumbes Region, Peru (Lightner & Redman, 1994).

Some reported mean values for ~~NHPB~~ Candidatus *H. penaei* prevalence in shrimp farms are between 0.6% and 1.3% in *P. vannamei* collected from shrimp farms in Belize, Brazil, Guatemala, Honduras, Mexico, Nicaragua and Venezuela (Morales-Covarrubias *et al.,* 2011).

2.3.3. Geographical distribution

~~NHPB~~ Candidatus *H. penaei* appears to have a Western hemisphere distribution in both wild and cultured penaeid shrimp (Aguirre-Guzman *et al.,* 2010; Del Río-Rodríguez *et al.,* 2006). In the Western Hemisphere, ~~NHPB~~ Candidatus *H. penaei* is commonly found in cultured penaeid shrimp in Belize, Brazil, Colombia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Peru, United States of America, and Venezuela (Frelier *et al.,* 1992; Ibarra-Gámez *et al.,* 2007; Lightner, 1996; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.,* 2011).

2.3.4. Mortality and morbidity

In *P. vannamei*, infection ~~by NHPB~~ with *H. penaei* results in an acute, usually catastrophic disease with mortalities approaching 100%.

2.3.5. Environmental factors

The replication rate of ~~NHPB~~ Candidatus *H. penaei* increases at lengthy periods of high temperatures (>29°C) and salinity changes (20–38%). In Mexico, ~~NHPB~~ Candidatus *H. penaei* has been detected at a low prevalence (<7%) in shrimp farms in the months of April, May, July and August. However, in the months of September and October when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

2.4. Control and prevention

Control

The use of the antibiotics, oxytetracycline and florfenicol 50%, in medicated feeds every 8 hours for 10 days is probably the best ~~NHPB~~ treatment currently available, particularly if infection with *H. paeni* is detected in the initial phase (Frelier *et al.,* 1995; Morales-Covarrubias *et al.,* 2012).

Prevention

a) Early detection (initial phase) of clinical ~~NHPB~~ infection with *H. penaei* is important for successful treatment because of the potential for cannibalism to amplify and transmit the infection.

b) Shrimp starvation and cannibalism of shrimps with ~~NHPB~~ infection with *H. penaei*, as well as positive conditions for ~~NHPB~~ Candidatus *H. penaei* cultivation, are important factors for ~~NHPB~~ Candidatus *H. penaei* propagation in *P. vannamei.*

Annex 26 (contd)

c) The use of quick hydrated lime (Ca(OH)$_2$) to treat pond bottoms during pond preparation before stocking can help reduce the incidence of ~~NHPB~~ infection with *H. penaei*.

d) Preventive measures can include raking, tilling and removing sediments from the bottom of the ponds, prolonged sun drying of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, and drying and extensive liming of ponds.

e) The use of specific pathogen-free (SPF) broodstock is an effective preventive measure.

2.4.1. Vaccination

No scientifically confirmed reports.

2.4.2. Chemotherapy

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Resistance breeding

No scientifically confirmed reports.

2.4.5. Restocking with resistant species

No scientifically confirmed reports.

2.4.6. Blocking agents

No scientifically confirmed reports.

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs and larvae is a good management practice (Lee & O'Bryen, 2003) and is recommended for its potential to reduce ~~NHPB~~ Candidatus *H. penaei* contamination of spawned eggs and larvae (and contamination by other pathogenic agents).

2.4.8. General husbandry practices

Some husbandry practices have been successfully applied to the prevention of ~~NHPB~~ Candidatus *H. penaei* infections and disease. Among these has been the application of PCR to pre-screening of wild or pond-reared broodstock.

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection ~~by NHPB~~ with *H. penaei* are life stages (postlarvae [PL], juveniles and adults).

3.2. 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.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, sub adults and adults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more ~~eggs or~~ larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs or tissues

~~NHPB~~ Candidatus *H. penaei* infects most enteric tissue. The principal target tissue for ~~NHPB~~ Candidatus *H. penaei* is hepatopancreas. Faeces may be collected and used for testing (usually by PCR, or dot-blot hybridisation with specific probes) when non-lethal testing of valuable broodstock is necessary (Bondad-Reantaso *et al.,* 2001; Bradley-Dunlop *et al.,* 2004; Briñez *et al.,* 2003; Frelier *et al.,* 1993; Lightner, 1996; Morales-Covarrubias *et al.*, 2012).

3.5. Samples/tissues those are not suitable

~~NHPB~~ Candidatus *H. penaei* do 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.

4. Diagnostic methods

4.1. Field diagnostic methods

The prevalence and severity of ~~NHPB~~ infection with *H. penaei* may be enhanced in a contained population by rearing shrimps in relatively crowded or stressful conditions. The 'crowding stress' factors may include high stocking densities, ablated, and marginal water quality (i.e. low dissolved oxygen, elevated water temperature, or elevated ammonia or nitrite) in the holding tank water. These conditions may encourage expression of low-grade ~~NHPB~~ infection with *H. penaei* and the transmission of the agent from carriers to previously uninfected hosts in the population. This results in increased prevalence and severity of infections that can be more easily detected using the available diagnostic and detection methods ~~for NHPB~~.

4.1.1. Clinical signs

A wide range of gross signs can be used to indicate the possible presence of ~~NHPB~~ 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 epicommensal 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.

4.1.2. Behavioural changes

In acute ~~NHPB~~ disease, *P. vannamei* may present behavioural changes including lethargy and reduced feeding activity.

4.2. Clinical methods

4.2.1. Gross pathology

~~NHPB~~ 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. Infected adults seldom show signs of the infection or mortalities (Aranguren *et al.,* 2006; 2010; Bastos Gomes *et al.,* 2010, Brock & Main, 1994; Morales-Covarrubias *et al.*, 2012). Gross signs are not ~~NHP~~ specific, but shrimp with acute ~~NHP~~ infection with *H. penaei* shows a marked reduction in food consumption, followed by changes in behaviour and appearance (see Section 4.1.1).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute and chronic ~~NHPB~~ infection with *H. penaei* in *P. vannamei* can be readily diagnosed using routine haematoxylin and eosin (H&E) stain histological methods (see Section 4.2.6).

4.2.3.1. Initial phase of ~~infection with H. penaei~~ ~~necrotising hepatopancreatitis~~

Initial ~~NHPB~~ infection with *H. penaei* is more difficult to diagnose using routine H&E histological methods. For diagnosis of initial infections, molecular methods are recommended for ~~NHPB~~ Candidatus *H. penaei* detection (e.g. by PCR or application of ~~NHPB~~ Candidatus *H. penaei* -specific DNA probes to dot-blot hybridisation tests or *in-situ* hybridisation (ISH) of histological sections).

4.2.3.2. The acute phase of ~~infection with H. penaei~~ ~~necrotising hepatopancreatitis~~

Acute ~~NHPB disease~~ 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 in to the tubular lumen. The tubular epithelial cell lipid content is variable.

4.2.3.3. Transition phase of ~~infection with H. penaei~~ ~~necrotising hepatopancreatitis~~

The transitional phase of ~~NHPB disease~~ 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 in morphology. They contain little or no stored lipid vacuoles, markedly reduced or no secretory vacuoles and masses of bacteria. At this phase haemocyte nodules were observed in the presence of masses of bacteria in the centre of the nodule

4.2.3.4. Chronic phase of ~~infection with H. penaei~~ ~~necrotising hepatopancreatitis~~

In the chronic phase of ~~NHPB~~ 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.

4.2.4. Wet mounts

Wet-mount squash examination of hepatopancreas ~~(HP)~~ tissue is generally conducted to detect presumptive ~~NHPB disease~~ infection with *H. penaei*. The hepatopancreas may be atrophied and have any of the following characteristics: soft and watery; fluid filled centre; pale with black stripes (melanised tubules); pale centre instead of the normal orange coloration. For wet mount analysis the shrimp must be in the intermolt stage, and have not undergone a treatment that could alter the tubules. This technique uses tubular deformation or atrophy, mainly of the apical region to indicate early stages of ~~NHPB~~ infection with *H. penaei*.

~~NHPB disease~~ Infection with *H. penaei* has four phases (a semiquantitative scale):

Initial phase: low presence of tubular deformation (1–5 field^{-1} organism^{-1}) and cell detachment.

Acute phase: infiltration of haemocytes, increased numbers of deformed tubules (6–10 field^{-1} organism^{-1}), encapsulation present in different regions of the sample, which is atrophied tubules surrounded by multiple layers of haemocytes.

Transition phase: infiltration of haemocytes, increased numbers of deformed tubules (11–15 field^{-1} organism^{-1}), melanised tubules, necrotic tubules and a high level of encapsulation present in different regions of the sample. ~~At this stage haemocyte nodules were observed with masses of bacteria in the centre of the nodule.~~

Chronic phase: areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells ~~with masses of bacteria in the cytoplasm.~~

4.2.5. Smears

Not applicable.

4.2.6. Electron microscopy/cytopathology

Not currently applicable for diagnostic purposes

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See section 4.2.4

4.3.1.1.2. Smears

Not applicable

4.3.1.1.3. Fixed sections

See section 4.2.3.

4.3.1.1.4. Bioassay method

Confirmation of ~~NHPB~~ infection with *H. penaei* may be accomplished by bioassay of ~~NHPB~~ suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the intracellular bacteria (Cock *et al.,* 2009; Johnson, 1990; Lee & O'Bryen, 2003; 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 ~~NHPB~~ Candidatus *H. penaei*, ~~NHPB~~ Candidatus *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 ~~NHPB disease~~ infection with *H. penaei* and unusual mortalities.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

~~NHPB~~ Candidatus *H. penaei* has not been grown *in vitro*. No crustacean cell lines exist (Morales-Covarrubias *et al.*, 2010; Vincent & Lotz, 2007).

4.3.1.2.2. Antibody-based antigen detection methods

Immunohistochemistry (IHC) tests using monoclonal antibodies (MAbs) to ~~NHPB~~ Candidatus *H. penaei* according to the methods described in Bradley-Dunlop *et al.* (2004).

4.3.1.2.3. Molecular techniques

ISH and PCR tests for ~~NHPB~~ infection with *H. penaei* have been developed, and PCR kits for ~~NHPB~~ are commercially available. PCR tests for infection with *H. penaei* have been developed and a number of methods and commercial products using these methods are available (Loy & Frelier, 1996; Loy *et al.,* 1996b). Gene probes and PCR methods provide greater diagnostic sensitivity than do classic histological approaches to ~~NHP~~ diagnosis of infection with *H. penaei*. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp.

4.3.1.2.3.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled probes for ~~NHPB~~ Candidatus *H. penaei* may be produced in the laboratory. The ISH method of Loy & Frelier (1996) and Lightner (1996) provides greater diagnostic sensitivity than do more traditional methods for ~~NHPB~~ Candidatus *H. penaei* detection and diagnosis of infection that employ classical histological methods (Johnson, 1990; Lightner, 1996; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2012). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled cDNA probe to ~~NHPB~~ Candidatus *H. penaei*, provides a definitive diagnosis of ~~NHPB~~ infection with *H. penaei* (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.*, 2006). Pathognomonic ~~NHPB~~ Candidatus *H. penaei* positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. (See Chapter 2.2.2 IHHN 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.)

4.3.1.2.3.2. PCR method

Hepatopancreas and faeces may be assayed for ~~NHPB~~ Candidatus *H. penaei* using PCR. 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) designed against the GenBank accession number corresponding to the 16S rRNA of ~~NHPB~~ Candidatus *H. penaei* (Nunan *et al.*, 2008). The PCR method outlined below generally follows the method described in Aranguren *et al.* (2010) with modifications by an OIE Reference Laboratory in the USA.

i)   *Preparation of* DNA *template:* DNA can be extracted from 25–50 mg of fresh, frozen and ethanol-preserved hepatopancreas. Extraction of DNA should be performed using commercially available DNA tissue extraction kits following the manufacturer's procedures for production of quality DNA templates. Other DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucelic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega)[5].

ii) The following controls should be included when perfomring the PCR assay ~~for NHPB~~ a) known ~~NHPB~~ Candidatus *H. penaei* negative tissue sample; b) a known ~~NHPB~~ Candidatus *H. penaei* -positive sample (hepatopancreas); and c) a 'no template' control.

iii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

iv) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of ~~NHPB~~ Candidatus *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.

v)   If the thermal cycler does not have a heated lid, then light mineral oil (50 µl) is overlaid on the top of the 25 µl reaction mixtures to prevent condensation or evaporation during thermal cycling.

vi) 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.

Note: The conditions should be optimised for each thermal cycler using known positive controls.

4.3.1.2.3.3. Real-time PCR method

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

The real-time PCR method using TaqMan chemistry described below for ~~NHPB~~ Candidatus *H. penaei* generally follows the method used in Aranguren *et al* (2010).

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

i) The PCR primers and TaqMan probe were selected from the 16S, rRNA gene of ~~NHPB~~ Candidatus *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) *Preparation of DNA template:* the extraction and purification of *DNA* template from hepatopancreas, is the same as that described in the section for traditional PCR.

iii) *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 of *DNA*, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) 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.

v) At the end of the reaction, real time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase in PCR product.

vi) 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 or in the heat block of the thermal cycler. A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from ~~NHPB~~ CHp-infected hepatopancreas.

4.3.1.2.3.4. Sequencing

PCR products may be cloned and sequenced or sequenced directly when necessary to confirm infection ~~by NHPB~~ with *H. penaei* or to identify false positives or nonspecific amplification (Aranguren *et al.,* 2010; Bustin *et al.,* 2009; Vincent & Lotz, 2005).

4.3.1.2.4. Agent purification

Methods for ~~NHPB~~ Candidatus *H. penaei* isolation and purification are available (Aranguren *et al.,* 2010; Nunan *et al., 2013;* Vincent *et al.,* 2004; Vincent & Lotz, 2005). ~~The NHPB bacterium~~ Candidatus *H. penaei* is unculturable using traditional bacteriological methods, thus ~~NHPB~~ infection with *H. penaei* must be maintained through continual exposure of uninfected *L. vannamei* stock to a population undergoing an ~~NHPB~~ infection with *H. penaei* epizootic.

4.3.2   Serological methods

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 ~~NHPB~~ Candidatus *H. penaei*.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of ~~NHPB~~ infection with *H. penaei* are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Annex 26 (contd)

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	d
Bioassay	d	d	d	d	c	d
Direct LM	d	d	c	d	c	d
Histopathology	d	b	b	c	a	b
In-situ DNA probes	a	a	a	a	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
Real-time PCR	a	a	a	a	a	a
PCR	a	a	a	a	a	a
Sequencing	d	d	d	d	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *H. penaei* ~~Necrotising hepatopancreatitis~~

As indicated in Table 5.1, real-time PCR (Section 4.3.1.2.3.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity. When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic ~~NHPB~~ Candidatus *H. penaei*-induced lesions in the hepatopancreas by histology (with or without confirmation by ISH with ~~NHPB~~ Candidatus *H. penaei*-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

The presence of ~~NHPB~~ infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

- Sudden high mortalities in late PL, juvenile or subadult *P. vannamei* ~~or *P. stylirostris*~~ in regions where ~~NHPB~~ infection with *H. penaei* is enzootic;

- Samples of cultured *P. vannamei* ~~or *P. stylirostris*~~ from ponds with feeding sea birds that present gross signs indicative of acute- or transition-phase infection with *H. penaei*, such as a general atrophied hepatopancreas, reddish colouration, lethargy, soft shells, empty guts, and the presence of numerous irregular black spots on the cuticle;

- Poor hatching success of eggs, and poor survival and culture performance of the larval and PL stages when broodstock are used from wild or farmed stocks where ~~NHPB~~ infection with *H. penaei* is enzootic.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

• Histological demonstration of diagnostic acute-phase ~~NHPB~~ infection with *H. penaei* lesions in (especially) the atrophied hepatopancreas with moderate atrophy of the tubule mucosa, presence of bacteria and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations).

• ISH positive histological signal to ~~NHPB~~ infection with *H. penaei*-type lesions.

• PCR positive results for ~~NHPB~~ infection with *H. penaei*.

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CHAPTER 2.2.5.

INFECTION WITH TAURA SYNDROME VIRUS

EU comment

The EU in general supports the proposed changes to this chapter. Comments are inserted in the text below.

1. Scope

Infection with Taura syndrome virus means infection with Taura syndrome virus (TSV) of the genus Aparavirus in the Family *Dicistroviridae.*

Taura syndrome (TS) is a viral disease of penaeid shrimp caused by infection with Taura syndrome virus (TSV) (Bonami *et al.,* 1997; Fauquet *et al.,* 2005; Lightner 1996a; Mari *et al.,* 1998).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is TSV, as described by Bonami *et al.* (1997) and Mari *et al.* (1998; 2002). At least four genotypes (strains) 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 (Chang *et al.,* 2004; Erickson *et al.,* 2002; 2005; 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 to a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (Mari *et al.,* 2002; Poulos *et al.,* 1999): 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. The MAB 1A1 non-reactors were subdivided into Types 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 (Erickson *et al.,* 2002; 2005), 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}. 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 nonstructural 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).

TSV has been assigned to the genus Aparavirus in the Family Dicistroviridae in the 9th report of the International Committee on Taxonomy of Viruses (ICTV; King *et al.,* 2012).

Other reported causes of Taura syndrome: Taura syndrome 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 (Bonami *et al.,* 1997; Hasson *et al.,* 1995; Lightner, 2005). Hence, several papers in the literature propose a toxic aetiology for Taura syndrome (Intriago *et al.,* 1997; Jimenez, 1992; Jimenez *et al.,* 2000).

2.1.2.   Survival outside the host

No information available.

2.1.3.   Stability of the agent (effective inactivation methods)

No information available.

2.1.4.   Life cycle

Not applicable.

2.2. Host factors

2.2.1.   Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with TSV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: white leg shrimp (*Penaeus vannamei*), blue shrimp (*P. stylirostris*), northern white shrimp (*P. setiferus*), greasyback shrimp (Metapenaeus ensis), giant tiger prawn (*P. monodon*) and northern brown shrimp (*P. aztecus*).

~~The principal host species for TSV are the Pacific white shrimp, *Penaeus vannamei*, and the Pacific blue shrimp, *P. stylirostris*. While the principal host species for TSV all belong to the penaeid subgenus *Litopenaeus*, other penaeid species can be infected with TSV by direct challenge, although disease signs do not develop. Documented natural and experimental hosts for TSV include: *P. setiferus, P. schmitti, P. monodon, P. chinensis, P. japonicus, P. aztecus, P. duorarum, P. indicus* and *Metapenaeus ensis* (Bondad-Reantaso et al., 2001; Brock, 1997; Brock et al., 1997; Chang et al., 2004; Lightner, 1996a, 1996b; Overstreet et al., 1997; Srisuvan et al., 2005; Stentiford et al., 2009; Wertheim et al., 2009).~~

2.2.2.   Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility to infection with TSV according to Chapter 1.5. of the *Aquatic Code* include: fleshy prawn (*P. chinensis*), giant river prawn (*Macrobrachium rosenbergii*), *Ergasilus manicatus, Chelonibia patula* and *Octolasmis muelleri*.

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: northern pink shrimp (*Panaeus duorarum*), kuruma prawn (*P. japonicas*), southern white shrimp (*P. schmitti*), *Fundulus grandis, Callinectes sapidus, Uca vocans, Sesarma mederi* and *Scylla serata*.

2.2.3~~2~~.   Susceptible stages of the host

TSV has been documented in all life stages (i.e. PL, juveniles and adults) of *P. vannamei* (the most economically significant of the two principal host species) except in eggs, zygotes and larvae (Lightner, 1996a).

2.2~~.4~~3.   Species or subpopulation predilection (probability of detection)

No data.

2.2.5~~4~~.   Target organs and infected tissue

TSV infects and has been shown to replicate (using ISH with specific DNA probes) principally in the cuticular epithelium (or hypodermis) of the general exoskeleton, foregut, hindgut, gills and appendages, and often in the connective tissues, the haematopoietic tissues, the lymphoid organ (LO), and antennal gland. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, striated muscle, and the ventral nerve cord, its branches and its ganglia typically show no histological signs of infection by TSV and are usually negative ~~for TSV~~ by ISH (Bondad-Reantaso *et al.,* 2001; Hasson *et al.,* 1997; 1999a; 1999b; Jimenez *et al.,* 2000; Lightner, 1996a; Lightner & Redman 1998a; 1998b; Lightner *et al.,* 1995; Srisuvan *et al.,* 2005).

2.2.6~~5.~~   Persistent infection ~~with lifelong carriers~~

Some members of populations of *P. vannamei* or *P. stylirostris* that survive TSV infections or epizootics may carry the virus for life (Hasson *et al.,* 1999a; 1999b) and, although not documented, are assumed to pass the virus to their progeny by vertical transmission.

2.2.<u>7.</u>~~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*) (Garza *et al.,* 1997); and chickens (*Gallus domesticus,* used as a laboratory surrogate for all shrimp-eating birds) (Vanpatten *et al.,* 2004), thus suggesting that the virus can retain infectivity when passed through the gastro-intestinal system of any bird species. These findings indicate that birds may act as a mechanical vector for the transmission of the virus. However, there is no epidemiological evidence to suggest that this is an important route of spread between farms or farming regions.

~~*Frozen TSV-infected commodity products:* TSV has been found in frozen commodity shrimp (*P. vannamei*) products in samples from markets in the USA that originated in Latin America and South East Asia. Improper disposal of wastes (liquid and solid, i.e. peeled shells, heads, intestinal tracts, etc.) from value added reprocessing of TSV infected shrimp at coastal locations may provide a source of TSV that may contaminate wild or farmed stocks near the point of the waste stream discharge (Lightner, 1996b; Nunan *et al.,* 2004).~~

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

~~No data.~~

2.3. Disease pattern

Taura syndrome 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 TS are typically small juveniles of from ~0.05 g to <5 g. 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.   Transmission mechanisms

Transmission of TSV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (Brock, 1997; Hasson *et al.,* 1995; Lightner, 1996a, 1996b; White *et al.,* 2002). Vertical transmission from infected adult broodstock to their offspring is strongly suspected but has not been experimentally confirmed.

2.3.2.   Prevalence

In regions where the virus is endemic in farmed stocks, the prevalence of TSV has been found in various surveys to range from 0 to 100% (Brock, 1997; Jimenez *et al.,* 2000; Laramore, 1997).

2.3.3.   Geographical distribution

TSV is now widely distributed in the shrimp-farming regions of the Americas, South-East Asia and the Middle East (Bondad-Reantaso *et al.,* 2001; Brock, 1997; Chang *et al.,* 2004; 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; Yu & Song, 2000).

The Americas: following its recognition in 1992 as a distinct disease of cultured *P. vannamei* in Ecuador (Brock *et al.,* 1995; Jimenez, 1992; Lightner *et al.,* 1995), TSV spread rapidly throughout many of the shrimp-farming regions of the Americas through shipments of infected PL and broodstock (Brock, 1997; Brock *et al.,* 1997; Hasson *et al.,* 1999a; Lightner, 1996a, 1996b; Lightner *et al.,* 2012). Within the Americas, infection withTSV have been reported from virtually every penaeid shrimp-growing country in the Americas and Hawaii (Aguirre Guzman & Ascencio Valle, 2000; Brock, 1997; Lightner, 2011; Lightner *et al.,* 2012; Robles-Sikisaka *et al.,* 2001). TSV is endemic in cultured penaeid shrimp stocks on the Pacific coast of the Americas from Peru to Mexico, and it has been occasionally found in some wild stocks of *P. vannamei* from the same region (Lightner & Redman, 1998a; Lightner *et al.,* 1995). TSV has also been reported in farmed penaeid stocks from the Atlantic, Caribbean, and Gulf of Mexico coasts of the Americas, but it has not been reported in wild stocks from the these regions (Hasson *et al.,* 1999a; Lightner, 1996a; 2005; 2011; Lightner *et al.,* 2012).

Asia and the Middle East: TSV was introduced into Chinese Taipei in 1999 with infected imported Pacific white shrimp, *P. vannamei*, from Central and South American sources (Tu *et al.,* 1999; Yu & Song, 2000). Since that original introduction, the virus has spread with movements of broodstock and PL to China (People's Rep. of), Thailand, Malaysia, and Indonesia where it has been the cause of major epizootics with high mortality rates in introduced unselected stocks of *P. vannamei* (Chang *et al.,* 2004; Lightner, 2011; Nielsen *et al.,* 2005; Tang & Lightner, 2005). ~~Recently~~ During 2010 and 2011, TSV has also been associated with significant mortalities in <u>farmed</u> *P. indicus* ~~being farmed~~ in Saudi Arabia. <u>By a phylogenetic analysis based on the viral capsid protein 2 (also named as VP1) sequence, the Saudi Arabian TSV clustered into a new, distinct group</u> (<u>Tang *et al.,* 2012</u>; Wertheim *et al.,* 2009).

2.3.4. Mortality and morbidity

In on-farm Taura syndrome epizootics involving unselected (i.e. not selected for TSV resistance) stocks of *P. vannamei*, the principal host species for TSV, 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; Moss *et al.,* 2001).

2.3.5. Environmental factors

Outbreaks of Taura syndrome are more frequent when salinities are below 30 ppt (Jimenez *et al.,* 2000).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccines for TSV are available.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Resistance breeding

After Taura syndrome emerged in Ecuador in 1992–1994, *P. stylirostris* were found that possessed resistance to TSV (genotype 1, MAb 1A1 Type A). Following from this discovery and due to TSV reaching 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; Erickson *et al.,* 2002; Fegan & Clifford, 2001; Lightner, 1999; 2005; Zarin-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; Moss *et al.,* 2001; White *et al.,* 2002). After the appearance of TS in Central America, improved TS resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region (Laramore, 1997).

2.4.5. Restocking with resistant species

Selected lines of TSV resistant *P. vannamei* have been developed and are commercially available (Clifford, 1998; Laramore, 1997; Moss *et al.,* 2001; White *et al.,* 2002).

2.4.6. Blocking agents

Resistance to TSV infection was reported by expression of the TSV coat protein antisense RNA in *P. vannamei* zygotes. Transgenic juveniles reared from zygotes protected in this manner showed improved resistance to TSV challenge by *per os* or intramuscular (IM) injection routes (Lu & Sun, 2005). Similar results have been produced by injection of short random double-stranded RNAi sequences into juvenile *P. vannamei* (Robalino *et al.,* 2004).

2.4.7. Disinfection of eggs and larvae

It is possible TSV might be transmitted vertically (transovarian transmission), despite no published report 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.

2.4.8. General husbandry practices

Some husbandry and disease control and management practices have been used successfully to reduce the risks TSV infections and disease occurring during farm grow-out. These include the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/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; Lotz *et al.,* 1995; Moss *et al.,* 2001; Pruder *et al.,* 1995; Wyban 1992; Wyban *et al.,* 2004). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of TS. Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF *P. vannamei* that were free not only of TSV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Rosenberry, 2004).

> **EU comment**
>
> **Please replace the words "polymerase chain reaction (PCR)" by the words "reverse transcription polymerase chain reaction (RT-PCR)" (the standard nucleic acid detection method for RNA viruses).**

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by 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 TSV.

3.2. 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.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and PL pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs and tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of TS is the cuticular epithelium. In chronic infections the LO is the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.5. Samples/tissues that are not suitable

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 by TSV.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only acute-phase disease can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase disease 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 Taura syndrome outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicate the presence of a serious disease outbreak (which is often either Taura syndrome or WSD when sea birds are observed) to the farm manager.

4.2. Clinical methods

4.2.1. Gross pathology

Taura syndrome has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b; 2011; Lightner *et al.,* 1995). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of TS are unique and provide a presumptive diagnosis of infection with TSV.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase TSV infection include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish coloration 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 Taura syndrome 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. If the affected shrimp are larger than ~1 g, moribund shrimp may be visible to sea birds at the pond edges and surface. Thus, during the peak of severe epizootics, hundreds of sea birds (gulls, terns, herons, cormorants, etc.) may be observed feeding on affected moribund shrimp that accumulate at the surface of the affected pond surface and edges (Brock, 1997; Brock *et al.,* 1995; 1997; Garza *et al.,* 1997; Lightner, 1996a; 1996b; 2011; Lightner *et al.,* 1995; Vanpatten *et al.,* 2004).

Transition (*recovery*) *phase:* although only present for a few days during epizootics, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of TSV infection. 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 resolving 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; 2011).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of the disease in which persistently infected shrimp show no obvious clinical signs of disease (Brock, 1997; Hasson *et al.,* 1999b; Lightner, 1996a; 1996b; 2011; 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 (Lotz *et al.,* 1995).

4.2.2.   Clinical chemistry

Not applicable.

4.2.3.   Microscopic pathology (for penaeid hosts)

Infection with TSV in the acute and chronic phases can be diagnosed most reliably using histological methods (Hasson *et al.,* 1999b; Lightner, 1996a). Pathognomonic TSV-induced pathology is unique in acute-phase infections (Brock *et al.,* 1995; Lightner, 1996a; 2011). In chronic TSV infections, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.,* 1999b; Lightner 2011), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When LOS are observed by routine histology and chronic TSV infection is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR [see Section 4.3.1.2.7]) is recommended for confirmation of TSV infection.

4.2.3.1. Acute phase of Taura syndrome

Diagnosis of infection with TSV in the acute phase of the disease is dependent on the histological demonstration (in haematoxylin and eosin [H&E] stained preparations) of 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, 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 Taura syndrome 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 Taura syndrome 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 TSV infections distinguishes it from acute-phase yellowhead disease 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 the disease from the transitional phase (Bondad-Reantaso *et al.,* 2001; Brock, 1997; Brock *et al.,* 1995; 1997; Erickson *et al.,* 2002; 2005; Hasson *et al.,* 1995; 1999a; 1999b; Lightner, 1996a; Lightner *et al.,* 1995).

4.2.3.2. Transition (recovery) phase of Taura syndrome

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; 2011). 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).

4.2.3.3. Chronic phase of Taura syndrome

Shrimp in the chronic phase of Taura syndrome 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; 2011).

4.2.4. 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 TSV infection) focal lesions of acute-phase TS TSV infection in cuticular epithelial cells. Preparations presenting TS TSV infection acute-phase lesions 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.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

See Section 4.2.3.

4.2.7. Electron microscopy/cytopathology

Not currently applicable for diagnostic purposes.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

TSV has not been grown in vitro, as no crustacean cell lines exist (Lightner, 1996a; Pantoja et al., 2004). Despite a publication that incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle et al., 2003), two other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (Luo et al., 2004; Pantoja et al., 2004).

4.3.1.2.2. Antibody-based antigen detection methods

An MAb for detection of TSV may be used to assay samples of haemolymph, tissue homogenates, or Davidson's AFA-fixed tissue sections from shrimp (Erickson *et al.,* 2002; 2005; Poulos *et al.,* 1999). TSV MAb 1A1 may be used to distinguish some variants or 'strains' of TSV from other strains (Erickson *et al.,* 2002; 2005).

4.3.1.2.3. Bioassay method

Confirmation of TSV infection may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the virus (Brock *et al.,* 1997; 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 a TSV epizootic. 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).

To perform the IM (injection) bioassay for TSV:

Note that tissues and the resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

i) Prepare a 1:2 or 1:3 ratio of TSV-suspect shrimp heads or whole shrimp with TN buffer (~~see Chapter 2.2.2, infectious hypodermal and haematopoietic necrosis [IHHN], for the composition of this buffer~~ 20 mM Tris-HCl, pH 7.4, 0.4 M NaCl) or sterile 2% saline prepared with distilled water.

ii) Homogenise the mixture using a tissue grinder or blender. Do not permit the mixture to heat up by excessive homogenisation or grinding.

iii) Clarify the homogenate by centrifugation at 3000 ***g*** for 10 minutes. Decant and save the supernatant fluid. ~~Discard the pellet.~~

iv) Centrifuge the supernatant fluid at 27,000 ***g*** for 20–30 minutes at 4°C. Decant and save the supernatant fluid. ~~Discard the pellet.~~

v) Dilute the supernatant fluid from step iv to 1/10 to 1/100 with sterile 2% saline. This solution may now be used as the inoculum to inject indicator shrimp (or filter sterilised as described in step vi).

vi) Filter the diluted supernatant fluid from step v using a sterile syringe (size depends on the final volume of diluted supernatant) and a sterile 0.45 µm syringe filter. ~~Multiple filters may have to be used as they clog easily.~~ Filtrate should be collected in a sterile test tube or beaker. The solution can ~~now~~ be stored frozen (~~recommend –20°C~~ at –20°C (or –80°C for ~~short-term [weeks] storage and –80°C for~~ a long-term ~~[months to years]~~ storage) or used immediately to inject indicator shrimp.

vii) Indicator shrimp should be from TSV-susceptible stocks of SPF *P. vannamei* (such as the 'Kona stock') (Moss *et al.,* 2001), which are commercially available from a number of sources in the Americas, and not from selected lines of known TSV-resistant stocks.

viii) Inject 0.01 ml per gram of body weight using a 1 ml tuberculin syringe. Indicator shrimp should be injected intramuscularly into the third tail segment. If the test shrimp begin to die within minutes post-injection, the inoculum contains excessive amounts of proteinaceous materials and should be further diluted prior to injecting additional indicator shrimp. Sudden death occurring post-injection is referred to as 'protein shock', and is the result of systemic clotting of the shrimp's haemolymph in response to the inoculum (Lightner, 1996a; White *et al.,* 2002).

ix) Haemolymph samples may be diluted (1/10 or 1/20 in TN buffer), filter sterilised (if necessary), and injected into the indicator shrimp without further preparation.

x)   If TSV was present in the inoculum, the indicator shrimp should begin to die within 24–48 hours post-injection. Lower doses of virus may take longer to establish a lethal infection and shrimp should be monitored for at least 10–15 days post-injection.

xi)   The presence (or absence) of TSV in the indicator shrimp should be confirmed by histological analysis (and/or ISH by gene probe, if available) of Davidson's fixed moribund shrimp. If additional confirmation is needed beyond demonstration of pathognomonic TSV lesions, RT-PCR with sequencing of the resulting amplicon can be carried out.

4.3.1.2.4. Sentinel shrimp bioassay method

As a variation to the bioassay technique, a 'sentinel shrimp' system may be used. For example, TSV-sensitive stocks of small juvenile SPF P. vannamei may be held in net-pens in tanks, or in the same water system, with other shrimp of unknown TSV status to bioassay for the presence of infectious agents such as TSV.

4.3.1.2.5. 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, South San Francisco, California [CA], USA)6.

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, Gibco BRL) and 2% Hammersten casein (Amersham Life Sciences, Arlington Heights, Illinois, USA).

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, South San Francisco, CA) 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, Corp.) in 100 mM Tris-HCl, 100 mM NaCl (100 mM each) buffer containing 50 mM MgCl2, 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.

4.3.1.2.6. Other antibody-based methods

The TSV MAb 1A1 may be applicable to other antibody-based test formats (i.e. indirect fluorescent antibody [IFAT] or immunohistochemistry [IHC] tests with tissue smears, frozen sections, or deparaffinised fixed tissues). MAb 1A1 is applicable for use in an IHC format using Davidson's AFA-fixed tissue sections (Erickson *et al.,* 2002; 2005).

It is recommended that unexpected results from MAb-based tests for TSV should be interpreted in the context of clinical signs, case history, and in conjunction with other test results (e.g. RT-PCR test results, or findings from histology or ISH with a TSV-specific DNA probe – see appropriate sections in this chapter).

4.3.1.2.7. Molecular techniques

ISH and RT-PCR tests for TSV have been developed, and kits of RT-PCR methods for TSV are commercially available. The dot-blot method for TSV detection is not available.

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

4.3.1.2.7.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled cDNA probes for 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 1998b; 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 TSV infection (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 2.2.2 IHHN 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 avoided through the use of neutral fixatives, including an 'RNA-friendly' fixative developed for shrimp, or by the proper use (avoiding fixation times over 24 hours) of Davidson's fixative (Hasson et al., 1997; Lightner, 1996a; Lightner & Redman 1998).

4.3.1.2.7.2. Reverse-transcription (RT)-PCR method

Tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9992F and 9195R, amplify a 231 base pair (bp) sequence of the TSV genome (Nunan *et al.*, 1998). The fragment amplified is from a conserved sequence located in the intergenic region and ORF 2 of TSV. Primer 9992F is located near the 3' end of intergenic region and 9195R is located on ORF 2 within VP2 (= CP1) (Mari *et al.*, 2002; Nunan *et al.*, 1998). A new pair of TSV primers (7171F and 7511R) has been developed and shown to have an improved sensitivity for TSV detection (Navarro *et al.*, 2009). ~~These replacement primers are 9992F/9195R and they are located within ORF 2.~~

Primer	Product	Sequence	Temperatue	~~G+C%~~
9992F	231 bp	5'-AAG-TAG-ACA-GCC-GCG-CTT-3'	69°C	~~55%~~
9195R		5'-TCA-ATG-AGA-GCT-TGG-TCC-3'	~~63°C~~	~~50%~~
7171F	341 bp	5'-CGA-CAG-TTG-GAC-ATC-TAG-TG-3'	<u>63°C</u>	~~50%~~
7511R		5'-GAG-CTT-CAG-ACT-GCA-ACT-TC-3'		~~50%~~

EU comment

In the table above, please replace the word "Temperatue" by the word "Temperature" (typographical error).

Furthermore, it is not clear what the temperatures mentioned in the table are for (it is presumed to be the melting temperatures; however this should be clarified in the table).

The RT-PCR method outlined below for TSV generally follows the method used in Nunan *et al.* (1998).

i) Preparation of RNA template: RNA can be extracted from fresh, frozen and ethanol-preserved tissues. ~~Extraction of RNA should be performed using commercially available RNA tissue extraction kits, such as the High Pure RNA Tissue Kit (Roche, Penzberg, Germany) and following the manufacturer's procedures for production of quality RNA templates.~~ <u>Viral RNA can be isolated using any commercially available RNA isolation kit. The amount of tissue required will depend on the kit selected (i.e. Qiagen RNA extraction kit, Promega and Roche RNA purification kit recommend using 25–50 mg of tissue). Depending on the kit used, the elution volume for Roche and Qiagen and low elution volume RNA isolation Promega extraction kit is 100 µl. Extracted RNA should be maintained at –20°C before testing, however, for long-term storage the RNA should be kept at –70°C.</u>

ii) The RT-PCR assay is carried out ~~in solution,~~ using ~~10~~ 5 µl of total RNA extracted ~~from haemolymph, frozen shrimp tissues, ethanol fixed tissue~~ as the template ~~(concentration of RNA = 1–100 ng ml⁻¹)~~.

iii) The following controls should be included in every RT-PCR assay for TSV: (a) known TSV-negative tissue sample; (b) a known TSV-positive sample (tissue or purified virus); and (c) a 'no-template' control.

iv) The ~~GeneAmp® EZ rTth RNA PCR kit (Applied Bioscience, Forster City, CA) was used~~ SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Life Technologies) can be used for all amplification reactions described here. ~~Alternative kits~~ Other commercially available equivalent reagent can also be used ~~and adjusted for use for this assay~~.

v) The optimised RT-PCR conditions (final concentrations in ~~50~~ 25 µl total volume) for detection of TSV in shrimp tissue samples are: ~~primers (0.62 µM each), dNTPs (300 µM each), rTth DNA polymerase (2.5 U 50 µl⁻¹), manganese acetate (2.5 mM), in 5 × EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2).~~

~~vi) If the thermal cycler does not have a heated lid, then light mineral oil (50 µl) is overlaid on the top of the 50 µl reaction mixtures to prevent condensation or evaporation during thermal cycling.~~

Reagent	Volume	Final concentration
dH₂O	5.5 µl	
2× Reaction Mix	12.5 µl	1×
Primer Forward/Reverse (10 M each)	1.0 µl	0.4 µM
RT/Taq enzyme Mix	1.0 µl	
RNA template*	5.0 µl	1–50 ng

vi) The RNA template and all the reagents are combined and reverse transcription is allowed to proceed at 60°C for 30 minutes, followed by ~~94°C for 2 minutes~~ 95°C for 2 minutes. At the completion of reverse transcription, the samples are amplified for 39 cycles under the following conditions: denaturation at 95°C for 45 seconds, and then annealing/extension at 62°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle. ~~in a 4°C soak file~~.

~~Note: The reaction conditions described here were optimised using an automatic Thermal Cycler GeneAmp 980 (Applied Biosystems). The conditions should be optimised for each thermal cycler using known positive controls.~~

vii) A 6 µl of the ~~completion of reverse transcription, the samples are~~ amplified ~~for 40 cycles under the following conditions: denaturation at 94°C for 45 seconds, and then annealing/extension at 60°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle and the process is terminated in a 4°C soak file.~~

~~ix) Following the termination of RT PCR, the amplified cDNA solutions are drawn off from beneath the mineral oil and placed into clean 0.5 ml microfuge tubes.~~

x) ~~A 10 µl sample of the amplified~~ products can then be added to the well of a ~~2.0~~ 1.5% agarose gel, stained with ethidium bromide (0.5 g ml⁻¹), and electrophoresed in 0.5 × TBE (Tris, boric acid, ethylene diamine tetra-acetic acid [EDTA]).

~~xi) A 1 kb DNA ladder (Invitrogen, Carlsbad, CA) is used as a marker.~~

~~xiii) Details of the composition of the reagents and buffers used here may be found in Chapter 2.2.2 IHHN.~~

4.3.1.2.7.3. Real-time RT-PCR (qPCR qRT-PCR) method for TSV

Quantitative RT-PCR methods have been developed for the detection of TSV. These methods have the advantages of speed, specificity and sensitivity. The sensitivity of qRT-PCR is ~100 copies of the target sequence from the TSV genome (Dahr *et al.,* 2002; Tang *et al.,* 2004).

The ~~real-time RT~~ qRT-PCR method using TaqMan chemistry described below for TSV generally follows the method used in Tang *et al.* (2004).

i) The PCR primers and TaqMan probe were selected from the ORF1 region of the TSV genomic sequence (GenBank AFAF277675) that encodes for nonstructural proteins. The primers and TaqMan probe were designed by the Primer Express software (~~Applied Biosystems~~ Life Technologies). The upstream (TSV1004F) and downstream (TSV1075R) primer sequences are: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3' and 5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'), respectively. The TaqMan probe, TSV-P1 (5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3'), which corresponds to the region from nucleotide 1024 to 1051, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end ~~(Applied Biosystems, catalog no. 450025)~~.

ii) Preparation of RNA template: the extraction and purification of RNA template from haemolymph, or shrimp tissue, is the same as that described in the section for ~~traditional~~ conventional RT-PCR.

iii) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of contaminants in the reaction mixture ~~or in the heat block of the thermal cycler~~. A positive control should also be included, and this can be an *in-vitro* transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV-infected tissue.

iv) The qRT-PCR reaction mixture contains: TaqMan ~~One step RT-PCR~~ Fast virus 1-Step Master Mix (~~Applied Biosystems, part no. 4309169~~ Life Technologies), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng of RNA, and water in a reaction volume of ~~25~~ 10 µl. ~~For optimal results, the reaction mixture should be vortexed and mixed well.~~

v) Amplification can be performed with the ~~GeneAmp 5700 Sequence Detection~~ StepOnePlus PCR System (~~Applied osystems; ABI PRISM 7000, 7300, 7500, or newer models~~ Life Technologies or equivalent ~~thermocycler~~ real-time PCR systems). The cycling consists of reverse transcription at ~~48~~ 50°C for 30 minutes and initial denaturation at 95°C for ~~10 minutes~~ 20 seconds, followed by 40 cycles of denaturation at 95°C for ~~15~~ 3 seconds and annealing/extension at 60°C for ~~1 minute. The levels of fluorescence are measured at the end of each annealing/extension cycle~~ 30 seconds.

vi) At the end of the reaction, real-time fluorescence measurements are analysed. A threshold will be set to be above the baseline ~~that begins to detect the increase in signal associated with an exponential increase in PCR product~~. Samples will be defined as negative if there is no Ct (threshold cycle) value after 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive.

4.3.1.2.7.4. Sequencing

RT-PCR products may be cloned and sequenced when necessary to confirm infection by TSV or to identify false positives or nonspecific amplification (Mari *et al.,* 2002; Nielsen *et al.,* 2005; Srisuvan *et al.,* 2005; Tang & Lightner, 2005; Wertheim *et al.,* 2009).

EU comment

The EU is of the opinion that cloning is not necessary for RT-PCR products, as they can be sequenced directly. Thus, the words "cloned and" should be deleted.

4.3.1.2.8. Agent purification

Methods for TSV isolation and purification are available (Bonami *et al.,* 1997; Hasson *et al.,* 1995; Mari *et al.,* 2002; Poulos *et al.,* 1999), but these are not recommended for routine diagnosis of TS.

4.3.2. Serological methods

Not applicable because shrimp are invertebrate animals which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to TSV.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of TSV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic

sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Infection with TSV surveillance, detection and diagnostic methods in penaeids

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	c
Bioassay	d	d	d	d	c	b
Direct LM	d	d	c	d	c	d
Histopathology	d	b	b	c	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
In-situ DNA probes	d	c	b	b	a	a
RT-PCR, qRT-PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy;
RT-PCR = reverse-transcriptase polymerase chain reaction; qPCR = quantitative PCR.

EU comment

Please replace the word "transcriptase" by the word "transcription" in the text under the table above (typographical error).

6. Test(s) recommended for targeted surveillance to declare freedom from Taura syndrome

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.7.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic TSV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with TSV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is represented by:

- Sudden high mortalities in late PL, juvenile or subadult *P. vannamei* or *P. stylirostris* in regions where TSV is enzootic;

- The sudden presence of numerous sea birds (gulls, cormorants, herons, terns, etc.) 'fishing' in one or more shrimp culture ponds;

- Samples of cultured *P. vannamei* or *P. stylirostris* from ponds with feeding sea birds that present gross signs indicative of acute- or transition-phase infection with TSV, such as a general reddish colouration, lethargy, soft shells, empty guts, and the presence of numerous irregular black spots on the cuticle; or

- Demonstration of foci of necrosis in the cuticular epithelium using low magnification (i.e. a ×10 hand lens or by direct microscopic examination of wet mounts) to examine the edges of appendages such as uropods or pleopods, or the gills.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- Histological demonstration of diagnostic acute-phase TSV lesions in (especially) the cuticular epithelia of the foregut (oesophagus, anterior, or posterior chambers of the stomach) and/or in the gills, appendages, or general cuticle. Such TSV lesions are pathognomonic for TSV only when they occur without accompanying severe acute necrosis (with nuclear pyknosis and karyorrhexis) of the parenchymal cells of the lymphoid organ tubules (which may occur in acute-phase yellowhead virus infections).

- ISH-positive (with a TSV-specific cDNA probe) signal to TSV-type lesions in histological sections (i.e. cuticular acute-phase Taura syndrome lesions) or to distinctive lymphoid organ spheroids (LOS) in the lymphoid organs of shrimp with chronic phase Taura syndrome lesions.

- RT-PCR positive results for TSV.

- Sequencing of PCR product encompassing CP2 may be accomplished, as needed, to determine the TSV genotype (Tang & Lightner, 2005; Wertheim *et al.,* 2009).

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CHAPTER 2.2.7.

INFECTION WITH
MACROBRACHIUM ROSENBERGII NODAVIRUS
(WHITE TAIL DISEASE)

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Infection with *Macrobrachium rosenbergii* nodavirus means infection with *Macrobrachium rosenbergii* nodavirus (*Mr*NV) (of the Family Nodaviridae. The disease is commonly known as white tail disease (WTD). or white muscle disease (WMD) is defined as a viral infection caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and its associate extra small virus (XSV). They cause a milky whitish appearance in larvae/postlarvae (PL)/early juveniles, and are responsible for large-scale mortalities in the freshwater prawn *M. rosenbergii*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agents are two viral pathogens, namely *Macrobrachium rosenbergii* nodavirus (*Mr*NV) (primary) and extra small virus (XSV) (associate) (Qian *et al.,* 2003; Romestand & Bonami, 2003). *Mr*NV is important in WTD outbreaks in prawns, although the role of XSV in pathogenicity remains unclear. Strains are not yet known. *Mr*NV belongs in the family Nodaviridae (Bonami *et al.,* 2005; Van Regenmortel *et al.,* 2000). XSV is the first sequenced satellite virus in animals and it is also the first record of a satellite-nodavirus association (Bonami *et al.,* 2005).

2.1.2. Survival outside the host

Survival outside the host is not known, however viral inoculum prepared from tissue homogenate stored at –20°C caused 100% mortality in PL of *M. rosenbergii* by immersion challenge (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.1.3. Stability of the agent (effective inactivation methods)

Agent stability is not known. However, heat treatment destroyed infectivity of *Mr*NV and XSV in challenge experiments (Qian *et al.,* 2003).

2.1.4. Life cycle

Not known.

2.2. Host factors

Infection with MrNV is responsible for huge mortalities in larvae and PL of the freshwater prawn, *M. rosenbergii,* in hatcheries with subsequent economic losses to nursery systems.

2.2.1. Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with *Mr*NV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: giant river prawn (*Macrobrachium rosenbergii*).

The giant freshwater prawn, ~~*Macrobrachium rosenbergii* (DeMan, 1879). Other proven or suspected hosts are not yet known.~~

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: white leg shrimp (*P. vannamei*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results (but an active infection has not been demonstrated) have been reported in the following organisms: kuruma prawn (*Penaeus japonicas*), Indian white prawn (*P. indicus*), giant tiger prawn (*P. monodon*), *Aesohna* sp., *Belostoma* sp., *Cybister* sp., *Notonecta* sp., *Macrobrachium rude, M. malcolmsonii, Artemia* sp. and *Cherax quadricarinatus.*

2.2.3~~2~~. Susceptible stages of the host

Larvae, PL and early juveniles are susceptible, whereas adults are resistant and act as carriers (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.2.3~~4~~. Species or subpopulation predilection (probability of detection)

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.,* 2006a).

2.2.4~~5~~. Target organs and infected tissue

*Mr*NV and XSV are confined to 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). The presence of both viruses in ovarian tissue indicates the possibility of vertical transmission of infection with *Mr*NV ~~WTD~~ from broodstock to larvae and PL. Experiments proved that pleopods would be a convenient source of RNA for non-destructive screening of *Mr*NV and XSV without stress to the prawns (Sahul Hameed *et al.,* 2004a).

2.2.5~~6~~. Persistent infection ~~with lifelong carriers~~

Challenge experiments indicate long-term persistent infection in adults and also the possibility of transmitting *Mr*NV ~~WTD~~ from broodstock to larvae and PL (Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a).

2.2.6~~7~~. Vectors

Not known. ~~Penaeid shrimp (*Penaeus indicus, P. monodon, P. japonicus*) (Sudhakaran *et al.,* 2006b), *Artemia* (Sudhakaran *et al.,* 2006c), and aquatic insects (*Belostoma* sp., *Aesohna* sp., *Cybister* sp., and *Notonecta* sp.) are vectors of WTD (Sudhakaran *et al.,* 2008).~~

~~2.2.8. Known or suspected wild aquatic animal carriers~~

~~Not known.~~

2.3. Disease pattern

A high prevalence of infection with *Mr*NV ~~WTD infection~~ has been reported in hatchery-reared larvae and PL of *M. rosenbergii*. The *Mr*NV may be transmitted both vertically and horizontally in culture systems.

2.3.1. Transmission mechanisms

Transmission is vertical (trans-ovum) and horizontal by the waterborne route (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a).

2.3.2. Prevalence

Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems, as well as in experimental infection by immersion challenge, and 100% mortality has been reported 5–7 days after the appearance of the first gross signs in PL in natural or experimental infection (Arcier *et al.,* 1999; Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; b).

2.3.3. Geographical distribution

WTD was first reported in the French West Indies (Arcier *et al.,* 1999), later in China (People's Rep. of) (Qian *et al.,* 2003), India (Sahul Hameed *et al.,* 2004b), Chinese Taipei (Wang & Chang, 2006), Thailand (Yoganandhan *et al.*, 2006) and Australia (Owens *et al.,* 2009).

2.3.4. Mortality and morbidity

Larvae, PL and juveniles of *M. rosenbergii* are highly 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 gross signs. Very few PL with WTD survive beyond 15 days in an outbreak, and PL that survive may grow to market size like any other normal PL. Adults are resistant to WTD, but can be persistently subclinically infected (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.3.5. Environmental factors

Not much is known about environmental factors. However, outbreaks of infection with *Mr*NV WTD may be induced by rapid changes in salinity, temperature and pH (Arcier *et al.,* 1999; Qian *et al.,* 2003).

2.4. Control and prevention

No work has been carried out on control and prevention of infection with *Mr*NV WTD. However, proper preventive measures, such as screening of brood stock and PL, and good management practices may help to prevent WTD in culture systems. As the life cycle of *M. rosenbergii* is completed under controlled conditions, specific pathogen free (SPF) brood stock and PL can be produced by screening using sensitive diagnostic methods such as reverse-transcription PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al.,* 2005).

2.4.1. Vaccination

Not yet available.

2.4.2. Chemotherapy

No known chemotherapeutic agents reported for infection with *Mr*NV WTD.

2.4.3. Immunostimulation

No reports available concerning the use of immunostimulants infection with *Mr*NV WTD.

2.4.4. Resistance breeding

None reported.

2.4.5. Restocking with resistant species

No report on the occurrence of resistant species.

2.4.6. Blocking agents

Not known.

2.4.7. Disinfection of eggs and larvae

Routine procedures followed for crustacean viral disease control are suggested. For example, application of formalin or iodophor helps to eliminate virus (Chen *et al.,* 1992).

2.4.8. General husbandry practices

Experimental infection confirmed the possibility of horizontal and vertical transmission of infection with *Mr*NV WTD in culture systems (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a). Good husbandry practices, such as proper disinfection of tanks, water and broodstock, and the use of RT-PCR negative broodstock in the hatchery grow-out ponds may be useful in the prevention of WTD in culture systems (Chen *et al.,* 1992; Sri Widada *et al.,* 2003; Sudhakaran *et al.,* 2008). There is no evidence of WTD prevention by crop rotation either with rice or polyculture with fish. Some farmers have considered either mixed culture of shrimp (*P. monodon*) with *M. rosenbergii* or crop rotation of these two species as a viable alternative for their sustenance and economic viability. This situation invites the possibility of transmitting pathologically significant organisms from native to non-native hosts as observed by Sudhakaran *et al.* (2006b) and Ravi *et al.* (2009) in their studies. Based on their results, it would seem that mixed culture of *M. rosenbergii* with *P. monodon* is to be avoided before adopting any preventive measures in the management of infection with *Mr*NV.

3. Sampling

3.1. Selection of individual specimens

Infection with *Mr*NV ~~WTD~~ of freshwater prawns is mainly diagnosed by the whitish coloration of abdominal and tail muscle (Arcier *et al.,* 1999; Romestand & Bonami, 2003; Sahul Hameed *et al.,* 2004b). However, this clinical sign is not specific to infection with *Mr*NV ~~WTD~~ and diagnosis is not easy, particularly in the earlier stages of infection. WTD-affected PL are more milky and opaque. Once this clinical sign appears, death usually follows; mortality rates are variable and reach up to 95%. The tissues most affected in moribund PLs/early juveniles are striated muscles of the abdomen, cephalothorax and tail. PLs with whitish muscle are suitable for diagnostic purposes (Sahul Hameed *et al.,* 2004a).

3.2. Preservation of samples for submission

Infected larvae/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, transported to the laboratory on dry ice and stored at –70°C until further use (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al.,* 2005). Frozen samples can be used for virus isolation and detection by RT-PCR or ELISA (Romestand & Bonami, 2003). Samples for virus detection by RT-PCR can be transported to the laboratory after fixing in 70% ethanol (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al.*, 2005). See also Chapter 2.2.0 *General information* (on diseases of crustaceans).

3.3. Pooling of samples

Infected larvae or PL (5 to 10 in number) can be pooled for screening tests. See also chapter 2.2.0.

3.4. Best organs or tissues

The whole PL body is preferred (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al.*, 2005). All the organs, except eyestalks and the hepatopancreas, of adult *M. rosenbergii* are best for screening the viruses by RT-PCR. Pleopods (swimming legs) would be a convenient source of RNA for non-destructive screening of *Mr*NV and XSV without stress to the broodstock (Sahul Hameed *et al.,* 2004a).

3.5. Samples/tissues that are not suitable

Eyestalks and the hepatopancreas of adult prawns are not suitable (Sahul Hameed *et al.,* 2004a; Sri Widada *et al.,* 2003).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

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. Mortality may reach a maximum in about 5 days after the appearance of the first gross signs.

4.1.2. Behavioural changes

PLs are highly susceptible to infection with *Mr*NV ~~WTD~~ and mortality reaches a maximum in about 5 days after the appearance of whitish coloration. 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).

4.2. Clinical methods

4.2.1. Gross pathology

The infection with *Mr*NV WTD of *M. rosenbergii, resulting from MrNV* and XSV infection, is mainly diagnosed by the whitish coloration of abdominal muscle. However, this clinical sign is not specific to WTD, but it is associated with high mortality rates.

4.2.2. Clinical chemistry

The prophenol oxidase activity significantly increased in *Mr*NV and XSV-injected prawns on day 3 and 5 post-injection (p.i.) and became normal on 10 day p.i. onwards. Superoxide anion concentration was found to be increased significantly on day 3, 5, and 10 p.i. whereas SOD activity decreased significantly up to 10 day p.i. and became normal after 15 day p.i. The total haemocyte count decreased significantly in *Mr*NV and XSV-injected prawns on day 1 and 3 p.i. and there was no significant change in the level of hemocyanin in *Mr*NV and XSV-injected and normal prawns (Ravi *et al.,* 2010).

4.2.3. Microscopic pathology

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). Pathognomonic oval or irregular basophilic cytoplasmic inclusion bodies are demonstrated in the target tissues by histology (Arcier *et al.,* 1999; Hsieh *et al.,* 2006).

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).

4.2.4. Wet mounts

None to date.

4.2.5. Smears

None to date.

4.2.6. Electron microscopy/cytopathology

Using transmission electron microscopy (TEM), infected cells appear necrotic, exhibiting a disorganised cytoplasm. TEM studies reveal the presence of two types of non-enveloped para-spherical virus particles of different sizes within the cytoplasm of connective cells and muscle cells. Large viral particles are five- to six-sided, with a diameter of 26–27 nm, and would be characteristic of *Mr*NV. Smaller viral particles similar in structure (five- to six-sided), but with a diameter of 14–16 nm, would be characteristic of XSV (Qian *et al.,* 2003).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Genome and antibody-based diagnostic methods are available to detect *Mr*NV/XSV (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al.*, 2005).

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

None to date.

4.3.1.1.2. Smears

None to date.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

*Mr*NV/XSV can be easily propagated in the C6/36 mosquito *Aedes albopictus* cell line (Sudhakaran *et al.,* 2007a) and this cell line can be cultured easily in Leibovitz L-15 medium containing 100 International Units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} fungizone supplemented with 10% fetal bovine serum at 28°C (Sudhakaran *et al.,* 2007a). Other cell lines, namely the fish SSN-1 cell line, partially support the multiplication of these viruses (Hernandez-Herrera *et al.,* 2007).

4.3.1.2.2. Antibody-based antigen detection methods

Antibody-based diagnostic methods for *Mr*NV include the ELISA described by Romestand & Bonami (Ravi *et al.,* 2009) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (Qian *et al.,* 2006).

4.3.1.2.2.1. ELISA protocol (Romestand & Bonami, 2003)

i)   Homogenise infected or healthy PL samples in 0.5 ml phosphate-buffered saline (PBS) and centrifuge at 10,000 *g* for 15 minutes. Collect and store the supernatant at –20°C for diagnostic purposes.

ii) Coat ELISA plates with 50 μl per well sample supernatant and incubate overnight at 4°C.

iii) Block with 250 μl 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C.

iv) Add 50 μl IgG anti-*Mr*NV with 1% BSA and incubate for 2 hours at room temperature.

v)   Add 50 μl of an anti-mouse IgG conjugated to peroxidase at 0.4 μg ml^{-1} and incubate for 1 hour at room temperature.

vi) Add 50 μl orthophenylene diamine chromogen at 0.4 mg ml^{-1} in substrate buffer (citric acid 0.1 M, sodium acetate 0.1 M, pH 5.4, H_2O_2 at a 0.33% final concentration).

vii) Stop the reaction after 15 minutes by adding 25 μl of H_2SO_4 to each well.

viii) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.2.2. TAS-ELISA protocol (Qian et al., 2006)

i)   Coat ELISA plates with rabbit polyclonal antibody raised against *Mr*NV and incubate for 2 hours at 37°C and keep at 4°C before use.

ii) Block with 250 μl 1% BSA in PBS for 1 hour at 37°C.

iii) Homogenise infected or healthy PL samples in 0.5 ml PBS and centrifuge at 10,000 *g* for 15 minutes. Collect and store the supernatant at –20°C for diagnostic purposes.

iv) Add 100 μl of sample to each well and incubate overnight at 4°C.

v)   Add 50 μl of a monoclonal antibody raised against *Mr*NV with 1% BSA and incubate for 2 hours at room temperature.

vi) Add 50 μl of an anti-mouse IgG conjugated to peroxidase at 0.4 μg ml^{-1} and incubate for 1 hour at room temperature.

vii) Add 50 μl orthophenylene diamine chromogen at 0.4 mg ml^{-1} in substrate buffer (citric acid 0.1 M, sodium acetate 0.1 M, pH 5.4, H_2O_2 at a 0.33% final concentration).

viii) Stop the reaction after 15 minutes by adding 25 µl H_2SO_4 to each well.

ix) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Reverse-transcription polymerase chain reaction (RT-PCR)

The protocol for the RT-PCR for detection of *Mr*NV/XSV developed by Sri Widada *et al.* (2003) and Sahul Hameed *et al.* (Sahul Hameed *et al.,* 2004a; 2004b) is recommended for all situations. *Mr*NV and XSV can be detected by 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). Nested RT-PCR (nRT-PCR) is also available and recommended for screening broodstock and seed (Sudhakaran *et al.,* 2006a).

Total RNA extraction

i) Collect 50 mg of PL or 100 mg of an organ piece (gill tissue, abdominal muscle, tail muscle or pleopods) from adult prawns and homogenate in 300 µl TN buffer (20 mM Tris/HCl, 0.4 M NaCl, pH 7.4).

ii) Centrifuge the homogenate at 12,000 ***g*** for 15 minutes at room temperature and collect the supernatant.

iii) Take 150 µl of supernatant and add 1 ml TRIzol. Mix thoroughly and incubate for 5 minutes at room temperature.

iv) After 5 minutes, add 200 µl chloroform to the sample, mix well and centrifuge at 12,000 ***g*** for 15 minutes at room temperature.

v) Collect the aqueous phase and transfer to a fresh tube, and precipitate RNA by mixing with 500 µl isopropanol.

vi) Incubate the sample for 10 minutes at room temperature and centrifuge at 12,000 ***g*** for 10 minutes at 4°C.

vii) Dissolve the RNA pellet in 50 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA [ethylene diamine tetra-acetic acid], pH 7.5) after a wash with 75% ethyl alcohol.

viii) Quantify the RNA by measuring the absorbance at 260 nm using UV spectrophotometer and check the purity by measuring the ratio of OD_{260nm}/OD_{280nm}.

RT-PCR protocol

Three RT-PCR methods are described to detect *Mr*NV and XSV. The first protocol is a one-step RT-PCR adapted from Sri Widada *et al.* (2003) and Sahul Hameed *et al.* (2004b), and this method can be used for confirmation of *Mr*NV and XSV in PL of prawns collected from suspected WTD outbreaks. The second protocol is a sensitive nRT-PCR protocol described by Sudhakaran *et al.* (2006a). This test can be used for screening healthy PL, juveniles and broodstock for viruses. The third protocol is a multiplex RT-PCR procedure adapted from Yoganandhan *et al.* (2005). It can be used for the simultaneous detection of *Mr*NV and XSV in disease outbreaks or for screening seeds and broodstock. In all the protocols described here, a commercial RT-PCR kit allowing reverse transcription and amplification in a single reaction tube is used.

Protocol 1: RT-PCR for specific detection of *Mr*NV or XSV in infected prawn PL or juveniles (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Sudhakaran *et al.,* 2007b):

The following controls should be included in every RT-PCR assay for *Mr*NV or XSV: a) a known *Mr*NV/XSV-negative tissue sample; b) a known *Mr*NV/XSV-positive sample (tissue or purified virus); and c) a 'no-template' control.

Annex 28 (contd)

For RT-PCR, a commercial RT-PCR kit is used. The reaction is performed in 50 µl RT-PCR buffer containing 20 pmol of each primer specific to *Mr*NV or XSV and RNA template (10–100 ng), using the following cycles: RT at 52°C for 30 minutes; denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 68°C for 1 minute, ending with an additional elongation step for 10 minutes at 68°C. Analyse the RT-PCR products by electrophoresis on a 1% agarose gel stain with ethidium bromide and a suitable DNA ladder marker and detect using an ultraviolet transilluminator.

A positive reaction will be indicated by a 425 bp product for *Mr*NV and a 546 bp product for XSV. The sensitivity of the assay is approximately 2.5 fg of total RNA.

PCR primer sequences for *Mr*NV (annealing temperature 55°C; product size 425 bp):

Forward: 5'-GCG-TTA-TAG-ATG-GCA-CAA-GG-3'
Reverse: 5'-AGC-TGT-GAA-ACT-TCC-ACT-GG-3'

PCR primer sequences for XSV (annealing temperature 55°C; product size 546 bp):

Forward: 5'-CGC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-TAA-3'
Reverse: 5'-CCG-GAA-TTC-CGT-TAC-TGT-TCG-GAG-TCC-CAA-3'

Protocol 2: the nRT-PCR is more sensitive and useful for screening seed and broodstock (Sudhakaran *et al.,* 2006a):

For the nRT-PCR, the first step of the RT-PCR, as described in protocol 1, should be performed with external primers and the nPCR should be carried out using an RT-PCR product as a template. For nRT-PCR, add 2 ml RT-PCR product to a PCR tube containing 20 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl$_2$, 0.1% Triton X-100, 200 µM of each dNTP, 20 pmol of each internal primer, 1.25 units of heat-stable DNA polymerase). The nRT-PCR protocol for both viruses comprise an initial 95°C for 10 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final extension at 72°C for 5 minutes. Analyse the nRT-PCR products by electrophoresis on a 1% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

If the viral load is sufficiently high, a 425 bp DNA will be amplified for *Mr*NV and 546 bp DNA for XSV in the first PCR step. In the nPCR step, a 205 bp product indicates detection of *Mr*NV and a 236 bp product indicates detection of XSV. The detection sensitivity of the nRT-PCR is ~1000-fold greater than the one-step RT-PCR.

The sequence of external primers for *Mr*NV and XSV is given in protocol 1 and the sequence of internal primers is given below:

The sequence of internal primers for *Mr*NV (annealing temperature 55°C; product size 205 bp):

Forward: 5'-GAT-GAC-CCC-AAC-GTT-ATC-CT-3'
Reverse: 5'-GTG-TAG-TCA-CTT-GCA-AGA-GG-3'

The sequence of internal primers for XSV (annealing temperature 55°C; product size 236 bp):

Forward: 5'-ACA-TTG-GCG-GTT-GGG-TCA-TA-3'
Reverse: 5'-GTG-CCT-GTT-GCT-GAA-ATA-CC-3'

Protocol 3: multiplex RT-PCR assay for simultaneous detection of *Mr*NV and XSV (Yoganandhan *et al.*, 2005).

To avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of *Mr*NV and XSV in a single-tube, one-step multiplex RT-PCR assay can be performed. The reaction is performed in 50 ml RT-PCR buffer containing 20 pmol of each primer specific to *Mr*NV and XSV, and RNA template (10–100 ng), using the following cycles: RT at 52°C for 30 minutes; denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 68°C for 1 minute, ending with an additional elongation step for 10 minutes at 68°C. Analyse the RT-PCR products by electrophoresis on a 1% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

If *Mr*NV and XSV are present in the sample, a 681 bp DNA for *Mr*NV and 500 bp DNA for XSV will be amplified. The presence of both 681 bp and 500 bp products indicates the presence of *Mr*NV and XSV. The detection sensitivity of the multiplex RT-PCR assay is approximately 25 fg of total RNA.

PCR primer sequences for *Mr*NV (annealing temperature 55°C; product size 681 bp):

Forward: 5'-GAT-ACA-GAT-CCA-CTA-GAT-GAC-C-3'
Reverse: 5'-GAC-GAT-AGC-TCT-GAT-AAT-CC-3'

PCR primer sequences for XSV (annealing temperature 55°C; product size 500 bp):

Forward: 5'-GGA-GAA-CCA-TGA-GAT-CAC-G-3'
Reverse: 5'-CTG-CTC-ATT-ACT-GTT-CGG-AGT-C-3'

Protocol 4: quantitative RT-PCR assay

Quantitative RT-PCR (RT-qPCR) assay can be performed to quantify the *Mr*NV/XSV in the infected samples using the SYBR Green dye based on the method described by Hernandez-Herrera *et al.* (2007) and Zhang *et al.* (2006).

i)   Extraction of total RNA from the samples as per the procedure mentioned above.

ii)   Incubate the RNA samples at 37°C for 1 hour in RT mixture (150 ng of total RNA, 8 U μl^{-1} M-MLV RT in buffer, 20 ng μl^{-1} hexaprimers and 0.2 mM dNTP) to obtain total cDNA and quantify the amount of cDNA by measuring the absorbance at 260 nm.

iii)   Perform RT-qPCR using q-PCR mixture (1 µl of cDNA [10 ng], 6 µl of sterile water, 0.5 µl of each primer specific to *Mr*NV and XSV [25 µM concentration] and 2 µl of reaction mixture containing Fast Start *Taq* polymerase, dNTP mix, SYBR Green, 10 mM $MgCl_2$ and 1 µl dye solution).

iv)   The PCR programme consists of initial *Taq* polymerase activation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 5 seconds at 60°C and 10 seconds at 72°C. Melting temperatures will be measured by returning to 70°C for 30 seconds and gradual heating to 95°C in 10 minutes. The negative control reactions should contain water in place of cDNA template in each run to ensure the absence of viruses.

v)   The number of viral cDNA copies in the sample will be determined using Light Cycler fit point method.

PCR primer sequences for *Mr*NV (annealing temperature 60°C; product size 211 bp):

Forward: 5'-AGG-ATC-CAC-TAA-GAA-CGT-GG-3'
Reverse: 5'-CAC-GGT-CAC-AAT-CCT-TGC-G-3'

PCR primer sequences for XSV (annealing temperature 58°C; product size 68 bp):

Forward: 5'-AGC-CAC-ACT-CTC-GCA-TCT-GA-3'
Reverse: 5'-CTC-CAG-CAA-AGT-GCG-ATA-CG-3'

4.3.1.2.3.2. *In-situ* hybridisation method *(Sri Widada et al., 2003; Zsikla et al., 2004)*

i)   Fix infected PL in neutral-buffered, modified Davidson's fixative without acetic acid (RNA friendly fixative) (Hasson *et al.,* 1997).

ii)   Embed the tissues in paraffin according to standard procedures (Bell & Lightner, 1988) and cut into 7 µm sections. Place sections on to positively charged microscope slides.

iii)   Dry the slides in an oven at 60°C. Remove paraffin and rehydrate through an ethanol series to water.

iv)   Incubate the sections twice for 5 minutes with diethylpyrocarbonate (DEPC)-treated Tris/HCl (0.2 M, pH 7.4) and 10 minutes with DEPC-treated Tris/HCl containing 100 mM glycine.

v)   Treat the sections for 5 minutes at 37°C with TE buffer (10 mM Tris/HCl, 5 mm EDTA, pH 8.0) containing 10 µg ml^{-1} RNAse-free proteinase K.

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vi) Post-fix the sections with DEPC-treated PBS containing 4% formaldehyde for 5 minutes.

vii) The sections are acetylated for 10 minutes with 0.1 M triethanolamine (TEA) buffer, pH 8, containing 0.25% (v/v) acetic anhydride.

viii) After dehydration, incubate the slides at 42°C for 16 hours in a humid chamber with hybridisation buffer containing 40% deionised formamide, 10% dextran sulphate, 1× Denhart's solution, 4× SSC (standard saline citrate), 10 mM dithiothreitol (DTT), 1 mg ml^{-1} yeast tRNA, 1 mg ml^{-1} denatured and sheared salmon sperm DNA and 40 ng ml^{-1} denatured digoxigenin-labelled DNA probe specific to *Mr*NV.

ix) Wash the slides at 37°C for 10 minutes with 1 × SSC, for 10 minutes with 0.5 × SSC and for 5 minutes twice with buffer III (100 mM Tris/HCl [pH 7.5], 150 mM NaCl).

x) Incubate for 20 minutes in buffer IV (buffer III, 1% normal goat serum) at room temperature.

xi) Incubate the slides for 1 hour in a humid chamber with buffer III containing 1% normal goat serum and 0.1% sheep anti-DIG alkaline phosphatase.

xii) Wash the slides successively for 10 minutes three times with buffer III and for 5 minutes twice with buffer V (100 mM Tris/HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl$_2$).

xiii) Develop the reaction by incubating the slides in buffer V containing NBT and BCIP in a dark and humid chamber for a minimum of 2 hours or overnight. Stop the reaction by incubating the slides in buffer III 2× for 15 minutes.

xiv) Counterstain the slides with 1% Brown Bismarck, mount with a cover-slip and examine with a bright field microscope.

xv) Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

4.3.1.2.3.3. Loop-mediated isothermal amplification (Haridas et al., 2010; Pillai et al., 2006; Puthawibool et al., 2010)

Haridas *et al.* (2010) and Pillai *et al.* (2006) have applied loop-mediated isothermal amplification (LAMP) for rapid diagnosis of *Mr*NV and XSV in the freshwater prawn. A set of four primers, two outer and two inner, have been designed separately for detection of *Mr*NV and XSV. In addition, a pair of loop primers specific to *Mr*NV and XSV has been used to accelerate LAMP reaction.

i) Extraction of total RNA from the samples as per the procedure mentioned above.

ii) Carry out the RT-LAMP reaction in the reaction mixture (2 µM each of inner primers FIP and BIP, 0.2 µM each of outer primers F3 and B3, 1400 µM of dNTP mix, 0.6 M betaine, 6 mM MgSO$_4$, 8 U of Bst DNA polymerase along with 1× of the supplied buffer, 0.125 U of AMV RTase and the specified amount of template RNA in a final volume of 25 µl) at 55, 60, 63 and 65°C for 1 each, followed by heat inactivation at 80°C for 2 minutes to terminate the reaction. Uninfected samples and reaction mix without template serve as the negative controls.

iii) Analyse the LAMP products by electrophoresis on a 2% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

iv) Without use of agarose electrophoresis, amplification of DNA can be detected by addition 1.0 µl of 10^{-1} diluted SYBR Green to the reaction mixture and observe the colour change.

4.3.1.2.3.4. Sequencing

For confirmation of suspected new hosts of *Mr*NV/XSV, the DNA fragment amplified from the PCR should be sequenced according to standard protocols (Sambrook & Russell, 2001).

4.3.1.2.4. Agent purification

*Mr*NV and XSV can be purified according to the protocol described by Bonami *et al.* (2005). The detailed procedure for viral purification is given below:

i) Collect sufficient quantity of infected PL and homogenate in PBS buffer (pH 7.4) using a tissue blender.

ii)   Centrifuge at 10,000 **g** for 25 minutes at 4°C. Collect supernatant and centrifuge again at 160,000 **g** for 4 hours at 4°C.

iii) Suspend the pellet in PBS and extract two or three times with freon (1,1,2-trichloro-2,2,1-trifluoroethane).

iv)   Collect the aqueous layer and centrifuge at 160,000 **g** for 4 hours at 4°C.

v)   Suspend the pellet in TN buffer and separate the two viruses with a 15–30% (w/v in PBS) sucrose gradient, followed by a CsCl gradient.

vi)   Examine the purity of the viruses by TEM using collodion-carbon-coated grids, negatively stained with 2% PTA (phosphotungstic acid), pH 7.0.

4.3.2.   Serological methods

None developed

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of infection with MrNV WTD are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	c	c	D	c	d
Bioassay	d	c	d	D	c	c
Direct LM	d	c	c	D	c	c
Histopathology	d	c	c	C	b	b
Transmission EM	d	d	d	D	d	a
Antibody-based assays	d	c	d	D	b	b
In-situ DNA probes	c	b	b	C	a	a
PCR	a	a	a	A	a	a
Sequence	d	d	d	A	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

The method for targeted surveillance to declare freedom from infection with MrNV WTD is nRT-PCR.

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Annex 28 (contd)

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Appearance of whitish muscle associated with mortality is a suspected case of infection with *Mr*NV WTD. It usually affects larval, PL and juvenile stages of *M. rosenbergii* and may appear as a cessation of feeding, reduced swimming activity and whitish coloration of the abdominal and tail muscles. Mortality reaches a maximum of up to 95% at 5 days after the appearance of the whitish coloration. Corroborative diagnostic criteria are summarised in Section 4.2 above.

7.2. Definition of confirmed case

Suspect cases should first be checked by RT-PCR and confirmed by nRT-PCR, sequencing, TEM and DNA probes.

8. References

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ASSESSMENT OF *BATRACHOCHYTRIUM SALAMANDRIVORANS* FOR LISTING IN THE *AQUATIC ANIMAL HEALTH CODE*

Overall Assessment

The Aquatic Animal Health Standards Commission assessed *Batrachochytrium salamandrivorans* (*Bsal*) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code*, and agreed that *Bsal* meets the OIE criteria for listing, notably A. Consequences: negative impact on wild amphibian populations, B. Spread: proven infectious aetiology, and high likelihood of spread via international trade, and zones free of the pathogen, and C. Diagnosis: availability of a robust diagnostic test (see Table 1 below).

Table 1. Summary of assessment of *Bsal*

	Listing criteria								Conclusion
	1	2	3	4	5	6	7	8	
Batrachochytrium salamandrivorans	NA	+	NA	+	NA	+	+	+?	List

NA = not applicable.

Background

It is well recognised that amphibian populations are in crisis across the globe due to a variety of factors, amongst them diseases. *Batrachochytrium dendrobatidis (Bd)*, a fungal infection, emerged as an important pathogen of amphibians in recent years and has resulted in declines of more than 200 amphibian populations and reductions in excess of 40% of amphibian species in Central America, and losses in Europe, Australia and North America (Fisher et al, 2012). *Bd* was added to the OIE list of diseases in 2008.

A rapid decline of free-living fire salmanders (*Salamandra salamandra*) in The Netherlands was reported in 2013 (Spitzen-van der Sluijs et al, 2013)). Initial investigations failed to identify a clear cause but subsequent investigations into the mortality of captive salamanders identified a new species of chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*) (Martel et al, 2013). Martel et al, (2014) conclude that the pathogen has co-existed with a clade of salamander hosts for millions of years in Asia. As a result of globalisation, and specifically international trade in salamanders, it was recently introduced to Europe where it has switched hosts with serious implications for biodiversity. Other emerging diseases which have caused serious declines in wild aquatic animal populations have been attributed to the movement of aquatic animals outside of their native range (Peeler et al, 2011).

Criteria for listing an aquatic animal disease (Article 1.2.2.)

A. Consequences

Criterion No. 1. *The disease has been shown to cause significant production losses at a national or multinational (zonal or regional) level.*

*Conclusion***:** Criteria is not applicable

OR

Criterion No. 2. *The disease has been shown to or scientific evidence indicates that it is likely to cause significant morbidity or mortality in wild aquatic animal populations.*

Annex 29 (contd)

Assessment:

Investigations by Martel *et al.* (2013) provides very solid evidence that *Bsal* is both a necessary and sufficient cause of disease in fire salamanders in the Netherlands. *Bsal* was isolated from the skin of fire salamanders in affected populations in Bunderos (the Netherlands). Analysis demonstrated that the *Bsal* is a novel chytrid fungus in a clade with *Bd*. Infected animals show severe pathology (multifocal erosions and ulcerations) and die within 7 days. Field observations and experimental studies indicate that case fatality approaches 100%. Between 2010 and 2013 the fire salamander in affected populations in the Netherlands was reduced by 96%.

Experimental challenge studies have demonstrated that 41 of 44 Western Paleartic salamander species are susceptible to *Bsal*, and it is lethal to at least some New World salmandrid species (Martel et al, 2014). Thus the disease has the potential to negatively impact many amphibian populations. Yap et al (2015) have modelled the likely impact of *Bsal* in North America and conclude that it is a serious threat to biodiversity there.

Conclusion: the criterion is satisfied

OR

Criterion No. 3. *The agent is of public health concern.*

Conclusion: Criteria is not applicable

AND

B. Spread

Criterion No. 4. *Infectious aetiology of the disease is proven*

Assessment:

Bsal was isolated from the skin of affected salamanders (Martel et al, 2013). Extensive screening was undertaken but no other pathogens were detected. By microscopy, high numbers of colonial thalli were observed. Transmission electron microscopic examination of skin lesions of clinically affected animals demonstrated presence of the pathogen (intracellular structures consistent with colonial thalli) (Martel et al, 2013). The infectious aetiology and role of *Bsal* is further proven by samples from declining and stable populations of fire salamanders (Martel et al, 2013). Thirteen of 33 swabs from live fire salmanders from declining populations tested positive for *Bsal* by PCR, in contrast to 0 of 51 swabs from a stable population.

Transmission studies provided further evidence of the infectious aetiology of the disease. Five salamanders were exposed to *Bsal* zoospores (Martel et al, 2013); all animals died. The pathogen was re-isolated from one animal and confirmed by PCR on all five.

Conclusion: the criterion is satisfied

OR

Criterion No. 5. *An infectious agent is strongly associated with the disease, but the aetiology is not yet known.*

Conclusion: Criteria is not applicable

AND

B. Spread

Criterion No. 6. *Likelihood of international spread, including via live aquatic animals, their products or fomites*

Assessment:

Martel et al (2014) speculated that *Bsal* originated in Asia and spread to Europe via the international salamander pet trade; and identified three actively traded Asian salamander species as reservoirs for *Bsal* (*Cynops cyanurus*, *Cynops pyrrhogaster*, and *Paramesotriton deloustali*) (Martel et al, 2015). The identification of *Bsal* in a collection of amphibians imported to the UK (Cunningham et al, 2015) demonstrates transboundary spread via movement of live animals. Skin samples from 1765 amphibians from pet shops, Heathrow Airport and an exporter in Hong Kong yielded 3 positive samples (2 of which were imported into Europe in 2010) (Martel et al, 2014). An analysis of the pet salamander trade by Yap et al (2015) concluded that it presents a high risk of *Bsal* introduction to N. America.

Conclusion: the criterion is satisfied

AND

Criterion No. 7. *Several countries or countries with zones may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4.*

Assessment:

Bsal was first described in 2013 and thus there has been limited opportunity to complete surveillance to evidence freedom or put in place sanitary measures to prevent introduction. *Bd* surveillance has been based on a *Bd*-specific qPCR, and cannot be used to assess the current worldwide distribution of *Bsal*. However, a *Bsal* specific PCR was developed by Martel et al (2013) and has been used to screen over 500 wild amphibians from 4 continents (Martel et al. 2014). Positive results were obtained from SE Asia and, the Netherlands and Belgium (where the pathogen was associated with disease). Two studies in North America found no evidence of *Bsal* in wild salamanders (Bales et al, 2015; Muletz et al, 2014). Yap et al (2015) also consider that North America is free but at risk of *Bsal* introduction. Given the susceptibility of fire salamander and its widespread distribution in central and southern Europe, it is reasonable to conclude that currently the pathogen has a restricted geographic distribution in Europe.

There is uncertainty regarding the global distribution of *Bsal*; however, based on available information it is likely that several countries may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4. However, it is unlikely at this point that countries have put in place measures to prevent introduction of *Bsal*.

Conclusion: the criterion is satisfied

AND

C. Diagnosis

Criterion No. 8. *A repeatable and robust means of detection/diagnosis exists*

Assessment:

Methods develop for the culture of *Bd* were successfully used to culture *Bsal*. Culture at various temperatures indicated that incubation at 20°C on tryptone-gelation hydrolactose-lactose (TGhL) broth produced the best results (Martel *et al.*, 2013).

Annex 29 (contd)

A PCR has been developed to amplify the 5.8S ribosomal RNA gene of *Bsal* and its flanking internal transcribed spacer regions (Martel et al, 2013). The PCR results showed that *Bsal* DNA was present in all five experimentally infected animals, and was associated with histopathological lesions (with very high numbers of colonial thalli of *Bsal*), consistent with the lesions found in wild animals. This provides evidence of the high sensitivity of the assay. The PCR has been demonstrated not to cross-react with *Bd,* providing evidence of specificity. However, given the limited validation studies it is not possible to assess the test characteristics (repeatability and robustness) of the PCR or culture methods.

Conclusion: *the criterion is partially satisfied.*

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REPORT OF THE MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 13–15 October 2015

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The OIE *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases (the *ad hoc* Group) met at OIE Headquarters on 13–15 October 2015.

The members of the *ad hoc* Group, the adopted agenda and the Terms of Reference are presented at Annex 1, Annex 2, and Annex 3, respectively.

Dr Gillian Mylrea, Deputy Head of the International Trade Department, welcomed members and thanked them for their willingness to work on this important topic. Dr Mylrea informed members that recommendations from their first meeting in February 2015 regarding the list of susceptible species for infection with yellow head virus had been considered by the Aquatic Animal Health Standards Commission at their March 2015 meeting.

The chair of the *ad hoc* Group, Dr Grant Stentiford, thanked the members for all their hard work prior to the physical meeting in undertaking literature reviews and preparing assessments for seven of the OIE listed crustaceans diseases (Acute hepatopancreatic necrosis disease; Crayfish plague; Infectious hypodermal and haematopoietic necrosis; Infectious myonecrosis; Necrotising hepatopancreatitis; Taura syndrome; and White tail disease). Dr Stentiford clarified that the purpose of this meeting was to review these assessments in order to finalise the lists of susceptible species for the pathogens associated with these diseases for inclusion in the OIE *Aquatic Animal Health Code (Aquatic Code)* and *Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual)*.

The *ad hoc* Group applied the 3-stage approach, outlined in Article 1.5.3. of Chapter 1.5 of the *Aquatic Code*, to assess susceptibility of a species to infection with a specified pathogenic agent . The "Criteria for listing species as susceptible to infection with a specific pathogen" in the *Aquatic Code* are as follows:

1) criteria to determine whether the route of transmission is consistent with natural pathways for the infection (as described in Article 1.5.4.);

2) criteria to determine whether the pathogenic agent has been adequately identified (as described in Article 1.5.5.);

3) criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection (as described in Article 1.5.6.).

Hosts that were classified as susceptible species (as described in Article 1.5.7.) were proposed for inclusion in Article X.X.2. of the *Aquatic Code.*

Hosts that were classified as species for which there is incomplete evidence for susceptibility (as described in Article 1.5.8.) were proposed for inclusion in a new Article 2.2.2. ' Species with incomplete evidence for susceptibility' of the *Aquatic Manual*.

In addition, the *ad hoc* Group identified hosts where there was only evidence for criteria in Article 1.5.4. ('natural pathways for infection') and 1.5.5. ('pathogenic agent has been adequately identified'), but not 1.5.6. ('presence of the pathogenic agent constitutes an infection'). The *ad hoc* Group proposed that these hosts be included in the relevant *Aquatic Manual* chapter under the proposed new sub-heading '2.2.2. Species with incomplete evidence for susceptibility' in section '2.2. Host factors' in the following manner:

'In addition, pathogen-specific positive PCR results have been reported in the following organisms but an active infection has not been demonstrated: …'

Annex 30 (contd)

The detailed assessments for each specific pathogenic agent assessed by the *ad hoc* Group are provided in Annexes 4 to 10.

Disease	Annex Number
Crayfish plague (*Aphanomyces astaci*)	Annex 4
Infectious hypodermal and haematopoietic necrosis	Annex 5
Infectious myonecrosis	Annex 6
Necrotising hepatopancreatitis	Annex 7
Taura syndrome	Annex 8
White tail disease	Annex 9
Acute hepatopancreatic necrosis disease	Annex 10

The *ad hoc* Group noted that some text in Section '2.2. Host Factors' of the *Aquatic Manual* includes text and references to susceptible species. Given the proposed revised lists of susceptible species, the *ad hoc* Group made the following recommendations:

1) Section '2.2.5. Persistent infection with lifelong carriers'. The *ad hoc* Group recommended this title be amended to 'Persistent infection carriers' because it is unknown whether persistence is lifelong. In addition they recommended that the relevant Reference Laboratory expert, includes in this section, a simple statement about persistent infection status supported by references, and that any text referring to susceptibility be deleted.

2) Section '2.2.7. Known or suspected wild aquatic animal carriers'. The *ad hoc* Group proposed that this section be deleted as it is covered under sections on susceptible hosts and persistent carriers and as currently written creates confusion.

The *ad hoc* Group noted that the only crustacean OIE listed disease yet to be assessed was white spot disease (WSD) caused by white spot syndrome virus (WSSV). The *ad hoc* Group agreed to commence this work electronically and requested that a physical meeting be held in early 2016 to finalise this work.

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.../Annexes

MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris (France), 13–15 October 2015

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**MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES
TO INFECTION WITH OIE LISTED DISEASES**

Paris (France), 13–15 October 2015

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Agenda

1. Welcome

2. Review assessments for species susceptibility as described in Chapter 1.5. of the *Aquatic Code* for:

    2.1. Crayfish plague (Chapter 9.1.)

    2.2. Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)

    2.3. Infectious myonecrosis (Chapter 9.4.)

    2.4. Necrotising hepatopancreatitis (Chapter 9.5.)

    2.5. Taura syndrome (Chapter 9.6.)

    2.6. White tail disease (Chapter 9.8.)

    2.7. Acute hepatopancreatic necrosis disease (Chapter 9.X.)

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**MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES
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Terms of Reference

Background

A new Chapter 1.5. 'Criteria for listing species as susceptible to infection with a specific pathogen' was introduced into the 2014 edition of the *Aquatic Code*. The purpose of this chapter is to provide criteria for determining which host species are listed as susceptible in Article X.X.2. of each disease-specific chapter in the *Aquatic Code*. The criteria are to be applied progressively to each disease-specific chapter in the *Aquatic Code*.

Assessments will be undertaken by *ad hoc* Groups and the assessments will be provided to Member Countries for comment prior to any change in the list of susceptible species in Article X.X.2. of the disease-specific chapters in the *Aquatic Code*.

For species where there is some evidence of susceptibility but insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3., information will be included in the relevant disease-specific chapter in the *Aquatic Manual*.

Purpose

The *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases will undertake this task for OIE listed crustacean diseases.

Terms of Reference

1)   Consider standards of evidence required to satisfy the criteria in Chapter 1.5.

2)   Review relevant literature documenting susceptibility of species

3)   Propose susceptible species for OIE listed diseases based on Article 1.5.7.

4)   Propose susceptible species for OIE listed diseases based on Article 1.5.8.

Expected outputs of the October 2015 meeting of the *ad hoc* Group

1)   Develop a list of susceptible species for inclusion in the relevant articles of crustacean disease-specific chapters in the *Aquatic Code* and *Manual* for Crayfish plague (Chapter 9.1.); Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.); Infectious myonecrosis (Chapter 9.4.); Necrotising hepatopancreatitis (Chapter 9.5.); Taura syndrome (Chapter 9.6.); White tail disease (Chapter 9.8.); and Acute hepatopancreatic necrosis disease (Chapter 9.X.).

2)   Draft a report for consideration by the Aquatic Animals Commission at their February 2016 meeting.

ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH *APHANOMYCES ASTACI*

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection with *Aphanomyces astaci* , the causative agent of crayfish plague, by applying the 3-stage approach as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with *A. astaci* (Stage 2) is based on Section 4 of ````Chapter 2.2.1. in the *Aquatic Manual* which states that a presumptive diagnosis of *A. astaci* can be made based on the presence of hyphae penetrating the cuticle resulting in a host tissue response (i.e. haemocytes and melanisation) and the presence of sporangia that morphologically correspond to *A. astaci* However, 'confirmation' of *A. astaci* should be based on PCR and sequence authentication.

Criteria for susceptibility to infection with *A. astaci* (stage 3) are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to be infected with *A. astaci* if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. Criteria for susceptibility to infection with A. astaci (Stage 3)

A: Replication♯	B: Viability/Infectivity	C: Pathology/Clinical Signs	D: Location
Presence of *A. astaci* developing hyphae with/without sporulation in the cuticle and/or underlying tissues;	*Aphanomyces* can be cultured in artificial media (Alderman and Polglase, 1986);	Presence of fungal hyphae 7 to 9 um wide in the cuticle and/or underlying tissues associated with haemocytic infiltration with/without melanisation.	Soft cuticle is usually the first tissue to be affected; however, *A. astaci* will eventually spread throughout connective tissue and haemal sinuses.
OR	OR		
Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Clinical signs include localised whitening of the muscle under the infected cuticle.	
AND			
1. Positive labelling of hyphae with ISH or IFAT;			
OR			
2. Demonstration of increased copy number over time with qPCR with confirmatory PCR/sequencing specific for *A. astaci*.			

Key:

♯   For this pathogen, the *ad hoc* Group agreed to forgo requirement for confirmation of replication using molecular or antibody labelling because these techniques were not utilized historically for this pathogen.

*   To demonstrate replication by this approach requires evidence for passage in confirmed pathogen-free hosts of the same species as being assessed.

**   To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

Annex 30 (contd)

Annex 4 (contd)

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with *A. astaci* is provided in Table 2 (nd - not determined).

Table 2. Outcome of assessment for host susceptibility to infection with A. astaci

Family	Genus	Species	Stage 1: Route of infection	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Outcome*	References
					A	B	C	D		
Astacidae	*Austropotamobius*	*pallipes*	Natural	PCR	Yes	Yes	Yes	Yes	1	1,3
Astacidae	*Austropotamobius*	*torrentium*	Natural	PCR and sequencing	nd	nd	nd	Yes	2	
Astacidae	*Astacus*	*leptodactylus*	Experiment non-invasive	PCR and sequencing	nd	Yes	Yes	Yes	1	1 6
Astacidae	*Astacus*	*astacus*	Natural; experimental non-invasive	No	Yes	Yes	Yes	Yes	1	4, 9, 15
Astacidae	*Pacifastacus*	*leniusculus*	Natural	PCR	Yes	Yes	Yes	Yes	1	7, 9, 14,16
Cambaridae	*Procambarus*	*clarkii*	Natural	No	Yes	Yes	Yes	Yes	2	4, 9, 14
Cambaridae	*Procambarus*	*alleni*	Natural	PCR and sequencing	nd	Yes	Yes	Yes	1	8
Cambaridae	*Procambarus*	*fallax virginalis*	Natural	PCR	nd	nd	nd	Yes	3	5, 8
Cambaridae	*Orconectes*	*limosus*	Natural	PCR	Yes	Yes	Yes	Yes	1	9, 14
Cambaridae	*Orconectes*	*cf. virilis*	Natural	PCR	nd	nd	nd	nd	3	14
Cambaridae	*Orconectes*	*immunis*	Natural	PCR	nd	nd	nd	Yes	2	10

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Family	Genus	Species	Stage 1: Route of infection	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Outcome*	References
					A	B	C	D		
Parastacidae	*Cherax*	*quadricarinatus*	Natural; experimental non-invasive	No**	Yes	nd	Yes	Yes	2	8, 15
Parastacidae	*Cherax*	*destructor*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Cherax*	*papuanus*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Euastacus*	*kershawi*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Euastacus*	*claydensis*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Euastacus*	*crassus*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Geocherax*	*gracilis*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Astacopsis*	*gouldi*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Parastacidae	*Astacopsis*	*fluviatilis*	Experimental non-invasive	No	Yes	nd	Yes	Yes	2	15
Palaemonidae	*Macrobrachium*	*dayanum*	Experimental non-invasive	PCR	Nd	nd	Nd	Nd	3	13
Varunidae	*Eriocheir*	*sinensis*	Natural; experimental	PCR and sequencing	nd	Yes	No	Yes	2	2, 14, 11, 13
Potamidae	*Potamon*	*potamios*	Natural	PCR and sequencing	Yes	nd	Yes	Yes	1	12

* * PCR positive in one study but could have been contamination and no stage 3.

Outcome Key*:
Outcome 1: Host species proposed to be listed in Article 9.1.2. of the *Aquatic Code.*
Outcome 2: Host species proposed to be listed in Chapter 2.2.1. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'.*
Outcome 3: Host species proposed to be listed in Chapter 2.2.1. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.
Note: No differentiation has been made between different groups (A-D) of *A. astaci* in this report because strain types are not generally reported in the literature.

Annex 30 (contd)

Annex 4 (contd)

Additional information relevant to *A. astaci*

Many of the early studies in the literature did not confirm the pathogen using molecular techniques that would differentiate it from other oomycetes or fungi. In most of these cases, with the exception of the crayfish species in Australia (i.e. *Cherax* spp.), the *ad hoc* Group were also able to confirm the susceptibility of the host taxa with more recent studies that utilised PCR and sequencing. Further, this pathogen infects the cuticle of crayfish so it is particularly difficult to establish whether an animal is infected with the pathogen versus surface contamination when no other diagnostic evaluation other than a molecular test was undertaken on the exoskeleton of the animal. The *ad hoc* Group relied on evidence of replication and invasion of the tissue to differentiate between these two scenarios but in many instances reference to pathology pertaining to crayfish plague was absent from the reports.

Host species to be included in Article 9.1.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.1.2. of the *Aquatic Code*: Noble crayfish (*Astacus astacus*), Danube crayfish (*Astacus leptodactylus*), Signal crayfish (*Pacifastacus leniusculus*), Red swamp crawfish (*Procambarus clarkii*), *Austropotamobius torrentium, Austropotamobius pallipes, Orconectes limosus*, *Orconectes immunis, Procambarus alleni* and *Potamon potamios.*

Note: The *ad hoc* Group found research indicating that several species of crayfish in the families Cambaridae, and Astacidae met our criteria for susceptibility (listed as '1' in Table 2). However, they also found research indicating several species in both of these families had evidence of susceptibility to infection with *A. astaci*, but the extent of the information provided was insufficient on its own to meet the criteria for listing these hosts in the *Aquatic Code*. Given the numerous species in both these families that either fully or partially met the criteria for susceptibility (listed as "1" or "2" in Table 2) the *ad hoc* Group recommends that all species belonging to these two families (Cambaridae and Astacidae) be included in the list of susceptible species in Article 9.1.2. of the *Aquatic Code*.

Host species to be included in Chapter 2.2.1. of the *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of Chapter 2.2. of the *Aquatic Manual* as species with only partially evidence for susceptibility to *A. astaci*: *Astacopsis fluviatilis*, *Astacopsis gouldi*, Red claw crayfish (*Cherax quadricarinatus*), Yabby crayfish (*Cherax destructor*), *Cherax papuanus*, *Euastacus crassus, Euastacus claydensis*, *Euastacus kershawi, Geocherax gracilis,* and Chinese mitten crab (*Eriocheir sinensis*).

Note: Crayfish belonging to the family Parastacidae did not meet the criteria for listing as susceptible species; however, there are very few studies that have evaluated these species. The only reports available did not confirm the presence of *A. astaci* using molecular tools and/or they did not clearly meet the criteria for infection. The *ad hoc* Group therefore recommends that species belonging to the family Parastacidae and the crab species *Eriocheir sinensis* be included in the *Aquatic Manual* under species with incomplete or partial evidence for susceptibility until new evidence is available. Given that at least one non-crayfish species (i.e. *Potamon potamios*) met the criteria for a susceptible host for *A. astaci* and a second crab species *(Eriocheir sinensis)* in the infraorder Brachyura met several of the criteria for susceptibility, the *ad hoc* Group recommends that text be added to the *Aquatic Manual* noting that any crustaceans from *A. astaci* positive watersheds could pose a risk of transmitting *A. astaci* either as vectors or potential susceptible hosts.

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ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS (IHHNV)

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with IHHNV infection is based on Section 4 of Chapter 2.2.2. in the *Aquatic Manual* which states that a confirmed diagnosis is:

"Infectious hypodermal and haematopoietic necrosis (IHHN) is considered to be confirmed if two of the following criteria are met:

i)   positive result by *in-situ* hybridization;

ii)   positive result by PCR (always genotype specific);

iii)   sequence analysis to confirm IHHNV nucleic acid sequence.

The two methods must target different areas of the genome."

Criteria for susceptibility to infection with IHHNV are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to be infected with IHHNV if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

***Table 1.** Criteria for susceptibility to infection with IHHNV*

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical Signs	D: Location
Presence of characteristic inclusion bodies and positive labelling of inclusion bodies by ISH or IFAT. Presence of virions in inclusion bodies by TEM. Demonstration of increasing copy number over time with qPCR with confirmatory PCR/ sequencing specific for infectious virus. Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Numerous necrotic cells with pyknotic nuclei or characteristic eosinophilic inclusion bodies, with within chromatin-marginated, hypertrophied nuclei of cells in target tissues and/or clinical signs (e.g. runt deformity syndrome)***.	Gills, cuticular epithelium (or hypodermis), all connective tissues, the haematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia****.

Key:

*   To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

**   To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

***   Clinical signs typical of IHHNV may provide evidence for fulfillment of this category when evidence from histopathology is not available. However, clinical signs according to the *Manual* chapter may not present equally in all host taxa and may not be specific for infection with IHHNV.

****   Lymphoid organ not present in most non-penaeid host taxa.

Annex 30 (contd)

Annex 5 (contd)

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with IHHNV is provided in Table 2.

Table 2. Outcome of assessment for host susceptibility to infection with IHHNV

Genus	Species	Stage 1: Transmission*	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Outcome**	Refs
				A	B	C	D		
Penaeus	*vannamei*	N, E (*per os*)	PCR	ISH; TEM	Yes	Yes	Yes	1	3, 8, 10, 13
	aztecus	N	PCR	No	No	No	Yes	2	1
	stylirostris	N, E (*per os*)	PCR	ISH; TEM	Yes	Yes	Yes	1	3, 7, 8
	californiensis	N	PCR	ISH	No	No	Yes	1	4, 5, 9
	setiferus	N	PCR	No	No	Yes	Yes	1	1
	duorarum	E	No	No	No	No	No	3	4
	monodon	N, E (*per os*)	PCR	ISH	No	Yes	Yes	1	8, 13
	occidentalis	N	No	No	No	No	No	3	4
	semisulcatus	N	No	No	No	No	No	3	4
	japonicus	N	No	No	No	No	No	3	4
Macrobrachium	*rosenbergii*	N	PCR	ISH	No	Yes	Yes	1	2
Hemigrapsus	*penicillatus*	N	PCR	No	No	No	No	3	14
Artemesia	*longinaris*	N	PCR	No	No	No	No	3	6
Callinectes	*arcuatus*	N	PCR	No	No	No	No	3	5
Achirus	*mazatlanus*	N	PCR	No	No	No	No	3	5
Gerres	*cinerus*	N	PCR	No	No	No	No	3	5
Oreochromis	sp.	N	PCR	No	No	No	No	3	5
Lile	*stolifera*	N	PCR	No	No	No	No	3	5
Centropomus	*medius*	N	PCR	No	No	No	No	3	5

Transmission Key*:
N: Natural infection
E: Experimental infection

Outcome Key**
Outcome 1:   Host species proposed to be listed in Article 9.3.2. of the *Aquatic Code.*
Outcome 2:   Host species proposed to be listed in Chapter 2.2.2. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'.*
Outcome 3:   Host species proposed to be listed in Chapter 2.2.2. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to IHHNV

Presence of IHHNV nucleic acid sequences integrated into the host genome were not considered to be infection with IHHNV and were not part of this assessment (Tang & Lightner, 2006; Tang *et al.*, 2007).

Host species to be included in Article 9.3.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.3.2. of the *Aquatic Code*:

Penaeus vannamei, *P. stylirostris*, *P. californiensis*, *P. setiferus*, *P. monodon* and *Macrobrachium rosenbergii*.

Host species to be included in Chapter 2.2.2. of *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of the *Aquatic Manual*:

P. aztecus, *P. duorarum*, *P. occidentalis*, *P. japonicus*, *P. semisulcatus*, *Hemigrapsus penicillatus*, *Artemesia longinarus*, *Callinectes arcuatus*, *Archirus mazatlanus*, *Gerres cinerus*, *Oreochromis* sp., *Lile stolifera* and *Centropomus medius*.

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Annex 30 (contd)

Annex 5 (contd)

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ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH INFECTIOUS MYONECROSIS VIRUS (IMNV)

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection with infectious myonecrosis virus (IMNV) by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with IMNV infection is based on Section 4 of Chapter 2.2.3. in the *Aquatic Manual* which states that a confirmed diagnosis is: "Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- histological demonstration of diagnostic acute, transition or chronic-phase IMNV lesions in the striated muscles and/or the LO;

- ISH positive (with an IMNV-specific cDNA probe) signal to IMNV-type lesions in striated necrotic muscle fibres or to distinctive LOS in the lymphoid organs of shrimp with transition or chronic-phase IMNV infections in histological sections;

- one step or nested RT-PCR, or real time RT-qPCR with positive results for IMNV."

Criteria for susceptibility to infection with IMNV are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to be infected by IMNV if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. Criteria for susceptibility to infection with IMNV

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical signs	D: Location
Presence of characteristic inclusion bodies and positive labelling of inclusion bodies by ISH or IFAT. Presence of virions in inclusion bodies by TEM. Demonstration of increasing copy number over time with RT-qPCR with confirmatory RT-PCR/ sequencing specific for infectious virus. Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Multifocal to diffuse with characteristic coagulative necrosis of skeletal muscle fibres, often with marked edema. The main sign is whitish opaque lesions in skeletal tail muscle; infected shrimp may present lethargy. Shrimp may present a mix of acute and older lesions. In these shrimp, the affected muscle fibres appear to progress from presenting coagulative necrosis to presenting liquefactive necrosis, which is accompanied by moderate infiltration and accumulation of haemocytes, fibrosis and presence of basophilic inclusion bodies within cytoplasm of haemocytes, muscle and connective tissue cells. Lymphoid organ spheroids and ectopic spheroids are also a frequent finding. 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***.	Striated muscle (skeletal and less often cardiac), connective tissue, haemocytes, and the lymphoid organ parenchymal cells****.

Key:

* To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

** To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

Annex 30 (contd)

Annex 6 (contd)

*** Clinical signs typical of IMNV may provide evidence for fulfillment of this category when evidence from histopathology is not available. However, clinical signs according to the *Manual* chapter may not present equally in all host taxa and may not be specific for infection with IMNV.

**** Lymphoid organ not present in most non-penaeid host taxa.

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with IMNV is provided in Table 2.

Table 2. Outcome of assessment for host susceptibility to infection with IMNV

Genus	Species	Stage 1: Transmission*	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Outcome**	Refs
				A	B	C	D		
Penaeus	*vannamei*	N, E (*per os*)	RT-PCR	ISH	Yes	Yes	Yes	1	3-5
	stylirostris	E (injection)	RT-PCR	ISH	No	Yes	Yes	2	4
	monodon	E (injection)	RT-PCR	ISH	No	No	Yes	2	4
	subtilis	E (*per os*)	RT-PCR	No	No	No	No	3	1
	esculentus	E (injection, immersion; *per os*)	RT-PCR	ISH	No	Yes	Yes	1	2
	merguiensis	E (injection; immersion; *per os*)	RT-PCR	ISH	No	Yes	Yes	1	2

Transmission Key*
N: natural infection
E: experimental infection

Outcome Key**:
Outcome 1: Host species proposed to be listed in Article 9.4.2. of the *Aquatic Code*.
Outcome 2: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'*.
Outcome 3: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to IMNV

Not applicable.

Host species to be included in Article 9.4.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.4.2. of the *Aquatic Code*:

Penaeus vannamei, *P. esculentus* and *P. merguiensis*.

Host species to be included in Chapter 2.2.3. of the *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of Chapter 2.2.3. of the *Aquatic Manual*:

P. monodon, *P. stylirostris* and *P. subtilis*.

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ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH CANDIDATUS HEPATOBACTER PENAEI

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection Candidatus *Hepatobacter penaei* (hereafter 'susceptibility to NHP') by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with NHP is based on Section 4 of Chapter 2.2.4. in the *Aquatic Manual* which states that a confirmed diagnosis is:

Histological demonstration of diagnostic acute-phase NHPB lesions in (especially) the atrophied hepatopancreas with moderate atrophy of the tubule mucosa, presence of bacteria and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations) AND, ISH positive histological signal to NHPB-type lesions, OR PCR positive result for the causative agent Candidatus *Hepatobacter penaei*.

Criteria for susceptibility to NHP are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This Table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to susceptible to NHP if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. *Criteria for susceptibility to infection with NHP*

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical Signs	D: Location
Presence of Candidatus *Hepatobacter penaei* colonies observed in HP epithelial cell cytoplasm observed by histology. Colonies confirmed to be C.*H.p.* via positive labelling by ISH or IFAT. Demonstration of increasing copy number of bacterial target genes (16s rRNA) over time with qPCR. Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) population of any susceptible host species (e.g. *P. vannamei*) and, confirmation of pathogen synonymy in donor and recipient population using PCR and sequencing of the 16s rRNA gene**.	Distinct phases of pathogenesis: **initial** (low presence of HP tubule epithelial desquamation), **acute** (atrophied hepatopancreas, increase in desquamation of epithelial cells, bacterial colonies and haemocytic infiltration), **transition** (widespread necrosis/sloughing of epithelial cells, edema, widespread haemocyte infiltration and encapsulation of HP tubules) and, **chronic** (HP lesions less abundant but organ is infiltrated by haemocytes and fibrosis apparent). Clinical signs (e.g slow growth, a soft cuticle and a flaccid body) and an acute, usually catastrophic disease with mortalities approaching 100% may indicate NHP but are not pathognomonic***.	Hepatopancreatic tubules. Intracellular infection of hepatopancreatic tubule epithelial cytoplasm by colonies of C.*H.p.* Pronounced encapsulation of infected hepatopancreatic tubules during acute and transition phases of the disease.

Key:

* To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

** To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage to a known susceptible SPF host is required.

Annex 30 (contd)

Annex 7 (contd)

***   Clinical signs typical of NHP may provide some evidence for fulfillment of this category when evidence from histopathology is not available. However, clinical signs according to the *Manual* chapter are not pathognomonic for NHP and further, may not present equally in all host taxa.

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with NHP is provided in Table 2.

Table 2. *Outcome of assessment for host susceptibility to infection with NHP*

Genus	Species	Stage 1: Transmission	Stage 2: Pathogen Identification	Stage 3: Evidence for infection				Outcome*	References
				A	B	C	D		
Penaeus	*vannamei*	Natural	Yes	Yes	Yes	Yes	Yes	1	1,2,3,4,5
	setiferus	Natural	Partial	No	No	No	No	2	6
	aztecus	Natural	Partial	No	No	No	No	2	6
	duorarum	Natural	Partial	No	No	No	No	2	6
	merguiensis	Natural	No	No	No	Yes	Yes	2	7
	marginatus	Natural	No	No	No	Yes	Yes	2	7
	stylirostris	Natural	No	n	n	y	y	2	7,8
	monodon	Natural	No	n	n	Y	y	2	7
Homarus	*americanus*	Experimental/ non-invasive	Yes	n	n	n	n	3	9

*Outcome key:

1:    Host species proposed to be listed in Article 9.5.2. of the *Aquatic Code*.

2:    Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'*.

3:    Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to NHP

Chapter 2.2.4. of the OIE *Aquatic Manual* states that the following may be used to confirm NHPB identity:

'any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results): Histological demonstration of diagnostic acute-phase NHPB lesions in (especially) the atrophied hepatopancreas with moderate atrophy of the tubule mucosa, presence of bacteria and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations); ISH positive histological signal to NHPB-type lesions; PCR positive results for NHPB.' A recent paper by Nunan *et al.* (2013) (10) has classified the causative agent of NHPB as Candidatus *Hepatobacter penaei*. Systematics based upon the 16S rRNA and gyrase B genes have placed the agent within the class *Alphaproteobacteria*, order *Rickettsiales*.

The authors state that 'classifying and provisionally naming the bacteria responsible for NHP will help eliminate future confusion with other pathogenic bacteria that can cause similar pathology of the HP in *P. vannamei*'. Lightner (1996) (7) previously stated that 'Similar, if not identical NHP bacteria, have been found to be associated with serious epizootic disease in shrimp farms in Peru, Ecuador, Venezuela, Brazil, Panama, and Costa Rica'.

Based upon this evidence, for the purposes of this exercise, 'Confirmation' of pathogen identification within a potential susceptible host is based upon characterisation of *Hepatobacter penaei* via the method of Nunan *et al.* (2013) (10), or by a previously published PCR (and sequencing) approach developed by the same group (Nunan *et al.*, 2008) (11).

Host species to be included in Article 9.5.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.5.2. of the *Aquatic Code*: *Penaeus vannamei*.

Host species to be included in Chapter 2.2.4. of the *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of Chapter 2.2.4. of the *Aquatic Manual*:

Penaeus aztecus, *Penaeus setiferus*, *Penaeus stylirostris*, *Penaeus duorarum*, *Penaeus merguiensis*, *Penaeus marginatus* and *Penaeus monodon*.

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ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH TAURA SYNDROME VIRUS (TSV)

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection with Taura syndrome virus (TSV) by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with TSV is based on Section 4 of Chapter 2.2.5. in the *Aquatic Manual* which states that a confirmed diagnosis is:

"Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

•   histological demonstration of diagnostic acute-phase TSV lesions in (especially) the cuticular epithelium of the foregut (oesophagus, anterior, or posterior chambers of the stomach) and/or in the gills, appendages, or general cuticle. Such TSV lesions are pathognomonic for TSV only when they occur without accompanying severe acute necrosis (with nuclear pyknosis and karyorrhexis) of the parenchymal cells of the lymphoid organ tubules (which may occur in acute-phase yellow head virus infections);

•   ISH-positive (with a TSV-specific cDNA probe) signal to TSV-type lesions in histological sections (i.e. cuticular acute-phase TS lesions) or to distinctive lymphoid organ spheroids (LOS) in the lymphoid organs of shrimp with chronic phase TS lesions;

•   RT-PCR positive results for TSV;

•   sequencing of PCR product encompassing CP2 may be accomplished, as needed, to determine the TSV genotype."

Criteria for susceptibility to infection with TSV are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical signs (C) and Location (D). Hosts were considered to be infected by TSV if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Annex 30 (contd)

Annex 8 (contd)

Table 1. *Criteria for susceptibility to infection with TSV*

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical Signs	D: Location
Presence of characteristic inclusion bodies and positive labelling of inclusion bodies by ISH or IFAT. Presence of virions in inclusion bodies by TEM. Demonstration of increasing copy number over time with qPCR with confirmatory RT-PCR/sequencing specific for infectious virus. Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Characteristic inclusion bodies, with pyknosis and karyorrhectic nuclei ("peppered" or "buckshot") in target tissues. No haemocytic infiltration. Clinical signs include lethargy, low feeding rate, static activity on pond edge when moribund, red pale body and appendage discoloration, intensely red tail fan and pleopods, soft shell, empty gut, multifocal irregular melanized cuticle lesions. TS in *P. vannamei* has three phases clinically and histologically differentiated: peracute-to-acute phase, displaying lethargy, anorexia, empty midgut, atactic swimming, flaccid bodies, soft cuticles, muscle opacity, and chromatophore expansion resulting in red or dark discoloration of uropods, antennae and general body. A transition/recovery phase with generalized multifocal irregular black melanized cuticular lesions; lethargy and anorexia may occur; mortality continues. Chronic, sub-clinical carrier phase starting post-molt and loss of melanized cuticle; this phase can persist for the remainder of the shrimp's life. Typical histological lesions can be observed only during acute phase. In severely infected shrimp, lymphoid organ spheroids are sometimes observed in association with tegmental glands and within connective tissues of the cephalothorax and appendages (ectopic spheroids)***.	Cells of tissues of ectodermic and endodermic origin that include cuticular epithelium (or hypodermis) of most exoskeleton, foregut, hindgut, gills, appendages, connective tissue, haematopoietic tissues, lymphoid organ and antennal gland****.

Key:

* To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

** To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

*** Clinical signs typical of TSV may provide evidence for fulfillment of this category when evidence from histopathology is not available. However, clinical signs according to the *Manual* chapter may not present equally in all host taxa and may not be specific for infection with TSV.

**** Lymphoid organ not present in most non-penaeid host taxa.

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with TSV is provided in Table 2.

Table 2. *Outcome of assessment for host susceptibility to infection with TSV*

Genus	Species	Stage 1 Transmission*	Stage 2 Pathogen identification	Stage 3 Evidence for infection				Outcome**	Refs
				A	B	C	D		
Penaeus	*vannamei*	N	RT-PCR	IHC	No	Yes	Yes	1	7, 8
	aztecus	E (*per os*)	RT-PCR	ISH	No	Yes	Yes	1	6
	stylirostris	N	RT-PCR	IHC	No	Yes	Yes	1	8
	setiferus	E (*per os*)	RT-PCR	ISH	No	Yes	Yes	1	6
	duorarum	E (*per os*)	RT-PCR	No	No	No	No	3	6
	monodon	N, E (*per os*)	RT-PCR	ISH	No	Yes	Yes	1	2, 7
	japonicus	E (injection)	RT-PCR	No	No	No	No	3	1
	ensis	N	RT-PCR	No	No	Yes	Yes	1	1
	chinensis	E (injection)	RT-PCR	No	No	Yes	Yes	2	6
	schmitti	N	RT-PCR	No	No	No	No	3	3
Macrobrachium	*rosenbergii*	E (injection)	RT-PCR	ISH	No	Yes	Yes	2	2
Fundulus	*grandis*	E (*per os*)	RT-PCR	No	No	No	No	3	5
Ergasilus	*manicatus*	E (*per os*)	RT-PCR	RT-qPCR	No	No	No	2	5
Chelonibia	*patula*	E (*per os*)	RT-PCR	ISH	No	No	No	2	5
Callinectes	*sapidus*	E (per os)	RT-PCR	No	No	No	No	3	5
Octolasmis	*muelleri*	E (*per os*)	RT-PCR	ISH	No	No	No	2	5
Uca	*vocans*	E (injection and *per os*)	RT-PCR	No	No	No	No	3	4
Sesarma	*mederi*	E (injection and *per os*)	RT-PCR	No	No	No	No	3	4
Scylla	*serrata*	E (injection and *per os*)	RT-PCR	No	No	No	No	3	4

Transmission Key*:
N: Natural infection
E: Experimental infection

Outcome Key**:
Outcome 1: Host species proposed to be listed in Article X.X.2. of the *Aquatic Code.*
Outcome 2: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'.*
Outcome 3: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to TSV

Not applicable.

Host species to be included in Article 9.6.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.6.2. of the *Aquatic Code*:

Penaeus vannamei, *P. aztecus*, *P. stylirostris*, *P. setiferus*, *P. monodon* and *P. ensis*.

Host species to be included in Chapter 2.2.5. of the *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of Chapter 2.2.5. of the *Aquatic Manual*:

P. duorarum, *P. japonicus*, *P. chinensis*, *P. schmitti*, *Macrobrachium rosenbergii*, *Fundulus grandis*, *Ergasilus manicatus*, *Chelonibia patula*, *Callinectes* sapidus, *Octolasmis* muelleri, *Uca vocans*, *Sesarma mederi* and *Scylla serrata*.

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ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH MACROBRACHIUM NODAVIRUS (MRNV)

The objectives of this assessment were (1) to determine susceptibility of given host taxa to infection with *Macrobrachium rosenbergii* nodavirus (hereafter 'susceptibility to WTD') by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to infection with WTD is based on Section 4 of Chapter 2.2.7. in the *Aquatic Manual* which states that a confirmed diagnosis is: Suspect cases should first be checked by RT-PCR and confirmed by nRT-PCR, sequencing, TEM and DNA probes.

Criteria for susceptibility to WTD are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This Table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to susceptible to NHP if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. *Criteria for susceptibility to infection with WTD*

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical signs	D: Location
Presence of characteristic lesions and positive labelling by ISH or IFAT; OR Presence of virions by TEM; OR Demonstration of increased copy number with RT-qPCR; OR Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**; OR Replication in C6/36 sub clones of *Aedes albopictus* mosquito cell line.	Characteristic pathology including progressive segmental myofibre degeneration of striated muscle and necrotic myopathy. Basophilic cytoplasmic inclusions in striated muscles of abdomen, cephalothorax and intratubular connective tissue of the hepatopancreas; AND/OR Clinical signs including lethargy, anorexia, opaqueness of abdominal muscle and degeneration of telson and uropods.	Striated muscle.

Key:

*   To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

**   To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

Annex 30 (contd)

Annex 9 (contd)

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with WTD is provided in Table 2.

Table 2. *Outcome of assessment for host susceptibility to infection with WTD*

Genus	Species	Stage 1: Transmission	Stage 2: Pathogen identification	Stage 3: Evidence for infection				Outcome*	References
				A	B	C	D		
Macrobrachium	*rosenbergii*	Experimental (immersion,, oral, injection)	Northern blotting, RT-PCR, real time RT-PCR, nested RT-PCR, ISH) ; Natural	Yes	Yes	Yes	Yes	1	3,4,5,7,10,13,17,18,19
Penaeus	*japonicus*	Experimental (oral & intramuscular injection)	RT-PCR	No	No	No	No	3	16
Penaeus	*indicus*	Natural & experimental infection	RT-PCR	No	Yes	No	No	3	6, 16
Penaeus	*monodon*	Natural & experimental infection	RT-PCR	No	Yes	Yes	No	3	6, 16
Penaeus	*vannamei*	Natural and Experimental (oral)	Nested RT-PCR	No	Yes	Yes	Yes	2	11, 12
Belostoma	sp.	Experimental challenges with C6/36 cells	RT-PCR, Nested RT-PCR, TEM	No	Yes	No	No	3	15
Aesohna	sp.	Experimental challenges with C6/36 cells	RT-PCR, Nested RT-PCR, TEM	No	Yes	No	No	3	15
Cybister	sp.	Experimental challenges with C6/36 cells	RT-PCR, Nested RT-PCR, TEM	No	Yes	No	No	3	15
Notonecta	sp.	Experimental challenges with C6/36 cells	RT-PCR, nested RT-PCR, TEM	No	yes	No	No	3	15
Macrobrachium	*malcolmsonii*	Experimental (oral and intramuscularly injection)	RT-PCR	No	No	No	No	3	8
Macrobrachium	*rude*	Experimental (oral and intramuscularly injection)	RT-PCR	No	No	No	No	3	8
Artemia	sp.	Experimental (oral)	RT-PCR, nested RT-PCR	No	No	No	No	3	16
Cherax	*quadricarinatus*	Experimental (feed & intramuscular injection)	qRT-PCR	No	No	Yes	No	3	2

*Outcome key:

1: Host species proposed to be listed in Article 9.5.2. of the *Aquatic Code.*

2: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'*.

3: Host species proposed to be listed in the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to WTD

Outbreaks of white tail disease have never been reported in any species of crustacean other than *Macrobrachium rosenbergii*. *Mr*NV has been observed in several species of crustacean but they did not show mortality or any clinical sign. These species could be reservoirs for *Mr*NV.

Only post larvae of *Macrobrachium rosenbergii* exhibited specific clinical signs. Muscle turned opaque from tail to head and mortality reached 100% within 2–5 days. No outbreaks have been reported in adult stages.

The *Aquatic Manual* for WTD states that suspect cases should first be checked by RT-PCR (9, 10, 21) and confirmed by nRT-PCR (14), sequencing, TEM and DNA probes (20, 21).

White tail disease is associated with two aetiological agents: *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV). The role of XSV in pathogenicity remains unclear. *Mr*NV genome is composed of two linear ss-RNAs present in equimolar ratio and exhibiting a length of ca 3200 (RNA-1) and ca 1250 nucleotides (RNA-2), respectively (1).

Host species to be included in Article 9.8.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.8.2. of the *Aquatic Code*: giant freshwater prawn *(Macrobrachium rosenbergii)*.

Host species to be included in Chapter 2.2.7. of the *Aquatic Manual*

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of Chapter 2.2.7. of the *Aquatic Manual*:

Penaeus vannamei, *Penaeus japonicus*, *Penaeus indicus*, *Penaeus monodon*, *Belostoma* sp., *Aesohna* sp., *Cybister* sp., *Notonecta* sp., *Macrobrachium rude*, *M. malcolmsonii*, *Artemia* sp. and *Cherax quadricarinatus*.

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ASSESSMENT OF HOST SUSCEPTIBILITY TO ACUTE HEPATO-PANCREATIC NECROSIS DISEASE (AHPND)

The objectives of this assessment were (1) to determine susceptibility of given host taxa for Acute Hepatopancreatic Necrosis Disease (AHPND) by applying the 3-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and *Aquatic Manual* with respect to host species susceptibility.

In this assessment the confirmation for susceptibility to AHPND is based on draft OIE *Aquatic Manual* chapter (in preparation) which states that a confirmed diagnosis is:

"in addition to the criteria in Section 7.1, two or more of the following criteria are met:

- histopathology indicative of AHPND;

- detection of PiR toxin gene by PCR and sequence analysis;

- positive results (clinical signs/mortality/histopathology/PCR and sequence) by bioassay."

Criteria for susceptibility to infection with AHPND causing bacteria are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D). Hosts were considered to be infected with AHPND causing bacteria if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. *Criteria for susceptibility to infection with AHPND causing bacteria*

A: Replication	B: Viability/Infectivity	C: Pathology/Clinical signs	D: Location
Presence of characteristic histopathology. Demonstration of increasing copy number over time with qPCR with confirmatory PCR/ sequencing specific for Pir toxin gene. Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Clinical signs and mortality can start as early as 10 days post-stocking. Clinical signs include a pale-to-white hepatopancreas (HP), significant HP atrophy, soft shells, guts with discontinuous, or no, contents, black spots or streaks visible within the HP (due to melanised tubules). Acute phase: Characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells. Terminal phase: 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***.	Gut-associated tissues and organs, such as hepatopancreas (HP), stomach, the midgut and the hindgut.

Key:

* To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the <u>same species</u> as being assessed.

** To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in <u>any</u> known susceptible SPF host is required.

*** Demonstration of terminal phase is insufficient evidence for fulfillment of this category when evidence from acute phase histopathology is not available.

Annex 30 (contd)

Annex 10 (contd)

ASSESSMENT FOR HOST SUSCEPTIBILITY

The assessment for host susceptibility to infection with AHPND causing bacteria is provided in Table 2.

Table 2. *Outcome of assessment for host susceptibility to AHPND*

Genus	Species	Stage 1: Transmission*	Stage 2: Toxin gene identification	Stage 3: Evidence for infection				Outcome**	References
				A	**B**	**C**	**D**		
Penaeus	*vannamei*	N, E (immersion and *per os*)	PCR	Yes	Yes	Yes	Yes	1	3, 5, 6
	monodon	N, E (immersion)	PCR	Yes	Yes	Yes	Yes	1	1, 2
	chinensis	N	nd	histo	No	Yes	Yes	2	4

Transmission Key*:
N: Natural infection
E: Experimental infection

Outcome Key**:
Outcome 1:   Host species proposed to be listed in Article 9.3.2. of the *Aquatic Code*.
Outcome 2:   Host species proposed to be listed in Chapter 2.2.2. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'*.
Outcome 3:   Host species proposed to be listed in Chapter 2.2.2. of the *Aquatic Manual* under the revised Section 2.2.2. *'Species with incomplete evidence for susceptibility'* where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.

Additional information relevant to AHPND

AHPND is caused by infection with unique strains of *Vibrio* species, including *V. parahaemolyticus* (VP$_{AHPND}$), *V. harveyi*, and possibly others, that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB.

Host species to be included in Article 9.3.2. of the *Aquatic Code*

The *ad hoc* Group proposed that the following host species be included in Article 9.3.2. of the *Aquatic Code*:

Penaeus vannamei and *P. monodon*.

Host species to be included in the new draft Chapter X.X.X. of the *Aquatic Manual* Chapter 2.2.2.

The *ad hoc* Group proposed that the following host species be included in the revised Section 2.2.2. of the *Aquatic Manual*:

P. chinensis.

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AQUATIC ANIMAL HEALTH STANDARDS COMMISSION WORK PLAN 2016–2017

Task	Feb 2016	Sept 2016
AQUATIC CODE		
Glossary	Propose some definitions for adoption and some for Members' comments	Review Member comments
Chapter 1.1. – Notification of diseases, and provision of epidemiological information	Review Member comments and propose for adoption	
Chapter 1.2. – Criteria for listing	Review Member comments, amend and circulate for comment	Review Member comments
Chapter 1.3. – List of diseases	Review assessments using the criteria for listing for *Batrachochytrium salamandrivorans* and *Marteilia cochillia.* Circulate for comment listing of *B.sal* Circulate for comment revised crustacean disease names	Review Member comments
New chapter on Biosecurity (4.X.)		Develop and review draft ToR for a new *ad hoc* Group to develop text for this new chapter
Revision of Chapters 4.2- 4.4		Prioritise this work after new chapter on Biosecurity underway
NEW chapter Emergency disease preparedness (Note Chile (Feb 16) requested a Fact Sheet on this topic. Does AAC wish to do this)		Prioritise this work after new chapter on Biosecurity underway
Chapter 4.3. – General recommendations on disinfection	Review AHG draft chapter and propose for adoption	
Chapter XX Salmon egg disinfection	Review comments and circulate for comment	Review comments
Possible development of other eggs and larvae disinfection chapters	Request priorities from members re further work	Review Members priorities
Revise X.X.8. to remove reference to ICES	Revise Article X.X.8 and circulate for comment	Review Member comments
Develop concept for a possible guidance document on how to use the *Aquatic Code* to facilitate trade.		Consider developing a concept note
Chapter 9.2. – YHV1	Review Member comments and propose for adoption	
AHPND (Chapter 9.X.)	Review Member comments and circulate for comment	Review Member comments
Develop revised lists of susceptible species – crustacean	Review AHG report and amend Article X.X.2. of relevant disease-specific chapters and circulate for comment	Review comments

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Annex 31 (contd)

Develop revised lists of susceptible species – fish	Request an *ad hoc* Group be convened to start work assessing susceptible species lists for OIE listed fish diseases	Finalise ToR
Periods to claim/reclaim freedom (in relation to Chapter 1.4.) Develop principles for determining surveillance periods in disease-specific chapters and provide advice on amendments for Chapter 1.4.		Develop criteria for an ad hoc Group to start this work. Consider an eAHG
AQUATIC MANUAL		
YHV Chapter	Review comments and propose for adoption	
AHPND chapter	Review draft chapter developed by ad hoc Group	Review comments
Develop revised lists of susceptible species – crustacean	Review AHG report and amend Section 2.2. of relevant disease-specific chapters and circulate for comment	Review comments
WSD chapter		
Manual chapter – review (case definitions and test validation tests)	AHG to meet 19-21 April 2016	Review AHG report
Develop revised lists of susceptible species – fish	Request an *ad hoc* Group be convened to start work assessing susceptible species lists for OIE listed fish diseases	Finalise ToR
REFERENCE LABS (in collaboration with BSC)		
Develop Guidelines for RL		Develop and review draft Guidelines
Identify gaps in the RL network		Identify gaps and develop strategy
OTHER WORK		
Guidance document for assessments of new listings (Joint Commission activity)		Develop a guide and circulate before Sept 2017