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

Paris, 22 February–1 March 2017

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at OIE Headquarters in Paris from 22 February to 1 March 2017. 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 for the *Aquatic Code* and *Aquatic Manual* circulated after the Commission's September 2016 meeting: Argentina, Australia, Canada, China (People's Rep. of), Chinese Taipei, Colombia, Costa Rica, Cuba, Fiji, Guatemala, Japan, New Zealand, Norway, Saudi Arabia, Singapore, Switzerland, Tanzania, 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 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 '~~strike through~~' and may be found in the Annexes to this report. The amendments made at this meeting are highlighted with a coloured background in order to distinguish them from those made at the September 2016 meeting.

The Aquatic Animals Commission considered all Member Country comments that were supported by a rationale. However, the Commission was not able to draft a detailed explanation of the reasons for accepting or not accepting every comment received.

The Aquatic Animals 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 relevant reports of *ad hoc* Groups, which include important information and are provided as annexes to Commission's reports. The Commission encourages Member Countries to review these reports together with the report of the Commission. The Commission reminded Member Countries that all of the OIE *ad hoc* Group reports (since 2016) are also provided on the OIE website as stand-alone documents (available at <http://www.oie.int/en/standard-setting/specialists-commissions-working-groups/scientific-commission-reports/ad-hoc-groups-reports/>).

The table below lists the texts as presented in the Annexes. Member Countries should note that texts in **Annexes 3 to 25** are proposed for adoption at the 85th General Session in May 2017; **Annexes 26 to 28** are presented for Member Country comment; and **Annexes 29 to 31** are presented for information.

The Aquatic Animals Commission strongly encourages Member Countries to participate in the development of the OIE's international standards by preparing to participate in the process of adoption of texts in Annexes **3 to 25** at the 85th General Session. The Commission also invites the submission of comments on **Annexes 26–28** of this report. Comments should be submitted as specific proposed text changes, supported by a scientific rationale. Proposed deletions should be indicated by '~~strike through~~' 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 26 to 28** of this report must reach OIE Headquarters by the **4th August 2017** to be considered at the September 2017 meeting of the Aquatic Animals Commission. Comments received after the due date will not be submitted to the Commission for consideration at their September 2017 meeting.

All comments should be sent to the OIE Standards Department at: standards.dept@oie.int.

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A. MEETING WITH THE DEPUTY DIRECTOR GENERAL

The Aquatic Animals Commission met with Dr Matthew Stone, Deputy Director General (International Standards and Science), on 22 February 2017. Dr Stone welcomed the Commission Members and thanked them for their support and commitment to achieving OIE objectives related to aquatic animal health.

Among other matters, Dr Stone informed the Commission of the forthcoming session of the Council and the proposals that it will consider in relation to the new procedure for the election of experts, the provisional budget in particular noting the increased costs of supporting the standard-setting functions of the OIE (e.g. convening of *ad hoc* Groups). Dr Stone also noted the efforts of OIE Headquarters to improve the efficiency across all of the Specialist Commissions through enhanced coordination systems that would provide stronger direction and support to the work programmes, improve internal communication and strengthen understanding of roles and responsibilities.

Dr Ernst, President of the Aquatic Animals Commission, thanked Dr Stone for his opening remarks and noted that the Aquatic Animals Commission has a challenging work programme that includes significant enhancements to both the OIE *Aquatic Code* and *Aquatic Manual*. Dr Ernst highlighted that the support of OIE Headquarters and the secretariat staff, including to support the operation of several *ad hoc* Groups, is essential for the Commission to implement its work programme and meet the expectations of Member Countries.

B. ADOPTION OF THE AGENDA

The draft agenda circulated prior to the meeting was discussed, updated, and agreed. The adopted agenda of the meeting is attached as **Annex 2**.

C. MEETING WITH THE PRESIDENT OF THE OIE TERRESTRIAL HEALTH STANDARDS COMMISSION

The President of the Aquatic Animals Commission met with the President of the Terrestrial Animal Health Standards Commission (Code Commission) during the week when both Commissions were meeting. The Presidents discussed issues of mutual interest in the *Aquatic* and *Terrestrial Codes*, notably: alignment of Glossary terms, in particular the definition for zoning and the ongoing review of definitions used in the *Terrestrial Code*; proposed revisions to Chapters 1.2. in the *Aquatic Code* (criteria for listing); and the proposed development of a guidance document on the application of the criteria for listing an OIE disease.

The Aquatic Animals Commission agreed that these meetings are important to facilitate harmonisation of relevant horizontal chapters in the two Codes.

D. OIE AQUATIC ANIMAL HEALTH CODE – EXAMINATION OF MEMBER COUNTRY COMMENTS

Item 1. General comments

Specific comments were received from Argentina, Australia, Colombia, Costa Rica, EU, Fiji, New Zealand, Norway, Thailand and EU.

The Aquatic Animals Commission agreed with a Member Country comment that although harmonisation of both the *Aquatic* and *Terrestrial Codes* is desirable, there will always be some areas where this is not appropriate due to a number of differences between terrestrial and aquatic animals, for example the large number of aquatic animal species, and the diversity of environmental factors and pathogens to which they are exposed.

In response to a Member Country comment regarding guidance on disease risks and stress associated with the transport of aquatic animals, the Aquatic Animals Commission noted that this is addressed in Chapter 5.5. *Control of aquatic animal health risks associated with transport of aquatic animals* and Chapter 7.2. *Welfare of farmed fish during transport*.

A Member Country commented that some words in the *Aquatic Code* were difficult to interpret, such as ‘is likely’, ‘the likelihood’ and ‘would be likely’. The Aquatic Animals Commission reviewed the use of these terms in the *Aquatic Code* and found that they were clear as written when read in the context of the relevant section of the *Code*. For example, the term ‘likelihood’ is used in the context of the risk analysis framework described in Chapter 2.1 of the *Aquatic Code*.

A Member Country requested the rationale for the inclusion of text in Articles X.X.9. and X.X.11. regarding ‘consideration of introducing internal measures to address the risks associated with the commodity being used for any purpose other than for human consumption’. The Aquatic Animals Commission noted that products referred to in Article X.X.11. are not necessarily free of viable pathogen and controls may be considered to address transmission pathways and prevent exposure to susceptible animals. Therefore, the text is intended to alert Member Countries to this potential risk and encourage them to put in place appropriate internal mitigation measures, where relevant.

Item 2. Glossary

Specific comments were received from China (People’s Rep. of), Colombia, Costa Rica, Fiji, Guatemala, New Zealand, Saudi Arabia, Tanzania and EU.

Aquatic animals

The Aquatic Animals Commission reminded Member Countries that the definition of aquatic animals currently includes both farmed and wild aquatic animals. Measures in the *Aquatic Code* apply to susceptible aquatic animals regardless of their origin, i.e. wild and farmed.

The Aquatic Animals Commission did not agree with a Member Country to further define that aquatic animals come from an aquatic ecosystem as the *Aquatic Code* does not address terrestrial species.

The Aquatic Animals Commission did not agree with a Member Country comment to delete ‘viable’ as they considered this concept was important to include to ensure clarity of the meaning of this defined term.

Zoning

In response to several Member Country comments and also amendments being proposed by the Code Commission to the term ‘zone’ in the *Terrestrial Code*, the Aquatic Animals Commission amended the definition in order to ensure alignment, where relevant, and improve readability and clarity. The Commission agreed with a Member Country comment that the term ‘contiguous hydrological system’ was unclear and agreed it was unnecessary text and should be deleted.

Biological Products

The Aquatic Animals Commission noted that the words ‘infectious agents’ would be changed to ‘pathogenic agents’ in line with the work to ensure consistent use of the defined term pathogenic agent throughout the *Aquatic Code* (see text below).

Disease

The Aquatic Animals Commission noted that the words ‘aetiological agents’ would be changed to ‘pathogenic agents’ in line with the work to ensure consistent use of the defined term pathogenic agent throughout the *Aquatic Code* (see text below).

Use of the term pathogenic agent

The Aquatic Animals Commission noted that although they have been progressively ensuring the consistent use of the defined term ‘pathogenic agent’ throughout the *Aquatic Code* there were still some places, particularly in the horizontal chapters (Sections 1–7) where other terms such as ‘pathogen’ and ‘aetiological agent’ needed to be replaced by the defined term ‘pathogenic agent’. The Commission agreed to make relevant amendments in a progressive manner as chapters in these sections come under review. The Commission reminded Member Countries that similar revisions within disease-specific chapters (Sections 8–11) will be made as these chapters are revised as part of the ongoing work to update the lists of susceptible species.

Other relevant information

The Aquatic Animals Commission reviewed the revised definitions in the glossary of the *Terrestrial Code* that will be proposed for adoption and those that will be circulated for Member Country comment to ensure alignment between relevant definitions used in both Codes. The Commission agreed to review relevant definitions in the *Aquatic Code* once any revised definitions are adopted in the *Terrestrial Code*.

The revised Glossary is attached as **Annex 3** and is proposed for adoption at the 85th General Session in May 2017.

Item 3. Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)

Specific comments were received from Australia, Canada, China (People’s Rep. of), Saudi Arabia, Tanzania, Thailand, the USA and EU.

The Aquatic Animals Commission noted that the majority of Member Country comments were supportive of the revised criteria and that other comments received had been submitted previously and had been addressed by the Commission at previous meetings.

In response to a Member Country comment regarding the difficulty of interpreting the criteria, the Aquatic Animals Commission noted that it will develop a guidance document to support application of the criteria. This guidance document will be used by the Commission when they are overseeing an assessment to ensure consistency in the interpretation of the criteria. The Commission will also share this document with the Code Commission.

The Aquatic Animals Commission reiterated that the rationale for not aligning criterion 1 of Article 1.2.2. with text used in the *Terrestrial Code*, i.e. ‘has been proven’ rather than ‘is likely’, was included in its September 2016 meeting:

“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 for listing a pathogenic agent 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 especially important for aquatic animal diseases because it is often not possible to eradicate them once they have spread.”

The Aquatic Animals Commission reiterated that it does not agree to change ‘may’ to ‘has’ regarding the demonstration of country freedom, noting that the same comment had been considered at its October 2015 meeting. The word ‘may’ is more appropriate because Member Countries are required to have basic biosecurity conditions in place for at least two years prior to making a self-declaration of freedom.

The Aquatic Animals Commission did not agree with a Member Country comment to include additional text regarding emerging diseases because there is an obligation to report emerging diseases through the OIE World Animal Health Information System (WAHIS) in accordance with Article 1.1.6. of the *Aquatic Code*. The Commission noted that this article is particularly important for the *Aquatic Code* given the regular emergence of diseases in aquatic animals.

The Aquatic Animals Commission did not agree with a Member Country comment to define the terms ‘precise’ and ‘reliable’ in criteria 3, noting that the use of these terms is consistent with the Oxford English Dictionary and Chapter 1.1.2. of the *Aquatic Manual*.

After consideration of all Member Country comments, the Aquatic Animals Commission agreed not to make any additional amendments to the text as no new issues had been raised.

The revised Chapter 1.2. *Criteria for the inclusion of diseases in the OIE list* is attached at **Annex 4A** (as clean text) and **Annex 4B** (with track changes) and is proposed for adoption at the 85th General Session in May 2017.

Item 4. Diseases listed by the OIE (Chapter 1.3.)

Specific comments were received from Canada, China (People’s Rep. of), Costa Rica, , Guatemala, Tanzania and EU.

In response to a Member Country comment to amend the text in the preamble in line with the amendments being proposed for Chapter 1.3. of the *Terrestrial Code*, the Aquatic Animals Commission agreed to consider aligning the text between the two Codes once the revised text is adopted in the *Terrestrial Code*.

4.1. Amended crustacean disease names

Member Country comments supported proposed amendments to the names of eight of the listed crustacean diseases in Article 1.3.3.

In light of a Member Country comment, the Aquatic Animal Commission did not agree to include ‘*Candidatus*’ in the listed disease name for ‘Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)’. However, the Commission agreed to include ‘*Candidatus*’ in Article 9.5.1. when referring to the taxonomic name for this bacterial agent.

4.2. *Batrachochytrium salamandrivorans*

Several Member Countries supported the listing of this disease; however, some Member Countries questioned whether criteria 8 of Article 1.2.2. had been met. The Aquatic Animals Commission re-evaluated the available real-time polymerase chain reaction (PCR) method (Bloo *et al.*, 2013) noting that stage 1 validation has been completed and that stage 2 has commenced and is ongoing. The Commission noted that as this new method is applied, it is expected that the additional information required for further validation will be generated. The Commission updated the text regarding criterion 8 in the assessment (see Annex 29).

In response to a Member Country comment, the Aquatic Animals Commission agreed that the presence of a Reference Laboratory for *Batrachochytrium* spp., is desirable, and they have taken steps to identify potential candidates.

The Aquatic Animals Commission reviewed the new USA regulatory controls (information available at: <https://www.federalregister.gov/documents/2016/01/13/2016-00452/injurious-wildlife-species-listing-salamanders-due-to-risk-of-salamander-chytrid-fungus>) and the European Food Safety Authority (EFSA) report for *Bsal*¹ and updated the assessment accordingly (see Annex 29).

¹(EFSA), Baláž V., Gortázar Schmidt C., Murray K., Carnesecchi E., Garcia A., Gervelmeyer A., Martino L., Munoz Guajardo I., Verdonck F., Zancanaro G. & Fabris C. (2017). Scientific and technical assistance concerning the survival, establishment and spread of *Batrachochytrium salamandrivorans* (*Bsal*) in the EU. *EFSA Journal* 2017;**15** (2), 4739, 77 pp. doi:10.2903/j.efsa.2017.4739).

The Aquatic Animals Commission reiterated its proposal to list *Batrachochytrium salamandrivorans*.

The revised assessment for *B. salamandrivorans* is provided for Member Country information at [Annex 29](#) in support of the proposed listing.

4.3. Infection with Ranavirus

The Aquatic Animals Commission further amended the listed name ‘infection with *Ranavirus* spp.’ to ‘infection with *Ranavirus* species’ because binomial nomenclature is not commonly used for viruses. The scope of this listed disease (described in Chapter 8.2.) is intended to include any species within the Genus *Ranavirus* (excluding epizootic haematopoietic necrosis, which is listed separately) and the Commission agreed that the revised name better reflects this scope.

In response to a Member Country comment that ranavirus species should be considered for listing separately, the Aquatic Animals Commission noted that the International Committee for Taxonomy of Viruses is considering classification of ranaviruses. The outcomes of this work would guide future consideration for amendments to the current listing of ranaviruses.

The revised Chapter 1.3. *Diseases listed by the OIE* is attached at [Annex 5](#) and is proposed for adoption at the 85th General Session in May 2017.

4.4. Assessment for a novel orthomyxo-like virus, tilapia lake virus (TiLV)

In light of a recent publication on diagnostic methods for tilapia lake virus (TiLV), the Aquatic Animals Commission reassessed TiLV against criterion 8 (availability of repeatable and robust diagnostic tests) of the criteria for listing (Chapter 1.2.). The Commission reviewed this information and agreed that the information did not support any change to its previous assessment that the disease could not be proposed for listing at this time, due to the lack of specific diagnostic methods for TiLV.

The Aquatic Animals Commission recognised the potential significance of TiLV to many countries given the worldwide importance of tilapia farming and trade in this species. The Commission noted that TiLV meets the definition of an “emerging disease” and, as such, should be reported in accordance with Article 1.1.4. of the *Aquatic Code*.

The Aquatic Animals Commission noted that recent disease events associated with TiLV have not been reported to the OIE in accordance with the obligations described in Chapter 1.1. Member Countries are encouraged to investigate mortality and morbidity events in tilapines as an understanding on the geographic distribution of TiLV is essential for efforts to control its possible spread.

In order to provide information for Member Countries on available detection methods and transmission risks for this virus, the Aquatic Animals Commission finalised the Technical Disease Card for TiLV. The Technical Disease Card for TiLV will be uploaded onto the OIE website in early April 2017 and can be accessed at: <http://www.oie.int/international-standard-setting/specialists-commissions-groups/aquatic-animal-commission-reports/disease-information-cards/>

The Technical Disease Card for TiLV is also presented at **Annex 30** for Member Country information.

Item 5. Criteria for listing species as susceptible (Chapter 1.5.)

Specific comments were received from Australia, Canada, New Zealand, Saudi Arabia, the USA and EU.

Several Member Countries supported the new Article 1.5.9.; however, some expressed concern regarding the lack of clarity on how a broad host range is defined and the implications of listing susceptible species at the level of a taxonomic group.

The Aquatic Animals Commission reminded Member Countries that the aim of the *Aquatic Code* is to prevent the spread of aquatic animal diseases and assure the sanitary safety of international trade in aquatic animals. Application of the current criteria to diseases with a proven broad host range (e.g. infection with *Aphanomyces astaci* and infection with white spot syndrome virus) would result in a substantial reduction in the list of susceptible species for these diseases. As a consequence, the *Aquatic Code* measures for these diseases would not apply to many host species that are likely to be susceptible but for which scientific information is lacking. The Commission noted that this circumstance would be contrary to the purposes of the *Aquatic Code* and could lead to the spread of listed diseases.

The Aquatic Animals Commission had proposed a new Article 1.5.9. to address this issue by including a mechanism to list taxonomic groups of species as susceptible. This mechanism is intended to apply only to diseases with a broad host range.

In light of comments from some Member Countries the Aquatic Animals Commission amended the new Article 1.5.9. to clarify when this article would be applied. The Commission also amended text in Articles 1.5.1. and 1.5.2. to ensure consistency with the new Article 1.5.9.

In addition, the Aquatic Animals Commission requested that the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases assess infection with *Aphanomyces astaci* using the revised Chapter 1.5. This assessment will provide Member Countries with an example of the application of the revised criteria, in particular the new Article 1.5.9. The Commission requested that the *ad hoc* Group work electronically and report back to the Commission by their September 2017 meeting.

The revised Chapter 1.5. *Criteria for listing species as susceptible* is attached at **Annex 26** for Member Country comments.

Item 6. Disinfection of aquaculture establishments and equipment (Chapter 4.3.)

Specific comments were received from China (People's Rep. of), Costa Rica, Guatemala, New Zealand, Saudi Arabia, Tanzania, Thailand, the USA and EU,.

The Aquatic Animals Commission considered Member Country comments received and made relevant amendments to improve clarity and readability. Noting that this new chapter had been circulated extensively for Member Country comments prior to adoption in 2016, the Commission only proposed amendments that significantly improved the clarity of the text.

The revised Chapter 4.3. *Disinfection of aquaculture establishments and equipment* is attached at [Annex 6](#) and is proposed for adoption at the 85th General Session in May 2017.

Item 7. Recommendations for surface disinfection of salmonid eggs (Chapter 4.4.)

Specific comments were received from Norway and the EU .

After consideration of several Member Country comments, the Aquatic Animals Commission agreed not to amend the text further as the Commission Members considered it was clear as proposed.

The Aquatic Animals Commission did not agree with a Member Country comment to include a recommendation regarding appropriate disposal of disinfectant solutions to protect the environment, noting that this was beyond the scope of this chapter.

The Aquatic Animals Commission agreed not to replace the term ‘pathogen’ with ‘pathogenic agent’ in Article 4.4.2. as the use of the term pathogen in the context of the chapter is not relevant to a specific pathogenic agent.

The revised Chapter 4.1. *Recommendations for surface disinfection of salmonid eggs* is attached at [Annex 7](#) and is proposed for adoption at the 85th General Session in May 2017.

Item 8. General obligations related to certification (Chapter 5.1.)

Specific comments were received from Argentina, Australia, Saudi Arabia, Thailand and EU.

After consideration of Member Country comments, the Aquatic Animals Commission agreed not to amend the text further as the Commission Members considered it was clear as proposed.

The Aquatic Animals Commission wished to reiterate that the term ‘incubation period’ that appears in a similar article in the *Terrestrial Code* was not included in this text because it is not used in the *Aquatic Code*. The Commission noted that unlike terrestrial animals, a wide variety of host species and environmental variables make determining the incubation period of aquatic animal pathogenic agents impractical.

The revised Chapter 5.1. *General obligations related to certification* is attached at [Annex 8](#) and is proposed for adoption at the 85th General Session in May 2017.

Item 9. Amendments to crustacean disease-specific chapters

Specific comments were received from Canada, Costa Rica, Guatemala, New Zealand, Norway, Tanzania and EU.

The Aquatic Animals Commission considered Member Country comments made in each chapter and made relevant amendments in each crustacean chapter to ensure alignment across these chapters.

The Aquatic Animals Commission agreed to i) delete the reference to ‘aquatic animals’ in Articles X.X.3. and X.X.11. noting that only aquatic animal products are listed in these articles; ii) add aquatic animal products to Article X.X.10. as this article also applies to aquatic animal products; iii) amend point 2 in Articles X.X.9. and X.X.10. to improve readability and include a cross reference to Chapter 5.5.; and iv) add a new point 3) in Articles X.X.9. and X.X.10. to address separately the management of effluent and waste materials.

The Aquatic Animals Commission did not agree with a Member Country comment to abbreviate the species name when it appears for the second time in Article X.X.2. because the susceptible species are listed in alphabetical order and inclusion of the Genus name in full ensures clarity.

The Aquatic Animals Commission noted that Article X.X.8. was amended as per the model Article X.X.8. (see Item 11).

9.1. Crayfish plague (*Aphanomyces astaci*) (Chapter 9.1.)

The Aquatic Animals Commission applied amendments of a horizontal nature as noted above.

The Aquatic Animals Commission reminded Member Countries that the listing of susceptible species in Article 9.1.2. will remain unchanged from the 2016 edition of the *Aquatic Code* until the issue of species with a broad host range is finalised (see Item 5).

The revised Chapter 9.1. *Crayfish plague* is attached at [Annex 9](#) and is proposed for adoption at the 85th General Session in May 2017.

9.2. Infection with yellow head virus genotype 1 (Chapter 9.2.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 9.2. *Infection with yellow head virus genotype 1* is attached at [Annex 10](#) and is proposed for adoption at the 85th General Session in May 2017.

9.3. Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The Aquatic Animals Commission did not agree with the proposal from a Member Country to remove *Macrobrachium rosenbergii* from the list of susceptible species in Article 9.3.2. as the Commission considered there was sufficient evidence to support the inclusion of this species in the list. In light of the reference provided, the Commission had asked the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases to review its previous assessment. The *ad hoc* Group reiterated that the paper by Hsieh *et al.* (2006) was scientifically sound; it was a natural infection that supported strongly susceptibility of *M. rosenbergii* to infectious hypodermal and haematopoietic necrosis virus (IHHNV); IHHNV was not only detected by PCR, it was also detected in infected nuclei by *in-situ* hybridisation; and there were clinical signs consistent with infection with IHHNV.

Reference: Hsieh C.Y., Chuang P.C., Chen L.C., Tu C., Chien M.S., Huang K.C., Kao H.F., Tung M.C. & Tsai S.S. (2006). Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture*, 258, 73–79.

On the basis of this expert opinion, the Aquatic Animals Commission agreed to leave *Macrobrachium rosenbergii* in the list of susceptible species in Article 9.3.2.

The revised Chapter 9.3. *Infectious hypodermal and haematopoietic necrosis* is attached at [Annex 11](#) and is proposed for adoption at the 85th General Session in May 2017.

9.4. Infectious myonecrosis (Chapter 9.4.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 9.4. *Infectious myonecrosis* is attached at [Annex 12](#) and is proposed for adoption at the 85th General Session in May 2017.

9.5. Necrotising hepatopancreatitis (Chapter 9.5.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

In light of a Member Country comment, the Commission agreed to include ‘*Candidatus*’ in Article 9.5.1. when referring to the taxonomic name for this bacterial agent. However, for ease of reference the term ‘*Candidatus*’ will not be used in the disease name ‘Infection with *Hepatobacter penaei*’ (see Item 4.1. above).

The revised Chapter 9.5. *Necrotising hepatopancreatitis* is attached at **Annex 13** and is proposed for adoption at the 85th General Session in May 2017.

9.6. Taura syndrome (Chapter 9.6.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 9.6. *Taura syndrome* is attached at **Annex 14** and is proposed for adoption at the 85th General Session in May 2017.

9.7. White spot disease (Chapter 9.7.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The Aquatic Animals Commission reminded Member Countries that the listing of susceptible species in Article 9.7.2. will remain unchanged from the 2016 edition of the *Aquatic Code* until the issue of species with a broad host range is finalised (see Item 5).

The revised Chapter 9.7. *White spot disease* is attached at **Annex 15** and is proposed for adoption at the 85th General Session in May 2017.

9.8. White tail disease (Chapter 9.8.)

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 9.8. *White tail disease* is attached at **Annex 16** and is proposed for adoption at the 85th General Session in May 2017.

Item 10. New chapter on Acute hepatopancreatic necrosis disease (Chapter 9.X.)

Specific comments were received from Canada, Costa Rica, Guatemala and New Zealand.

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

The Aquatic Animals Commission agreed to apply amendments of a horizontal nature as noted in Item 9 above.

The Aquatic Animals Commission did not agree with a Member Country comment to change acute hepatopancreatic necrosis disease (AHPND) to 'infection with $V_{p_{AHPND}}$ ' because the Commission considered it likely that the species of *Vibrio* associated with this disease will change over time. Using the disease approach will enable new pathogenic agents to be added to the definition of the disease in Article 9.X.1.

The Aquatic Animals Commission did not agree with several Member Country comments to broaden the scope in Article 9.X.1. beyond the inclusion of *Vibrio parahaemolyticus*. The Commission noted that, at the time of this meeting, there was insufficient published data to support other *Vibrio* species as causative agents of AHPND. The Commission will monitor AHPND research and amend 9.X.1., if supported by relevant scientific data.

The Aquatic Animals Commission corrected an error identified by a Member Country in the August 2016 electronic *ad hoc* Group report on safety of products derived from aquatic animals that pasteurised crustacean products had been assessed against criteria in Article 5.4.1. The *ad hoc* Group report is available at: <http://www.oie.int/en/standard-setting/specialists-commissions-working-groups/scientific-commission-reports/ad-hoc-groups-reports/>.

The revised Chapter 9.X. *Acute hepatopancreatic necrosis disease* is attached at **Annex 17** and is proposed for adoption at the 85th General Session in May 2017.

Item 11. Revised Article X.X.8.

Specific comments were received from Australia, Costa Rica, Guatemala and New Zealand.

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

In response to a Member Country comment to amend the term quarantine in relation to lifelong holding, the Aquatic Animals Commission reminded Member Countries that quarantine is a defined term in the *Aquatic Code* and includes 'a specified length of time'. When importing aquatic animals from a country not free of a specific pathogenic agent, a lifelong period would be appropriate. The Commission also added the word 'imported' in several places to clarify the reference to imported aquatic animals.

The Aquatic Animals Commission clarified that point 3a)ii) on high health status refers to the best possible health status with respect to a specific listed disease, noting that this article will apply to countries that are not free from the specific disease.

The Aquatic Animals Commission noted that if adopted, this model article will be applied to Article X.X.8. of all disease-specific chapters.

The revised model Article X.X.8. is attached at **Annex 18A** (as clean text) and **Annex 18B** (with track changes) and is proposed for adoption at the 85th General Session in May 2017.

Item 12. Inclusion of recommendations for surface disinfection of eggs for species where it is practised other than salmonids, and important for ensuring safe trade

The Aquatic Animals Commission welcomed suggestions received from several Member Countries regarding the possible expansion of disinfection protocols for eggs of non-salmonid fish species. However, the Commission noted that no validated disinfection protocols had been provided and decided that it could not proceed with the development of species-specific egg disinfection recommendations.

However, given the significant risk associated with this pathway, the Aquatic Animals Commission encourages Member Countries to provide relevant information on validated protocols in order for the Commission to reconsider this issue. The Commission encourages Member Countries to support validation of standard commercial methods used for disinfection of aquatic animal eggs and larvae in their respective countries.

Item 13. Red sea bream iridoviral disease (Chapter 10.8.)

A specific comment was received from the EU.

The Aquatic Animals Commission noted an error in the *Aquatic Code* and *Manual* regarding the incorrect reference to albacore as the common name for *Thunnus thynnus*. The Commission found that this issue could not be resolved simply by correcting a taxonomic name or common name and noted that it will be addressed as part of the work currently being undertaken by the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases.

**E. OTHER OIE AQUATIC ANIMAL HEALTH CODE ISSUES AND
WORK OF RELEVANT AD HOC GROUPS**

Item 14. Criteria to assess the safety of aquatic animal commodities (Chapter 5.4.)

The Aquatic Animals Commission noted that there were a number of inconsistencies between Chapter 5.4. *Criteria to assess the safety of aquatic animal commodities* and related Articles X.X.3. and X.X.11./12. in the disease-specific chapters. Relevant amendments were made, including the deletion of references to aquatic animals, as this chapter is intended to apply to aquatic animal products.

The revised Chapter 5.4. *Criteria to assess the safety of aquatic animal commodities* is attached at **Annex 27** for Member Country comments.

Item 15. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases

The Aquatic Animals Commission was informed that the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases held its first meeting in December 2016 and had undertaken assessments of the susceptibility of fish species (as outlined in Article 1.5.3. of Chapter 1.5. of the *Aquatic Code*) to i) epizootic haematopoietic necrosis disease; ii) infection with *Gyrodactylus salaris*; and iii) koi herpesvirus disease. The *ad hoc* Group will finalise these three assessments and commence work on spring viraemia of carp, infection with salmonid alphavirus and infection with infectious salmon anaemia virus when it next meets in April 2017.

The Aquatic Animals Commission appreciated the excellent work done to date by the *ad hoc* Group and requested that it submit a report with the revised lists of susceptible species for the six diseases for the Commission to consider at its September 2017 meeting.

Item 16. *Ad hoc* Group on Demonstration of disease freedom

The Aquatic Animals Commission reviewed the draft report of the *ad hoc* Group on Demonstration of disease freedom, which met from 17–19 January 2017. The Commission also held a tele-conference with the chair of the *ad hoc* Group. The Commission recognised that this is an important and complex area of work and touches on many other parts of the *Aquatic Code*. The Commission requested that the *ad hoc* Group meet again to complete the development of model text for a disease-specific chapter and report back to the Commission meeting in September 2017.

Item 17. *Ad hoc* Group on Aquatic animal biosecurity

The Aquatic Animals Commission was informed that an *ad hoc* Group on Aquatic animal biosecurity for aquaculture establishments will be convened to commence this work prior to the next Commission meeting in September 2017.

F. OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS – EXAMINATION OF MEMBER COUNTRY COMMENTS

Comments had been received from Canada, China (People’s Rep. of), Chinese Taipei, Japan, New Zealand, Norway, Singapore, Switzerland, Thailand, the USA and EU.

The Aquatic Animals Commission reviewed all Member Country comments on the crustacean disease chapters and made relevant amendments.

The Aquatic Animals Commission considered a Member Country comment to amend the definition of a confirmed case of infection with salmonid alphavirus. This chapter is not currently under review; however, the Commission agreed that the comment will be addressed when the chapter is revised to bring it into the format of the new template being developed by the *ad hoc* Group on the *Aquatic Manual* (see Item 26).

A Member Country had proposed in several chapters to change the genus name of shrimp species from *Penaeus* to *Litopenaeus*. The Aquatic Animals Commission refers the Member Country to Item 14 of the Commission’s September 2016 meeting report, *Review of prawn taxonomy literature for Penaeus*. This gives the rationale for the Commission’s decision to maintain the name *Penaeus*.

In reply to several proposals to amend the titles of certain sections of the *Aquatic Manual* chapters, the Aquatic Animals Commission reminded Member Countries that section titles will be revised once the new disease chapter template developed by the *ad hoc* Group on the *Aquatic Manual* (see Item 26) is approved and implemented.

On a number of occasions amendments to the protocols for polymerase chain reaction assays were proposed. The Aquatic Animals Commission agreed that Member Countries proposing changes to published protocols must provide the rationale for these amendments with evidence of equivalency of test performance under the new test conditions. It would also be preferable if the amended protocols had been validated and published.

The Aquatic Animals Commission agreed to ask Reference Laboratory experts to carefully assess the test protocols in each of the chapters and remove all unnecessary references to commercial products.

Several requests were received to add or delete species from the sections on *Susceptible host species* and *Species with incomplete evidence for susceptibility*. The Aquatic Animals Commission referred all such requests, and supporting references, to the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases for review.

Item 18. Acute hepatopancreatic necrosis disease (new draft Chapter 2.2.X.)

Comments had been received from Canada, China (People's Rep. of), Japan, New Zealand, Singapore, Thailand and the USA.

A Member Country proposed to broaden the scope of the chapter on acute hepatopancreatic necrosis disease (AHPND) to include other *Vibrio* strains. The Aquatic Animals Commission discussed this issue in the context of the *Aquatic Code* chapter for AHPND. Although some other *Vibrio* species have been isolated in association with AHPND, at the time of this meeting there was insufficient evidence to demonstrate that these species can cause the disease.

In the Section of the chapter on 'Disease information, agent factors', a Member Country requested replacement of one of the references with another that it believes is the first report of the disease. The Aquatic Animals Commission did not agree with this proposal as the suggested reference describes the pathogenicity of a strain of *Vibrio parahaemolyticus* but not in the context of AHPND.

In response to a request for references in the sections on *Susceptible host species* and *Species with incomplete evidence for susceptibility*, the Aquatic Animals Commission pointed out that all the necessary references can be found in the report of the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases.

The revised Chapter 2.2.X. *Acute hepatopancreatic necrosis disease* is attached as **Annex 19** and is proposed for adoption at the 85th General Session in May 2017.

Item 19. Crayfish plague (*Aphanomyces astaci*) (Chapter 2.2.1.)

Comments had been received from Canada, China (People's Rep. of), New Zealand, Norway, Singapore, Switzerland and EU.

In reply to a comment requesting a further update of this chapter, the Commission reminded Member Countries that once the new disease chapter template developed by the *ad hoc* Group on the *Aquatic Manual* (see Item 26) is approved and implemented, all the chapters would be revised in the format of the new template and the relevant information will be updated accordingly.

The Aquatic Animals Commission confirmed that as per Article 9.1.2. of the *Aquatic Code* all crayfish species in the families Cambaridae, Astacidae and Parastacidae are considered to be susceptible to infection with *Aphanomyces astaci*, and the measures of the *Aquatic Code* apply to these species when they are traded internationally.

The Aquatic Animals Commission reviewed Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert.

The revised Chapter 2.2.1. *Crayfish plague* is attached as **Annex 20** and is proposed for adoption at the 85th General Session in May 2017.

Item 20. Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)

Comments had been received from New Zealand, Singapore, Thailand and the USA.

A Member Country requested deletion of a species (giant river prawn, *Macrobrachium rosenbergii*) from the section on *Susceptible host species*. The Aquatic Animals Commission noted that this issue had been referred previously to the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases and the Group had confirmed its previous assessment.

A Member Country pointed out that commercial products are referred to frequently in the protocols for molecular tests. The Aquatic Animals Commission agreed to ask the Reference Laboratory expert to remove references to commercial products unless strictly necessary and to reduce the length of the protocols by removing superfluous details.

A number of changes were proposed to the protocols for molecular tests. The OIE Reference Laboratory would be asked to review these proposals. If these changes are optimisations of published protocols, it will be necessary to add a sentence on how any effects on the performance of the assay have been assessed.

A Member Country comment regarding the definition of a confirmed case will be addressed once the new disease chapter template developed by the *ad hoc* Group on the *Aquatic Manual* (see Item 26) is approved and implemented.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory experts.

The revised Chapter 2.2.3. *Infectious hypodermal and haematopoietic necrosis* is attached as **Annex 21** and is proposed for adoption at the 85th General Session in May 2017.

Item 21. Infectious myonecrosis (Chapter 2.2.4.)

Comments had been received from Japan, New Zealand, Singapore and the USA.

A number of changes were proposed to the molecular test methods. The Aquatic Animals Commission requested that rationale be provided for such changes along with evidence of equivalency of test performance under the new test parameters.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert.

The revised Chapter 2.2.4. *Infectious myonecrosis* is attached as **Annex 22** and is proposed for adoption at the 85th General Session in May 2017.

Item 22. Necrotising hepatopancreatitis (Chapter 2.2.5.)

Comments had been received from Canada, New Zealand, Singapore and the USA.

A Member Country proposed creating a new subsection following Section 2.2.2 *Species with incomplete evidence for susceptibility*, for species with PCR positive results only. The Aquatic Animals Commission did not agree with this proposal as positive PCR results are already mentioned in Section 2.2.2. and creating a dedicated section would give them more importance than justified.

A Member Country had proposed amendments to the PCR protocol, including the addition of faeces as one of the samples on which the test can be undertaken. The Aquatic Animals Commission agreed that faeces could be included as a sample type if information on validation of the assay for this purpose is provided.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert.

The revised Chapter 2.2.5. *Necrotising hepatopancreatitis* is attached as **Annex 23** and is proposed for adoption at the 85th General Session in May 2017.

Item 23. Taura syndrome (Chapter 2.2.6.)

Comments had been received from Canada, New Zealand, Singapore, Thailand, the USA and EU.

A number of changes were proposed to the molecular test methods. The Aquatic Animals Commission requested that rationale be provided for such changes along with evidence of equivalency of test performance under the new test parameters.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert.

The revised Chapter 2.2.6. *Taura syndrome* is attached as **Annex 24** and is proposed for adoption at the 85th General Session in May 2017.

Item 24. White tail disease (Chapter 2.2.8.)

Comments had been received from Canada, China (People's Rep. of), New Zealand, Singapore, Thailand and EU.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert.

The revised Chapter 2.2.8. *White tail disease* is attached as **Annex 25** and is proposed for adoption at the 85th General Session in May 2017.

Item 25. White spot disease (Chapter 2.2.7.)

Comments had been received from China (People's Rep. of).

At its last meeting in September 2016, the Aquatic Animals Commission agreed to delay revision of this chapter pending a review of the list of susceptible species, but did propose a revised title and scope in line with the amendments being proposed in the other crustacean chapters.

The Aquatic Animals Commission reviewed the chapter and applied the new disease name, 'infection with white spot syndrome virus' throughout. The Commission decided to revise the case definition for this chapter. While the case definition will be revised by the *ad hoc* Group on the *Aquatic Manual* as a part of its ongoing work, the Commission found them to be unsatisfactory and in need of immediate revision.

The revised Chapter 2.2.7. *White spot disease* is attached as **Annex 28** for Member Country comments.

**G. OTHER OIE OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS ISSUES
AND WORK OF RELEVANT AD HOC GROUPS**

Item 26. Review of the report of the *ad hoc* Group on the *Aquatic Manual*

In January 2017, the *ad hoc* Group on the OIE *Aquatic Manual* met for a second time. The purpose of the meeting was to further refine the new disease chapter template and the three model chapters that the Group had developed at its first meeting in April 2016, in accordance with feedback provided by the Aquatic Animals Commission. The new chapter template included proposed improvements to the structure of Sections 4 *Diagnostic methods*, which incorporated a revised table of test methods available for surveillance and diagnosis, and 6 *Corroborative diagnostic criteria*.

The Aquatic Animals Commission thanked the *ad hoc* Group for its work, which would result in important improvements to the consistency, quality, clarity and value of the information presented in the *Aquatic Manual*. The Commission reviewed the *ad hoc* Group's report and its recommendations along with the application of the new template for a mollusc disease, and provided further feedback for the Group.

The Aquatic Animals Commission recommended that the Group further refine the template using the three model chapters to refine their approach. Once the template is finalised, members of the *ad hoc* Group will assist Reference Laboratory experts to apply the new template to all crustacean disease chapters. At its next meeting in September 2017, the Commission would review the finalised template and the available revision of crustacean disease chapters.

H. OIE REFERENCE CENTRES

Item 27. Review reports from relevant Reference Laboratories and Collaborating Centres

Headquarters staff provided the Aquatic Animals Commission with an analysis of the Reference Centre activities based on the annual reports submitted by OIE Reference Centres for aquatic animals. As of 27 February 2017, 33 out of 44 (75%) Reference Laboratories and 2 out of 2 (100%) Collaborating Centres had submitted annual reports for 2016 to the OIE. Those laboratories that had not yet submitted an annual report would be reminded of their obligation. Submission of annual reports is one of the criteria for evaluating the performance of OIE Reference Laboratories.

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 increase in the number of positive responses indicating internationally recognised quality management systems are in place.

Item 28. 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 Acute hepatopancreatic necrosis disease

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.

The Delegate of the Member Country concerned had submitted to the OIE the following nomination for change of expert at two OIE Reference Laboratories. The Commission recommended their acceptance:

Epizootic haematopoietic necrosis and Infection with ranavirus

Dr Paul Hick to replace Professor Richard Whittington at the University of Sydney, Faculty of Veterinary Science, Australia.

Item 29. Withdrawal of OIE Reference Laboratory status

The Aquatic Animals Commission noted that the OIE Reference Laboratory for Viral encephalopathy and retinopathy at the Graduate School of Biosphere Science, Hiroshima University (Japan), had requested to be removed from the list following the retirement of the designated expert Professor Toshihiro Nakai. The Commission wished to thank Professor Nakai for his valuable contribution to the OIE since the OIE Reference Laboratory gained its designation in 2000.

Item 30. Progress of Reference Laboratories toward achieving quality management system accreditation

The Aquatic Animals Commission considered the progress of Reference Laboratories toward achieving accreditation with ISO 17025 or an equivalent quality management system. Reference Laboratory reports submitted by 27 February 2017 and previous Reference Laboratories responses were used to evaluate progress toward achieving accreditation. The following points summarise the progress:

- 61.9% are accredited to ISO 17025;
- 19.0% are in the process of achieving accreditation to ISO 17025;
- 4.8% are accredited to an equivalent quality management system;
- 14.3% have not and would not achieve accreditation to ISO 17025 or equivalent quality management system and would therefore lose their OIE Reference Laboratory status at the end of 2017.

The Aquatic Animals Commission appreciated the effort made by so many of the laboratories to achieve their accreditation status.

The Aquatic Animals Commission was informed that the Biological Standards Commission had reviewed documents received from OIE Reference Laboratories in North America that demonstrate that their quality management system is equivalent to ISO 17025.

Item 31. Further development of standard operating procedures for the approval and maintenance of Reference Laboratory status

The Aquatic Animals Commission reviewed the standard operating procedures for designation of OIE Reference Laboratories, which had been developed by the Biological Standards Commission in consultation with the Aquatic Animals Commission. The document now has five performance criteria for laboratory evaluations:

- i) submission of an annual report;
- ii) achievement of accreditation to ISO 17025 or equivalent quality management system, ideally with relevant tests included in the scope of the accreditation;
- iii) sufficient diagnostic activity or production and supply of reference material related to the disease or pathogen;
- iv) ready response to requests from the OIE Headquarters for scientific expertise;
- v) ready response to requests from the OIE for administrative issues relating to transparency and confidentiality (e.g. renewing the potential conflict of interest declaration and providing a confidentiality undertaking).

The Aquatic Animals Commission noted that the words “with relevant tests included in the scope of the accreditation” had been added to criterion ii) and point 5 of the *Guidelines for applicants for OIE Reference Laboratory status* after the words “accreditation to the ISO 17025 or equivalent quality management system”. The Commission noted that this addition would be problematic for some aquatic laboratories when assays are being developed and validated for new diseases or in circumstances where existing assays require improvement to address new information about the disease (e.g. new strains of the pathogen). The Commission considered that it would not be appropriate to remove designation in these circumstances. To address this concern, the Aquatic Animals Commission proposed that the word ‘ideally’ be added to the beginning of the clause. This proposed amendment was conveyed to the President of the Biological Standards Commission who agreed to make this amendment.

The document will be submitted for endorsement by the OIE Council before being presented for adoption by Resolution of the World Assembly at the General Session in May 2017.

Item 32. Twinning Projects

The Aquatic Animals Commission was updated on the status of aquatic animal disease twinning projects.

As of February 2017, two projects have been completed (Canada with Chile for infectious salmon anaemia; USA with China for infectious haematopoietic necrosis) and 5 projects are currently underway (Norway with Brazil for infectious salmon anaemia; Japan with Indonesia for Koi Herpesvirus; USA with Indonesia and Saudi Arabia for Shrimp disease; Denmark with Republic of Korea for viral haemorrhagic septicaemia). The Commission noted the outputs of the recently completed project between USA and China for infectious haematopoietic necrosis.

The Aquatic Animals Commission noted that a new proposal between the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy (Parent Laboratory) and Institut de la Recherche Vétérinaire de Tunisie, Tunisia (Candidate Laboratory) for viral encephalopathy and retinopathy had been received. The Commission reviewed the proposal and provided technical comments.

I. OTHER ISSUES

Item 33. Update on OIE activities on antimicrobial resistance

The Aquatic Animals Commission was updated on OIE activities concerning antimicrobial resistance, including the current work of the OIE *ad hoc* Group on Antimicrobial resistance. The Commission agreed that it is important to continue to follow this important topic, and update the *Aquatic Code* and *Manual* as relevant.

Item 34. Self-Declaration of disease freedom

The Aquatic Animals Commission were informed that the OIE has commenced a review of the OIE procedures related to self-declaration of disease freedom for a country, zone or compartment for OIE listed terrestrial and aquatic animal diseases. Although the OIE does not endorse a self-declaration of freedom claim, a Member Country may publish information in the OIE *Bulletin* in order to inform other OIE Member Countries. The OIE is undertaking this work in an effort to improve the procedures for submission, screening and publication of self-declarations.

The Aquatic Animals Commission commented that this is an important topic for Member Countries because an official status recognition process does not exist for OIE listed aquatic animal diseases. The Commission will continue to follow this important project and has requested that it be kept informed about work on this project.

Item 35. Nagoya protocol

The Aquatic Animals Commission was updated by OIE Headquarters on the Nagoya Protocol, which was passed in October 2010 by the United Nations Convention on Biological Diversity, and mandates the terms of Access-and-Benefit-Sharing agreements between countries for any exchange of research samples containing genetic material. The Commission was advised that both the OIE Biological Standards Commission and OIE Working Group on Wildlife had discussed this item at their respective meetings in 2016 with the aim of determining what experience respective members had regarding this protocol and the possible consequences on the international transport of animal specimens.

The Aquatic Animals Commission was informed that an OIE *ad hoc* Group on Transport of biological materials will be convened in 2017 to update Chapter 1.1.3. *Transport of specimens of animal origin* of the *Manual of Diagnostic Tests and Vaccines of Terrestrial Animals*, including consideration of the Nagoya Protocol.

J. WORK PLAN OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION FOR 2017/2018

The Aquatic Animals Commission reviewed and updated its work programme, taking into account Member Country comments, Headquarters' comments, and completed work.

The revised work programme is attached as [Annex 31](#) for Member Country information.

K. ACTIVITIES OF THE MEMBERS OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

The Aquatic Animals Commission wished to inform Member Countries of activities that Commission Members have undertaken in their role as Commission Members since the last meeting in September 2016.

Members of the Commission have participated in the following activities:

Dr Ingo Ernst held a teleconference on 8 December 2016 for OIE Delegates and Aquatic Animal Focal Points in the Asia Pacific region, organised by the OIE Regional Representative for Asia and the Pacific. The purpose of the teleconference was to brief Member Countries on the report of the September 2016 meeting of the Aquatic Animals Commission, particularly annexes that had been provided for Member Country comment.

Dr Joanne Constantine participated in a webinar on 20 December 2016 for OIE Delegates, Aquatic Animal Focal Points and other regional participants in the Americas, organised by the OIE Regional Representative for the Americas. The purpose of the webinar meeting was to brief participants on the report of the September 2016 meeting of the Aquatic Animals Commission, particularly the annexes that had been provided for Member Country comment. Dr Constantine gave a presentation summarising the highlights of the September report and responded to questions from Members. The report of the September 2016 meeting of the Terrestrial Animal Health Standards Commission was also discussed.

Dr Peeler accepted an invitation extended by the European Commission to attend an expert working group meeting held on November 24 2016 to coordinate an EU response to the report of the September 2016 meeting of the Aquatic Animals Commission. Dr Peeler answered questions that arose and discussed the future work plan of the Commission.

Professor Mohamed Shariff Bin Mohamed Din represented the OIE at the 15th meeting of the Network of Aquaculture Centres in Asia-Pacific's (NACA) Asia Regional Advisory Group on Aquatic Animal Health which was held in Bangkok from 21–23 November 2016. He provided presentations to the Advisory Group on OIE aquatic animal health standards adopted at the 84th General Session in May 2016 and outcomes of the September 2016 meeting of the Aquatic Animals Commission. The reports of Advisory Group meetings are available on the NACA website (www.enaca.org).

L. COLLABORATION

Item 36. FAO update

Dr Melba Reantaso, representing the Food and Agricultural Organization, joined the Aquatic Animals Commission meeting by teleconference and provided an update on relevant FAO Technical Cooperation Programmes underway, in particular those focused on tilapia lake virus in Egypt and Asia, and an FAO Project strengthening capacities, policies and national action plans on prudent and responsible use of antimicrobials in fisheries to be held in India in April 2017. Dr Ernst provided an update on relevant activities of the Commission.

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

M. NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 12–19 September 2017 inclusive.

/Annexes

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

Paris, 22 February–1 March 2017

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**MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION
Paris, 22 February–1 March 2017**

Adopted agenda

- A. MEETING WITH THE DEPUTY DIRECTOR GENERAL**
- B. ADOPTION OF THE AGENDA**
- C. MEETING WITH THE PRESIDENT OF THE OIE TERRESTRIAL ANIMAL HEALTH STANDARDS COMMISSION**
- D. OIE *AQUATIC ANIMAL HEALTH CODE* – EXAMINATION OF MEMBER COUNTRY COMMENTS**
 - Item 1. General comments
 - Item 2. Glossary
 - Item 3. Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)
 - Item 4. Diseases listed by the OIE (Chapter 1.3.)
 - 4.1. Amended crustacean disease names
 - 4.2. *Batrachochytrium salamandrivorans*
 - 4.3. Infection with Ranavirus
 - 4.4. Assessment for a novel orthomyxo-like virus, tilapia lake virus
 - Item 5. Criteria for listing species as susceptible (Chapter 1.5.)
 - Item 6. Disinfection of aquaculture establishments and equipment (Chapter 4.3.)
 - Item 7. Recommendations for surface disinfection of salmonid eggs (Chapter 4.4.)
 - Item 8. General obligations related to certification (Chapter 5.1.)
 - Item 9. Amendments to crustacean disease-specific chapters
 - 9.1. Crayfish plague (*Aphanomyces astaci*) (Chapter 9.1.)
 - 9.2. Infection with yellow head virus genotype 1 (Chapter 9.2.)
 - 9.3. Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)
 - 9.4. Infectious myonecrosis (Chapter 9.4.)
 - 9.5. Necrotising hepatopancreatitis (Chapter 9.5.)
 - 9.6. Taura syndrome (Chapter 9.6.)
 - 9.7. White spot disease (Chapter 9.7.)
 - 9.8. White tail disease (Chapter 9.8.)
 - Item 10. New chapter on Acute hepatopancreatic necrosis disease (AHPND) (Chapter 9.X.)

Annex 2 (contd)

- Item 11. Revised Article X.X.8.
 - Item 12. Inclusion of recommendations for surface disinfection of eggs for species where it is practised other than salmonids, and important for ensuring safe trade
 - Item 13. Red sea bream iridoviral disease (Chapter 10.8.)
- E. OTHER OIE AQUATIC ANIMAL HEALTH CODE ISSUES AND WORK OF RELEVANT AD HOC GROUPS**
- Item 14. Criteria to assess the safety of aquatic animal commodities (Chapter 5.4.)
 - Item 15. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases
 - Item 16. *Ad hoc* Group on Demonstration of disease freedom
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- F. OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS – EXAMINATION OF MEMBER COUNTRY COMMENTS**
- Item 18. Acute hepatopancreatic necrosis disease (new draft Chapter 2.2.X.)
 - Item 19. Crayfish plague (*Aphanomyces astaci*) (Chapter 2.2.1.)
 - Item 20. Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)
 - Item 21. Infectious myonecrosis (Chapter 2.2.4.)
 - Item 22. Necrotising hepatopancreatitis (Chapter 2.2.5.)
 - Item 23. Taura syndrome (Chapter 2.2.6.)
 - Item 24. White tail disease (Chapter 2.2.8.)
 - Item 25. White spot disease (Chapter 2.2.7.)
- G. OTHER OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS ISSUES AND WORK OF RELEVANT AD HOC GROUPS**
- Item 26. Review of the report of the *ad hoc* Group on the *Aquatic Manual*
- H. OIE REFERENCE CENTRES**
- Item 27. Review reports from relevant Reference Laboratories and Collaborating Centres
 - Item 28. Applications for OIE Reference Centre status or changes of experts
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I. OTHER ISSUES

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J. WORK PLAN OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION FOR 2017/2018**K. ACTIVITIES OF THE MEMBERS OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION****L. COLLABORATION**

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M. NEXT MEETING

GLOSSARY

AQUATIC ANIMALS

means all viable 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.~~

BIOLOGICAL PRODUCTS

means:

- a) biological reagents for use in the *diagnosis* of certain *diseases*;
- b) sera for use in the prevention and treatment of certain *diseases*;
- c) inactivated or modified vaccines for use in preventive vaccination against certain *diseases*;
- d) genetic material of **infectious pathogenic** agents;
- e) endocrine tissues from fish or used in fish.

DISEASE

means clinical or non-clinical *infection* with one or more **aetiological pathogenic** agents.

ZONE

means **a clearly defined part of an area in one or more countries that consists of a contiguous hydrological system containing an aquatic animal population with a distinct specific aquatic animal health status with respect to a specific disease(s) for disease, in which required surveillance and control measures are applied and basic biosecurity conditions are applied met. Such zones must be clearly documented. The zone should be defined by the Competent Authority(ies).**

means a portion of one or more countries comprising:

- a) ~~an entire water catchment from the source of a waterway to the estuary or lake, or~~
- b) ~~more than one water catchment, or~~
- c) ~~part of a water catchment from the source of a waterway to a barrier that prevents the introduction of a specific disease or diseases, or~~
- d) ~~part of a coastal area with a precise geographical delimitation, or~~
- e) ~~an estuary with a precise geographical delimitation,~~

~~that consists of a contiguous hydrological system with a distinct health status with respect to a specific disease or diseases. The zones must be clearly documented (e.g. by a map or other precise locators such as GPS co-ordinates) by the Competent Authority(ies).~~

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CHAPTER 1.2.

CRITERIA FOR THE INCLUSION OF DISEASES IN THE OIE LIST

Article 1.2.1.

Introduction

This chapter describes the criteria for the inclusion of *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*.

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* are detailed in Chapter 1.1.

Principles and methods of validation of diagnostic tests are 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 may demonstrate country or zone freedom from the *disease* in susceptible *aquatic animals*, based on provisions of Chapter 1.4.

AND

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

AND

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 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 or mortality at a population level, reduced productivity or ecological impacts.

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 validation selection of diagnostic tests are provided described described in Chapter 1.1.2. of the Aquatic Manual.

Article 1.2.2.

The cCriteria 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		
4-QR b.	The disease has been shown to 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. affect cause a significant production losses at a national or multinational (zonal or regional) level	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-QR c.Or	The disease has been shown to, or scientific evidence indicates that it is likely to would, 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. affect cause a significant impact on the health of morbidity or mortality in wild aquatic animals	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 4B (contd)

No.		Criteria for listing	Explanatory notes
And-B. Spread			
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 international 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-B. Spread			
7.2.	And	At least one Several countries 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-C. Diagnosis			
8.3.		A repeatable and robust A precise case definition is available and a rReliable means of detection/ and diagnosis exists exist 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.

 — Text deleted.

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

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 *Hepatobacter penaei* (Necrotising necrotising hepatopancreatitis)
- Infection with ~~Infectious~~ *infectious* hypodermal and haematopoietic necrosis virus
- Infection with ~~Infectious~~ *infectious* myonecrosis virus
- Infection with *Macrobrachium rosenbergii* nodavirus (White white tail disease).
- Infection with Taura syndrome virus
- Infection with ~~White white~~ spot syndrome virus disease
- Infection with yellow head virus genotype 1

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* ~~species spp.~~ *Ranavirus*

– Text deleted.

CHAPTER 4.3.

DISINFECTATION OF AQUACULTURE ESTABLISHMENTS AND EQUIPMENT

Article 4.3.1.

Purpose

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

Article 4.3.2.

Scope

This chapter describes recommendations for *disinfection of aquaculture establishments* and equipment 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 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.

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

Article 4.3.4.

General principles

Disinfection is a structured process that uses physical and chemical procedures to remove organic material and destroy or inactivate *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. 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.

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

a) Cleaning and washing

Cleaning and washing of surfaces and equipment is necessary to remove solid waste, organic matter (including biofouling) and chemical residues as these may reduce the efficacy of disinfectants. is an important part of the disinfection process and should always precede the application of disinfectants. The use of detergent is also important to break down biofilms. It is necessary to remove solid waste, organic matter (including biofouling) and chemical residues as these may reduce the efficacy of disinfectants. The detergent used should be compatible with the *disinfectant* and the surface being treated. After cleaning procedures, any excess water should be drained and before the application of *disinfectants* all surfaces and equipment should be inspected to ensure there is no remaining organic material.

Annex 6 (contd)

Where treatment of water is required, the presence of suspended solids may also reduce the 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*.

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.

b) Application of disinfectants

This phase involves the application of chemical compounds or physical processes that are appropriate to inactivate the *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 (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, **therefore** ~~so~~ proper maintenance of **surfaces and 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 **require** 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 people, *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 *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 transmission pathways, the type of material to be disinfected, the *pathogenic agents* to be inactivated, the health and safety precautions and control measures required, 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. The *disinfection* plan should be regularly reviewed to ensure the *disinfection* process remains effective and efficient. 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 likelihood 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.

An inventory of all items requiring *disinfection* should be developed when practical. An assessment should be made of the materials used in construction, their surface porosity and resistance to chemical damage, and accessibility for *disinfection*. 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 an 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 transmission pathways rather than relying on *disinfection*. Equipment should not be moved from an infected *aquaculture establishment* unless effective *disinfection* has been achieved. In some circumstances, equipment or material 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.

Article 4.3.7.

Types of disinfectants

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

1. Oxidising agents

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.

Annex 6 (contd)

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. pH modifiers (alkalis and acids)

Modification of pH can be achieved through the use of either alkaline or acidic compounds. Advantages of using pH modifiers include the ease of determining their concentrations and that they are not inactivated by organic matter. Also, they can be used in areas where the application of other effective *disinfectants* is not possible, such as in pipes or on biofilter surfaces. pH modifiers consist of either alkalis or acid compounds used to modify ambient pH. They have the advantage Advantages of using pH modifiers include that the concentration is easily measured and they are not inactivated by organic matter, and therefore pH modifiers can be used in areas where an effective cleaning phase is not possible such as in pipes and biofilters.

3. Aldehydes

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. Glutaraldehyde is used in the liquid form as a cold sterilant, particularly for heat-sensitive equipment. Formaldehyde may be used as a mist or a gas for fumigation.

4. Biguanides

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 skin surfaces 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

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:

- direct sunlight and 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;
- 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:

- 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 environment; 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 All 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*. Scraping, ploughing or tilling tilling of the base of unlined ponds will also aid in incorporation of liming compounds and drying.

Annex 6 (contd)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. 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 ~~Before~~ application of *disinfectants* any excess cleaning water should be drained and disinfected or disposed of in a biosecure manner.

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 ~~For cleaning to be effective,~~ *disinfection* in pipes requires the removal of biofilms must be removed 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

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. Nets should be dedicated to a single *aquaculture establishment* or area because they have a high likelihood of contamination and may be difficult to disinfect.

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. Water and organic matter should be disposed of in a biosecure manner.

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. Treatment may have a detrimental impact upon the strength of nets. This must be considered when deciding upon the treatment method to be applied to ensure net integrity is not compromised.~~ 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*.

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 likelihood of *vehicle* contamination 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 areas likely to be contaminated such as the internal surface of *containers*, pipes, transportation water and waste. The application of corrosive *disinfectants* to *vehicles* 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*.

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 highly likely to be contaminated. 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 areas likely to be contaminated such as in and around machinery, holding tanks, bilges and pipes. All loose equipment should be removed, and cleaned and disinfected separately from prior to *disinfection* of the boat. Additional procedures should be developed for well-boats because of their potential to transfer *pathogenic agents* through the discharge of contaminated water. Contaminated effluent water should be disinfected prior to discharge (refer to Article 4.3.11.).

Where possible, boats should be placed on land or dry-docked for *disinfection* in order to limit waste water entering the aquatic environment and to allow access to hull and niche areas. Biofouling organisms, that may act as vectors, and fomites should be removed.

Where boats cannot be removed to land or dry-docked, a *disinfection* method should be chosen that minimises the discharge of toxic chemicals into the aquatic environment. Divers should 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.

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. e.g. plastic, stainless 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 and disposed of in a biosecure manner. 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 highly likely to be contaminated. Organic material should be regularly removed from decks and work areas.~~

Annex 6 (contd)

~~As part of the *disinfection* planning process, an assessment should be made to identify areas likely to be contaminated 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. Contaminated effluent water should be disinfected prior to discharge (refer to Article 4.3.11.).~~

~~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 vectors and fomites 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.~~

9.8. 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 may 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.

When disinfecting biofilters and their substrates, the system should be drained, organic residues removed and surfaces cleaned. *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 *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 biofilter 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.

40.9. Husbandry **and harvesting** equipment

Aquaculture establishments will normally have a range of husbandry **and harvesting** 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 **and harvesting** 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 likelihood and degree level of 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 not inactivated by organic matter and be regularly refreshed with a new solution.

~~Highly contaminated **Contaminated** Some types of personal equipment such as dive equipment **may require** **requires** special attention **because they are difficult to disinfect, may be moved from site to site and are is** often 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 *pathogenic agents* are minimised.~~

Article 4.3.11.

Disinfection of water

Aquaculture establishments may need to disinfect intake and effluent water to eliminate *pathogenic agents*. 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~~ ~~transmittance, reducing the 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 *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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CHAPTER 4.4.

RECOMMENDATIONS FOR
SURFACE DISINFECTION OF SALMONID EGGS

Article 4.4.1.

Introduction

The practice of disinfecting salmonid *eggs* at hatcheries is an essential part of ensuring that ~~endemic diseases~~ *pathogenic agents* 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 maximum of 1:4; then
- 3) rinsed again ~~in pathogen-free~~ 0.9% to 1.1% ~~pathogen free~~ saline for 30–60 seconds; then
- 4) ~~held~~ hold 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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CHAPTER 5.1.
GENERAL OBLIGATIONS RELATED TO
CERTIFICATION

[...]

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* 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 should be informed 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*.
- 34) In case of suspicion, on reasonable grounds, that an *international aquatic animal health certificate* may be fraudulent, the *Competent Authorities* of the *importing country* and *exporting country* should conduct an investigation. Consideration should also be given to notifying any third country 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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CHAPTER 9.1.

INFECTION WITH APHANOMYCES ASTACI
(CRAYFISH PLAGUE)

Article 9.1.1.

For the purposes of the *Aquatic Code*, infection with *Aphanomyces astaci* crayfish plague means infection with the pathogenic agent *Aphanomyces astaci* Schikora. This organism is a member of a group commonly known as of the Family Leptolegniaceae, Phylum 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 is provided in the *Aquatic Manual*.

Article 9.1.2.

Scope

The recommendations in this chapter apply to 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 or 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* derived from of a 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 that has been demonstrated to inactivate *A. astaci*);
 - 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 that 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 that 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.

Annex 9 (contd)

- 2) When authorising the importation or transit of aquatic animals and or aquatic animal products derived from 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 or aquatic animal products derived from of a species not referred to covered in Article 9.1.2. but which could reasonably be expected to pose a *risk of transmission spread* of *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 analysis 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~~ infection with *A. astaci* 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 ten years;

OR

- 3) the ~~disease~~ infection with *A. astaci* 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 *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 *A. astaci*~~, the affected area was declared an *infected zone* and a *protection zone* was established; and
 - b) ~~infected populations have been destroyed or removed from within the infected zone~~ have been killed and disposed of by means that minimise the risk likelihood of further transmission spread of infection with *A. astaci* the disease, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

Annex 9 (contd)

- c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with *A. astaci* ~~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 infection with *A. astaci* ~~crayfish plague~~.

In the meantime, part or all of the unaffected ~~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 infection with *A. astaci* ~~crayfish plague~~

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from infection with *A. astaci* ~~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 infection with *A. astaci* ~~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 infection with *A. astaci* ~~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~~ infection with *A. astaci* 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 *A. astaci* ~~crayfish plague~~;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with *A. astaci* ~~crayfish plague~~ and subsequently lost its ~~disease~~ free status due to the detection of *A. astaci* ~~crayfish plague~~ in the *zone* but the following conditions have been met:
 - a) on detection of *A. astaci* ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the *risk likelihood* of further transmission spread of *A. astaci* ~~the disease~~, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

Annex 9 (contd)

- c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with *A. astaci* 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 *A. astaci* crayfish plague.

Article 9.1.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with *A. astaci* 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 infection with *A. astaci* crayfish plague provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with *A. astaci* 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 free status as free from crayfish plague provided that conditions that are conducive to clinical expression of infection with *A. astaci* crayfish plague, 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 *A. astaci* crayfish plague, *targeted surveillance* should ~~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 or~~ aquatic animal products from a country, zone or compartment declared free from infection with *A. astaci* crayfish plague

When importing *aquatic animals* of a species referred to in Article 9.1.2. ~~and~~ or aquatic animal products derived thereof, from a country, *zone* or *compartment* declared free from infection with *A. astaci* 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*. The international aquatic animal health certificate should state 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 or~~ *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with *A. astaci* crayfish plague.

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

This Article does not apply to commodities aquatic animal products listed in point 1 of Article 9.1.3.

Article 9.1.8.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with *A. astaci*

4) When importing for aquaculture *live aquatic animals* of a species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from infection with *A. astaci* crayfish plague, 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) 1 and 3) 2~~ below.

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

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; 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 *A. astaci* in accordance with Chapters 4.3, 4.7 and 5.5.

OR

2) 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.

2) 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 infection with *A. astaci*.

b) In the importing country:

- i) import the F-0 population into a *quarantine* facility;
 - ii) test the F-0 population for *A. astaci* in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in *quarantine*;
 - iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with *A. astaci* (as described in Chapter 2.2.1. of the *Aquatic Manual*) and test for *A. astaci* in accordance with Chapter 1.4.;
 - v) if *A. astaci* is not detected in the F-1 population, it may be defined as free from infection with *A. astaci* and may be released from *quarantine*;
 - vi) if *A. astaci* is detected in the F-1 population, those animals should not be released from *quarantine* and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- 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 free from infection with *A. astaci* 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*.

Annex 9 (contd)

- 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 listed in point 1 of Article 9.1.3.~~

Article 9.1.9.

Importation of aquatic animals ~~and~~ or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with *A. astaci* crayfish plague

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.1.2. ~~and~~ or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with *A. astaci* 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 processed 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) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of *A. astaci* or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species;
- 3) all processing effluent and waste materials are treated to ensure inactivation of *A. astaci* or disposed of in a biosecure manner in accordance with Chapters 4.3 and 4.7.

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

Article 9.1.10.

Importation of live aquatic animals or aquatic animal products intended for use uses other than human consumption including in animal feed, or for agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with *A. astaci* crayfish plague

When importing, for use in animal *feed* or for agricultural, industrial, research or pharmaceutical use, ~~live~~ *aquatic animals* of a species referred to in Article 9.1.2. ~~and~~ or aquatic animal products derived thereof, ~~of~~ from a country, zone or compartment not declared free from infection with *A. astaci* crayfish plague, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to and held in quarantine or containment facilities for slaughter and until processed processing into one of the products referred to in point 1 of Article 9.1.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that to ensure ensures inactivation of *A. astaci* or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and
- 3) all processing effluent and waste materials are treated to ensure inactivation of *A. astaci* or disposed of in a biosecure manner in accordance with Chapters 4.3 and 4.7.

~~This Article does not apply to commodities referred to in point 1 of Article 9.1.3.~~

Article 9.1.11.

Importation (or transit) of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from regardless of the infection with *A. astaci* crayfish plague status of the exporting country, zone or compartment

- 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 commodities aquatic animal products which ~~that~~ have been prepared and packaged for retail trade and ~~which~~ comply with Article 5.4.2.:
 - no commodities aquatic animal products listed.
- 2) When importing aquatic animals or aquatic animal products, other than those referred to in point 1 above, of derived from a of species referred to in Article 9.1.2. from a *country, zone or compartment* not declared free from infection with *A. astaci* crayfish plague, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

CHAPTER 9.2.

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

Article 9.2.1.

For the purposes of the *Aquatic Code*, infection with yellow head virus genotype 1 means infection with the pathogenic agent yellow head virus genotype 1 (YHV1), of the Genus *Okavirus*, Family *Roniviridae*, Order *Nidovirales*, Family *Roniviridae*, Genus *Okavirus* in the Family *Roniviridae* and the Order *Nidovirales*.

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

Article 9.2.2.

Scope

The recommendations in this chapter apply to the following susceptible species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: Jinga shrimp (*Metapenaeus affinis*), giant tiger prawn (*Penaeus monodon*), dagger blade grass shrimp (*Palaemonetes pugio*), blue shrimp (*Penaeus stylirostris*) and whiteleg shrimp (*Penaeus vannamei*), 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*).

Article 9.2.3.

Importation or transit of aquatic animals and or aquatic animal products for any purpose regardless of the infection with YHV1 status of the exporting country, zone or compartment

- 1) *Competent Authorities* should not require any conditions related to ~~infection with~~ YHV1, regardless of the infection with YHV1 status of the *exporting country, zone or compartment*, when authorising the importation or transit of the following *aquatic animal products* derived from of a 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 any time/temperature equivalent that has been demonstrated to inactivate YHV1);
 - 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 that has been demonstrated to inactivate YHV1);
 - 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 that has been demonstrated to inactivate YHV1);
 - d) crustacean oil;
 - e) crustacean *meal*;
 - f) chemically extracted chitin.
- 2) When authorising the importation or transit of aquatic animals and or *aquatic animal products* derived from 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 YHV1 status of the *exporting country, zone or compartment*.

Annex 10 (contd)

- 3) When considering the importation or transit of aquatic animals and/or aquatic animal products derived from ~~of~~ a species not referred to covered in Article 9.2.2. but which could reasonably be expected to pose a *risk* of transmission of infection with 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 analysis assessment.

Article 9.2.4.

Country free from infection with yellow head virus genotype 1

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

OR

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

- a) on detection of YHV1 ~~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~~ within the infected zone have been killed and disposed of by means that minimise the likelihood of further transmission spread of YHV1 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 infection with YHV1 ~~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~~ YHV1.

Annex 10 (contd)

In the meantime, part or all of the ~~unaffected 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 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 YHV1 may be declared free by the *Competent Authority* of the country 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 infection with YHV1 ~~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~~ infection with YHV1 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~~ YHV1;

OR

- 4) it previously made a *self-declaration of freedom for a zone* from infection with YHV1 ~~for a zone~~ and subsequently lost its ~~disease free~~ status due to the detection of ~~infection with~~ YHV1 in the *zone* but the following conditions have been met:
 - a) on detection of YHV1 ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the likelihood of further transmission spread of YHV1 ~~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 infection with YHV1 ~~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~~ YHV1.

Annex 10 (contd)

Article 9.2.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with 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 YHV1 provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with 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 free status ~~as free from infection with YHV1~~ provided that conditions that are conducive to clinical expression of infection with 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 YHV1, *targeted surveillance* should ~~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 or~~ aquatic animal products from a country, zone or compartment declared free from infection with yellow head virus genotype 1

When importing *aquatic animals* of a species referred to in Article 9.2.2. ~~and~~, or aquatic animal products derived thereof, from a country, *zone* or *compartment* declared free from infection with 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 *exporting country* or a *certifying official* approved by the *importing country*. The international aquatic animal health certificate should state ~~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 or~~ *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with YHV1.

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

This Article does not apply to commodities aquatic animal products listed in point 1 of Article 9.2.3.

Article 9.2.8.

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

4) When importing, for *aquaculture*, ~~live~~ *aquatic animals* of a species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with YHV1, 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)1 and 3) 2 below.

1)2 If the intention is to grow out and harvest the imported aquatic animals, consider applying the following:

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; ~~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 YHV1 in accordance with Chapters 4.3, 4.7 and 5.5.

OR

2) ~~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.~~

2) 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 infection with YHV1.
 - b) In the importing country:
 - i) import the F-0 population into a *quarantine* facility;
 - ii) test the F-0 population for YHV1 in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in *quarantine*;
 - iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with YHV1 (as described in Chapter 2.2.2. of the *Aquatic Manual*) and test for YHV1 in accordance with Chapter 1.4.;
 - v) if YHV1 is not detected in the F-1 population, it may be defined as free from infection with YHV1 and may be released from *quarantine*;
 - vi) if YHV1 is detected in the F-1 population, those animals should not be released from *quarantine* and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
 - 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 YHV1 and perform general examinations for pests and general health/disease status;
 - g) if 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 YHV1 or specific pathogen free (SPF) for infection with 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.

Annex 10 (contd)

Article 9.2.9.

Importation of aquatic animals ~~and~~ or 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* of a species referred to in Article 9.2.2. ~~and~~ or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with 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 processed 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) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that ensures~~ to ensure inactivation of YHV1 ~~or is disposed of~~ in a biosecure manner in accordance with Chapters 4.3, 4.7, and 5.5; and that prevents contact of waste with susceptible species
- 3) all processing effluent and waste materials are treated to ensure inactivation of YHV1 or disposed of in a biosecure manner in accordance with Chapters 4.3 and 4.7.

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

Article 9.2.10.

Importation of live aquatic animals or aquatic animal products intended for ~~use~~ uses other than human consumption including in animal feed, or for agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with yellow head virus genotype 1

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

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities ~~for slaughter and until processed~~ processing into one of the products referred to in point 1 of Article 9.2.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that~~ to ensure ~~ensures~~ inactivation of YHV1 ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3, 4.7, and 5.5.; and~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of YHV1 or disposed of in a biosecure manner in accordance with Chapters 4.3 and 4.7.

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

Article 9.2.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption ~~from a country, zone or compartment not declared free from~~ regardless of the infection with yellow head virus genotype 1 status of the exporting country, zone or compartment

- 1) *Competent Authorities* should not require any conditions related to ~~infection with~~ YHV1, regardless of the infection with 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 that have been prepared and packaged for retail trade and which comply with Article 5.4.2.

Annex 10 (contd)

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

— Text deleted.

CHAPTER 9.3.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

Article 9.3.1.

For the purposes of the *Aquatic Code*, infection with infectious hypodermal and haematopoietic necrosis virus (IHHN) means infection with the pathogenic agent infectious hypodermal and haematopoietic necrosis virus (IHHNV). IHHNV is classified as the species *Penaeus stylirostris densovirus* in of the of the Family Parvoviridae. Genus genus Brevidensovirus and Family Parvoviridae in the Family family Parvoviridae.

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

Article 9.3.2.

Scope

The recommendations in this chapter apply to the following species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: giant river prawn (*Macrobrachium rosenbergii*), yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*Penaeus stylirostris*) and whiteleg shrimp (*Penaeus vannamei*), giant tiger prawn (*Penaeus monodon*), Pacific white shrimp (*Penaeus vannamei*), and blue shrimp (*P. stylirostris*). 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 or~~ aquatic animal products for any purpose regardless of the infection with IHHNV status of the exporting country, zone or compartment 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 IHHNV IHHN, regardless of the infection with IHHNV IHHN status of the *exporting country, zone or compartment*, when authorising the importation or transit of the following *aquatic animal products* derived from of a 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 that has been demonstrated to inactivate IHHNV);
 - 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 that has been demonstrated to inactivate IHHNV);
 - c) crustacean oil;
 - d) crustacean *meal*.
- 2) When authorising the importation or transit of aquatic animals and or *aquatic animal products* derived from 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 infection with IHHNV IHHN status of the *exporting country, zone or compartment*.
- 3) When considering the importation or transit of aquatic animals and or *aquatic animal products* derived from of a species not referred to covered in Article 9.3.2. but which could reasonably be expected to pose a *risk of spread* of transmission of IHHNV 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 analysis assessment.

Annex 11 (contd)

Article 9.3.4.

Country free from infection with IHHNV

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with IHHNV ~~IHHN~~ if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with IHHNV ~~IHHN~~ (see Article 9.3.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with IHHNV ~~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~~ infection with IHHNV 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~~ infection with IHHNV 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 IHHNV ~~IHHN~~;

OR

- 4) it previously made a *self-declaration of freedom* from infection with IHHNV ~~IHHN~~ and subsequently lost its ~~disease~~ free status due to the detection of IHHNV ~~IHHN~~ but the following conditions have been met:
 - a) on detection of ~~the disease~~ IHHNV, the affected area was declared an *infected zone* and a *protection zone* was established; and
 - b) ~~infected populations have been destroyed or removed from within the infected zone~~ infected populations have been killed and disposed of by means that minimise the ~~risk likelihood~~ risk likelihood of further ~~spread~~ transmission of IHHNV ~~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 infection with IHHNV ~~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 IHHNV ~~IHHN~~.

In the meantime, part or all of the unaffected 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 infection with IHHNV

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from infection with IHHNV ~~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 IHHNV ~~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 infection with IHHNV ~~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~~ infection with IHHNV-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 IHHNV ~~IHHN~~;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with IHHNV ~~IHHN~~ and subsequently lost its ~~disease~~ free status due to the detection of IHHNV ~~IHHN~~ in the *zone* but the following conditions have been met:
 - a) on detection of IHHNV ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the risk likelihood of further ~~spread~~ transmission of IHHNV ~~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 infection with IHHNV ~~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 IHHNV ~~IHHN~~.

Annex 11 (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 free status as free from 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~~ should 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~~ or aquatic animal products from a country, zone or compartment declared free from infection with IHHNV

When importing *aquatic animals* of a species referred to in Article 9.3.2. ~~and~~ or *aquatic animal products derived thereof*, 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*. ~~The *international aquatic animal health certificate* should state~~ 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~~ or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with IHHNV ~~IHHN~~.

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

This Article does not apply to commodities aquatic animal products 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 IHHNV

1) ~~4)~~ When importing, for *aquaculture*, live aquatic animals of a 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: in accordance with Chapter 2.1. and consider the risk mitigation measures in points~~ 2) 1 and 3) 2 below.

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

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; ~~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 IHHNV in accordance with Chapters 4.3., 4.7 and 5.5.

OR

~~2)~~ 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.

2) 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 infection with IHHNV.
- b) In the importing country:
 - i) import the F-0 population into a *quarantine* facility;
 - ii) test the F-0 population for IHHNV in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in *quarantine*;
 - iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with IHHNV (as described in Chapter 2.2.3. of the *Aquatic Manual*) and test for IHHNV in accordance with Chapter 1.4.;
 - v) if IHHNV is not detected in the F-1 population, it may be defined as free from infection with IHHNV and may be released from *quarantine*;
 - vi) if IHHNV is detected in the F-1 population, those animals should not be released from *quarantine* and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- a) ~~identify stock of interest (cultured or wild) in its current location;~~
- b) ~~evaluate stock health and disease history;~~
- e) ~~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 free from infection with IHHNV IHHN free 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.~~
- 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 9.3.3.

Annex 11 (contd)

Article 9.3.9.

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

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.3.2. ~~and~~ or aquatic animal products derived thereof, 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, require that:

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing processed 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) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that ensures~~ to ensure inactivation of IHHNV ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species;~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of IHHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.3.10.

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

When importing, for use in animal *feed* or for agricultural, industrial, research or pharmaceutical use, ~~live~~ *aquatic animals* of a species referred to in Article 9.3.2. ~~and~~ or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with IHHNV-IHHN, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities ~~for slaughter and until processed~~ processing into one of the products referred to in point 1 of Article 9.3.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that to ensure~~ ensures inactivation of IHHNV ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of IHHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

~~This Article does not apply to commodities referred to in point 1 of Article 9.3.3.~~

Article 9.3.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption ~~from a country, zone or compartment not declared free from~~ regardless of the infection with IHHNV status of the exporting country, zone or compartment

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

Annex 11 (contd)

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~~ *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the ~~commodity~~ *aquatic animal products* 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~~ *derived from a* species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from ~~infection with IHNV~~ *IHHN*, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

CHAPTER 9.4.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

Article 9.4.1.

For the purposes of the *Aquatic Code*, infection with infectious myonecrosis virus (IMNV) means infection with the pathogenic agent infectious myonecrosis virus (IMNV) of the This virus that is similar to members of the family Totiviridae *Totiviridae* (tentative classification).

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

Article 9.4.2.

Scope

The recommendations in this chapter apply to the following species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: brown tiger prawn (*Penaeus esculentus*), banana prawn (*Penaeus merguensis*), Pacific white shrimp and whiteleg 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 or~~ 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 IMNV, regardless of the infection with IMNV status of the *exporting country, zone or compartment*, when authorising the importation or transit of the following *aquatic animal products* derived from the a 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 that has been demonstrated to inactivate IMNV);
 - 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 that 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 or~~ *aquatic animal products* derived from ~~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 or~~ *aquatic animal products* derived from ~~of~~ a species not referred to covered in Article 9.4.2. but which could reasonably be expected to pose a *risk of spread of transmission of IMNV*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis assessment.

Annex 12 (contd)

Article 9.4.4.

Country free from infection with IMNV

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom from infection with IMNV* if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with IMNV (see Article 9.4.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom from infection with IMNV* 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 infection with IMNV ~~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~~ infection with IMNV 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 IMNV;

OR

- 4) it previously made a *self-declaration of freedom from infection with IMNV* and subsequently lost its ~~disease~~ free status due to the detection of IMNV but the following conditions have been met:
 - a) on detection of ~~the disease~~ IMNV, the affected area was declared an *infected zone* and a *protection zone* was established; and
 - b) infected populations ~~have been destroyed or removed from within the infected zone~~ have been killed and disposed of by means that minimise the ~~risk likelihood~~ of further transmission spread of IMNV, 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 infection with IMNV ~~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 IMNV.

In the meantime, part or all of the unaffected 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 infection with IMNV

If a *zone* or *compartment* extends over more than one country, it can only be declared ~~a~~ an IMN-free zone or compartment free from infection with IMNV 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 IMNV 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 infection with IMNV ~~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 infection with IMNV ~~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 IMNV;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with IMNV and subsequently lost its ~~disease~~ free status due to the detection of IMNV in the *zone* but the following conditions have been met:
 - a) on detection of IMNV ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the *risk likelihood* of further transmission spread of IMNV ~~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 infection with IMNV ~~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 IMNV

Annex 12 (contd)

Article 9.4.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with IMNV 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 infection with IMNV provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with IMNV following the provisions of point 3 of Articles 9.4.4. or 9.4.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status as free from IMNV provided that conditions that are conducive to clinical expression of infection with IMNV 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 IMNV, *targeted surveillance* ~~should~~ 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~~ or aquatic animal products from a country, zone or compartment declared free from infection with IMNV

When importing *aquatic animals* of a species referred to in Article 9.4.2. ~~and~~ or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with IMNV, 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*. The international aquatic animal health certificate should state ~~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~~ or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with IMNV.

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

This Article does not apply to commodities aquatic animal products 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 infection with IMNV

4) When importing, for *aquaculture*, ~~live~~ *aquatic animals* of a species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from infection with IMNV, 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)1 and 3) 2 below.

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

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; 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 IMNV in accordance with Chapters 4.3, 4.7. and 5.5.

OR

4) ~~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.~~

2) 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 infection with IMNV.
 - b) In the importing country:
 - i) import the F-0 population into a quarantine facility;
 - ii) test the F-0 population for IMNV in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in quarantine;
 - iv) culture F-1 population in quarantine under conditions that are conducive to the clinical expression of infection with IMNV (as described in Chapter 2.2.4. of the Aquatic Manual) and test for IMNV in accordance with Chapter 1.4.;
 - v) if IMNV is not detected in the F-1 population, it may be defined as free from infection with IMNV and may be released from quarantine;
 - vi) if IMNV is detected in the F-1 population, those animals should not be released from quarantine and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- 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 free from infection with IMNV, 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~~ or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with IMNV

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.4.2. ~~and~~ or *aquatic animal products* derived thereof, from a country, zone or compartment not declared free from infection with IMNV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

- 1) the consignment is delivered directly to 1 and held in 1 *quarantine* or containment facilities until processing processed into one of the products referred to in point 1 of Article 9.4.3. 1 or products described in point 1 of Article 9.4.11., or other products authorised by the *Competent Authority*; and

Annex 12 (contd)

- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of IMNV or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species;
- 3) all processing effluent and waste materials are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.4.10.

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

When importing, for use in animal *feed* or for agricultural, industrial, research or pharmaceutical use, live aquatic animals of a species referred to in Article 9.4.2. and, or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with IMNV, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to, and held in, quarantine or containment facilities for slaughter and until processed processing into one of the products referred to in point 1 of Article 9.4.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of IMNV or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and
- 3) all processing effluent and waste materials are treated to ensure inactivation of IMNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

~~This Article does not apply to commodities referred to in point 1 of Article 9.4.3.~~

Article 9.4.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption ~~from a country, zone or compartment not declared free from~~ regardless of the infection with IMNV status of the exporting country, zone or compartment

- 1) *Competent Authorities* should not require any conditions related to infection with IMNV, regardless of the infection with IMNV status of the *exporting country, zone or compartment*, when authorising the importation (or transit) of frozen peeled shrimp (shell off, head off) which that 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 aquatic animal products Member Countries may wish to consider introducing internal measures to address the risks associated with the commodity aquatic animal products being used for any purpose other than for human consumption.

Annex 12 (contd)

- 2) When importing ~~aquatic animals or~~ *aquatic animal products*, other than those referred to in point 1 above, ~~of~~ derived from a species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from infection with IMNV, 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)

Article 9.5.1.

For the purposes of the *Aquatic Code*, infection with *Hepatobacter penaei* necrotising hepatopancreatitis (NHP) means infection with the pathogenic agent *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 species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: Pacific white whiteleg 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 or 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 *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 derived from a 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 that has been demonstrated to inactivate *H. penaei*);
 - 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 that 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 that 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 or aquatic animal products derived from 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 infection with *H. penaei* NHP status of the exporting country, zone or compartment.

Annex 13 (contd)

- 3) When considering the importation or transit of aquatic animals and or aquatic animal products derived from of a species not referred to covered in Article 9.5.2. but which could reasonably be expected to pose a risk of spread of transmission of *H. penaei* 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 analysis assessment.

Article 9.5.4.

Country free from infection with *H. penaei* necrotising hepatopancreatitis

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with *H. penaei* NHP if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with *H. penaei* NHP (see Article 9.5.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with *H. penaei* 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~~ infection with *H. penaei* 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~~ infection with *H. penaei* 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 *H. penaei* NHP;

OR

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

- a) on detection of ~~the disease~~ *H. penaei*, the affected area was declared an *infected zone* and a *protection zone* was established; and
- b) ~~infected populations have been destroyed or removed from within the infected zone have been killed and disposed of~~ by means that minimise the ~~risk likelihood~~ of further transmission spread of *H. penaei* ~~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 infection with *H. penaei* ~~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 *H. penaei* NHP.

Annex 13 (contd)

In the meantime, part or all of the ~~unaffected 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 infection with *H. penaei* ~~necrotising hepatopancreatitis~~

If a zone or compartment extends over more than one country, it can only be declared ~~a an NHP free zone~~ or compartment free from infection with *H. penaei* 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 infection with *H. penaei* 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 infection with *H. penaei* ~~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~~ infection with *H. penaei* 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 *H. penaei* NHP;

OR

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

- a) on detection of ~~the disease~~ *H. penaei*, the affected area was declared an *infected zone* and a *protection zone* was established; and
- b) ~~infected populations have been destroyed or removed from within the infected zone~~ infected populations have been killed and disposed of by means that minimise the ~~risk~~ likelihood of further transmission spread of *H. penaei* ~~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 infection with *H. penaei* ~~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 *H. penaei* NHP.

Annex 13 (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 free status as free from 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* should ~~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 or~~ aquatic animal products from a country, zone or compartment declared free from infection with *H. penaei* necrotising hepatopancreatitis

When importing *aquatic animals* of a species referred to in Article 9.5.2. ~~and~~ or aquatic animal products derived thereof, 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*. ~~The international aquatic animal health certificate should state 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 or~~ *aquatic animal products* is a country, zone or compartment declared free from infection with *H. penaei* NHP.

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

This Article does not apply to commodities aquatic animal products 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

4) When importing, for *aquaculture*, ~~live~~ *aquatic animals* of a 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: in accordance with Chapter 2.1. and consider the risk mitigation measures in points 2)1 and 3) 2 below.~~

~~1)-2)~~ If the intention is to grow out and harvest the imported aquatic animals, consider applying the following:

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a *quarantine facility*; 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 *H. penaei* in accordance with Chapters 4.3, 4.7, and 5.5.

OR

4) 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.

2) 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 infection with *H. penaei*.
- b) In the importing country:
- i) import the F-0 population into a quarantine facility;
 - ii) test the F-0 population for *H. penaei* in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in quarantine;
 - iv) culture F-1 population in quarantine under conditions that are conducive to the clinical expression of infection with *H. penaei* (as described in Chapter 2.2.5. of the Aquatic Manual) and test for *H. penaei* in accordance with Chapter 1.4.;
 - v) if *H. penaei* is not detected in the F-1 population, it may be defined as free from infection with *H. penaei* and may be released from quarantine;
 - vi) if *H. penaei* is detected in the F-1 population, those animals should not be released from quarantine and should be destroyed killed and disposed of in a biosecure manner.
- ~~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 free from infection with *H. penaei* 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.

Annex 13 (contd)

Article 9.5.9.

Importation of aquatic animals ~~and~~ or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with *H. penaei* necrotising hepatopancreatitis

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.5.2. ~~and~~ or aquatic animal products derived thereof, 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, require that:

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing processed 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) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated in a manner that ensures to ensure inactivation of *H. penaei* ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species;~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of *H. penaei* or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.5.10.

Importation of ~~live~~ aquatic animals or aquatic animal products intended for use uses other than human consumption including in animal feed, or for agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with *H. penaei* necrotising hepatopancreatitis

When importing, for use in animal *feed* or for agricultural, industrial, research or pharmaceutical use, *live aquatic animals* of a species referred to in Article 9.5.2. ~~and~~ or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with *H. penaei* NHP, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities ~~for slaughter and until processed~~ processing into one of the products referred to in point 1 of Article 9.5.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated in a manner that to ensure ensures inactivation of *Candidatus H. penaei* ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of *H. penaei* or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

~~This Article does not apply to commodities referred to in point 1 of Article 9.5.3.~~

Article 9.5.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from regardless of the infection with *H. penaei* necrotising hepatopancreatitis status of the exporting country, zone or compartment

- 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 frozen peeled shrimp (shell off, head off) which that have been prepared and packaged for retail trade and which comply with Article 5.4.2.

Annex 13 (contd)

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 aquatic animal products Member Countries may wish to consider introducing internal measures to address the risks associated with the commodity aquatic animal products 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 derived from a 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 apply appropriate risk mitigation measures.

— Text deleted.

CHAPTER 9.6.

INFECTION WITH TAURA SYNDROME VIRUS

Article 9.6.1.

For the purposes of the *Aquatic Code*, infection with Taura syndrome virus (TS) means infection with the pathogenic agent Taura syndrome virus (TSV). ~~Taura syndrome virus is classified as a species of the of the Genus *Aparavirus*, Family *Dicistroviridae* and Order *Picornavirales*, Family *Dicistroviridae*, 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~~ is provided in the *Aquatic Manual*.

Article 9.6.2.

Scope

The recommendations in this chapter apply to the following susceptible species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*P. Penaeus aztecus*), giant tiger prawn (*P. Penaeus monodon*), northern white shrimp (*P. Penaeus setiferus*), blue shrimp (*P. Penaeus stylirostris*) and whiteleg shrimp (*Penaeus vannamei*). ~~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 (*Metapenaeus 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 or~~ 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* derived from the a 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 that has been demonstrated to inactivate TSV);
 - 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 that 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 that 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 or *aquatic animal products* derived from 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*.

Annex 14 (contd)

- 3) When considering the importation or transit of aquatic animals and of aquatic animal products derived from of a species not referred to covered in Article 9.6.2. but which could reasonably be expected to pose a *risk of spread of transmission of 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 analysis assessment.

Article 9.6.4.

Country free from infection with TSV

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~~ infection with TSV 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~~ infection with TSV 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 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 TSV TS but the following conditions have been met:

- a) on detection of TSV 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 within the infected zone~~ have been killed and disposed of by means that minimise the *risk likelihood* of further transmission spread of TSV 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 infection with TSV 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 TSV TS.

In the meantime, part or all of the unaffected 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.

Article 9.6.5.

Zone or compartment free from infection with TSV

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from infection with TSV 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 TSV 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 infection with TSV ~~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~~ infection with TSV 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 TSV;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with TSV and subsequently lost its ~~disease~~-free status due to the detection of TSV in the *zone* but the following conditions have been met:
 - a) on detection of TSV ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the *risk likelihood* of further *transmission* spread of TSV ~~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 infection with TSV ~~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 TSV.

Article 9.6.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TSV 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 provided that *basic biosecurity conditions* are continuously maintained.

Annex 14 (contd)

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 free status as free from 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* should 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 or aquatic animal products from a country, zone or compartment declared free from infection with TSV

When importing *aquatic animals* of a species referred to in Article 9.6.2. and or *aquatic animal products* derived thereof, 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*. The international aquatic animal health certificate should state 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 or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with TSV TS.

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

This Article does not apply to commodities aquatic animal products 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 TSV

4) When importing, for *aquaculture*, live aquatic animals of a 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: in accordance with Chapter 2.1. and consider the risk mitigation measures in points 2)1 and 3) 2 below.

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

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; 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 TSV in accordance with Chapters 4.3., 4.7 and 5.5.

OR

~~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.~~

2) 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 infection with TSV.

- b) In the importing country:
- i) import the F-0 population into a quarantine facility;
 - ii) test the F-0 population for TSV in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in quarantine;
 - iv) culture F-1 population in quarantine under conditions that are conducive to the clinical expression of infection with TSV (as described in Chapter 2.2.6. of the Aquatic Manual) and test for TSV in accordance with Chapter 1.4.;
 - v) if TSV is not detected in the F-1 population, it may be defined as free from infection with TSV and may be released from quarantine;
 - vi) if TSV is detected in the F-1 population, those animals should not be released from quarantine and should be destroyed killed and disposed of in a biosecure manner.
- 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 free from infection with TSV 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~~ or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with TSV

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.6.2. ~~and~~ or *aquatic animal products* derived thereof, 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, require that:

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing processed 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) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of TSV or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species
- 3) all processing effluent and waste materials are treated to ensure inactivation of IHHNV TSV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

Annex 14 (contd)

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

Article 9.6.10.

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

When importing, for use in animal feed or for agricultural, industrial, research or pharmaceutical use, live aquatic animals of a species referred to in Article 9.6.2. and, or aquatic animal products derived thereof, from a country, zone or compartment not declared free from infection with TSV TS, the Competent Authority of the importing country should require that:

- 1) the consignment is delivered directly to, and held in, quarantine or containment facilities for slaughter and until processed processing into one of the products referred to in point 1 of Article 9.6.3. or other products authorised by the Competent Authority; and
- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of TSV or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and
- 3) all processing effluent and waste materials are treated to ensure inactivation of TSV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

~~This Article does not apply to commodities referred to in point 1 of Article 9.6.3.~~

Article 9.6.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from regardless of the infection with TSV status of the exporting country, zone or compartment

- 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 frozen peeled shrimp or decapod crustacea (shell off, head off) which that 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 aquatic animal products Member Countries may wish to consider introducing internal measures to address the risks associated with the commodity aquatic animal products 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 derived from a 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 apply appropriate risk mitigation measures.

 — Text deleted.

CHAPTER 9.7.

INFECTION WITH WHITE SPOT SYNDROME VIRUS DISEASE

Article 9.7.1.

For the purposes of the *Aquatic Code*, infection with white spot syndrome virus disease (WSD) means infection with the pathogenic agent white spot syndrome virus (WSSV). ~~White spot syndrome virus 1 is classified as a species in the genus *Whispovirus* of the family~~ of the Genus *Whispovirus*, Family *Nimaviridae*, Genus *Whispovirus*. Common synonyms are listed in the corresponding chapter of the *Aquatic Manual*.

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

Article 9.7.2.

Scope

The recommendations in this chapter apply to all decapod (order *Decapoda*) crustaceans from marine, brackish and freshwater sources. 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 9.7.3.

Importation or transit of ~~aquatic animals and or~~ aquatic animal products for any purpose regardless of the infection with WSSV status of the exporting country, zone or compartment

- 1) *Competent Authorities* should not require any conditions related to WSSV ~~WSD~~, regardless of the infection with WSSV ~~WSD~~ status of the *exporting country, zone or compartment*, when authorising the importation or transit of the following *aquatic animal products* derived from ~~the a~~ species referred to in Article 9.7.2., 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 that has been demonstrated to inactivate WSSV);
 - b) cooked crustacean products that have been subjected to heat treatment at 60°C for at least one minute (or any time/temperature equivalent which that has been demonstrated to inactivate WSSV);
 - 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 that has been demonstrated to inactivate WSSV);
 - d) crustacean oil;
 - e) crustacean *meal*;
 - f) chemically extracted chitin.
- 2) When authorising the importation or transit of ~~aquatic animals and or~~ *aquatic animal products* derived from ~~of~~ a species referred to in Article 9.7.2., other than those referred to in point 1 of Article 9.7.3., *Competent Authorities* should require the conditions prescribed in Articles 9.7.7. to 9.7.11. relevant to the infection with WSSV ~~WSD~~ status of the *exporting country, zone or compartment*.

Annex 15 (contd)

- 3) When considering the importation or transit of aquatic animals and/or aquatic animal products derived from ~~of~~ a species not referred to covered in Article 9.7.2. but which could reasonably be expected to pose a *risk* of transmission spread of WSSV WSD, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis assessment.

Article 9.7.4.

Country free from infection with WSSV 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 WSSV if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with WSSV WSD (see Article 9.7.5.).

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

- 1) none of the *susceptible species* referred to in Article 9.7.2. 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 9.7.2. are present and the following conditions have been met:

- a) there has been no ~~observed~~ occurrence of infection with WSSV 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~~ infection with WSSV 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 WSSV WSD;

OR

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

- a) on detection of WSSV 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 within the infected zone~~ infected populations have been killed and disposed of by means that minimise the *risk likelihood* of further transmission spread of infection with WSSV 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 infection with WSSV 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 WSSV WSD.

Annex 15 (contd)

In the meantime, part or all of the unaffected ~~non-affected~~ area may be declared a free zone provided that such a part meets the conditions in point 3 of Article 9.7.5.

Article 9.7.5.

Zone or compartment free from infection with WSSV disease

If a *zone* or *compartment* extends over more than one country, it can only be declared a ~~WSD free zone~~ or *compartment* free from infection with WSSV 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 WSSV ~~WSD~~ 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 9.7.2. 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 9.7.2. are present in the *zone* or *compartment* and the following conditions have been met:

- a) there has not been any ~~observed~~ occurrence of infection with WSSV ~~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~~ infection with WSSV 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 WSSV ~~WSD~~;

OR

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

- a) on detection of WSSV ~~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 within the infected zone~~ infected populations have been killed and disposed of by means that minimise the ~~risk~~ likelihood of further transmission ~~spread~~ of WSSV ~~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 infection with WSSV ~~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 WSSV ~~WSD~~.

Annex 15 (contd)

Article 9.7.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with WSSV ~~WSD~~ following the provisions of points 1 or 2 of Articles 9.7.4. or 9.7.5. (as relevant) may maintain its status as free from infection with WSSV ~~WSD~~ provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with WSSV ~~WSD~~ following the provisions of point 3 of Articles 9.7.4. or 9.7.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status ~~as free from WSD~~ provided that conditions that are conducive to clinical expression of infection with WSSV ~~WSD~~, 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 WSSV ~~WSD~~, *targeted surveillance* ~~should~~ needs to be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Article 9.7.7.

Importation of aquatic animals ~~and or~~ aquatic animal products from a country, zone or compartment declared free from infection with WSSV ~~disease~~

When importing *aquatic animals* of a species referred to in Article 9.7.2. ~~and~~ or *aquatic animal products* derived thereof, ~~of~~ from a country, *zone* or *compartment* declared free from infection with WSSV ~~WSD~~, 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*. ~~The international aquatic animal health certificate should state~~ certifying that, on the basis of the procedures described in Articles 9.7.4. or 9.7.5. (as applicable) and 9.7.6., the place of production of the *aquatic animals* ~~and or~~ *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with WSSV ~~WSD~~.

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

This Article does not apply to ~~commodities~~ aquatic animal products listed in point 1 of Article 9.7.3.

Article 9.7.8.

Importation of ~~live~~ aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with WSSV ~~disease~~

~~4)~~ 4) When importing, for *aquaculture*, ~~live~~ *aquatic animals* of a species referred to in Article 9.7.2. from a country, *zone* or *compartment* not declared free from infection with WSSV ~~WSD~~, 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)1 and 3) 2 below.

~~1)-2)~~ 1)-2) If the intention is to grow out and harvest the imported aquatic animals, consider applying the following:

- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; ~~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 WSSV in accordance with Chapters 4.3, 4.7 and 5.5.

OR

~~4)~~ 4) ~~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.~~

Annex 15 (contd)

~~2) 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 infection with WSSV.
 - b) In the importing country:
 - i) import the F-0 population into a quarantine facility;
 - ii) test the F-0 population for WSSV in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in quarantine;
 - iv) culture F-1 population in quarantine under conditions that are conducive to the clinical expression of infection with WSSV (as described in Chapter 2.2.7. of the Aquatic Manual) and test for WSSV in accordance with Chapter 1.4.;
 - v) if WSSV is not detected in the F-1 population, it may be defined as free from infection with WSSV and may be released from quarantine;
 - vi) if WSSV is detected in the F-1 population, those animals should not be released from quarantine and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
 - 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 WSSV, 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 WSSV and perform general examinations for pests and general health/disease status;~~
 - g) ~~if WSSV 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 WSD free or specific pathogen free (SPF) for WSSV;~~
 - 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 9.7.3.

Annex 15 (contd)

Article 9.7.9.

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

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.7.2. ~~and or~~ *aquatic animal products* derived thereof, from a country, zone or compartment not declared free from infection with WSSV WSD, 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 processed into one of the products referred to in point 1 of Article 9.7.3. ~~or products described~~ in point 1 of Article 9.7.11., or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated in a manner that ensures to ensure inactivation of WSSV ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species.~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of WSSV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.7.10.

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

When importing, for use in animal *feed* or for agricultural, industrial, research or pharmaceutical use, *live aquatic animals* of a species referred to in Article 9.7.2. ~~and or~~ *aquatic animal products* derived thereof, from a country, zone or compartment not declared free from infection with WSSV WSD, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities ~~for slaughter and until processed processing~~ into one of the products referred to in point 1 of Article 9.7.3. or other products authorised by the *Competent Authority*; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated in a manner that to ensure ensures inactivation of WSSV ~~or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and~~
- 3) all processing effluent and waste materials are treated to ensure inactivation of WSSV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.7.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption ~~from a country, zone or compartment not declared free from~~ regardless of the infection with WSSV disease status of the exporting country, zone or compartment

- 1) *Competent Authorities* should not require any conditions related to WSSV WSD, regardless of the infection with WSSV WSD 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 that have been prepared and packaged for retail trade and which comply with Article 5.4.2.

Annex 15 (contd)

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 aquatic animal products Member Countries may wish to consider introducing internal measures to address the *risks* associated with the commodities aquatic animal products 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 9.7.2. from a country, *zone* or *compartment* not declared free from infection with WSSV ~~WSD~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

CHAPTER 9.8.

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

Article 9.8.1.

For the purposes of the *Aquatic Code*, infection with *Macrobrachium rosenbergii* nodavirus means infection with the pathogenic agent *Macrobrachium rosenbergii* nodavirus (MrNV), of the of the Family *Nodaviridae* *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~~ is provided in the *Aquatic Manual*.

Article 9.8.2.

Scope

The recommendations in this chapter apply to the following susceptible species which that meet the criteria for listing species as susceptible in accordance with Chapter 1.5.: the giant fresh water river 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 or~~ 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 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* derived from the a 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 that has been demonstrated to inactivate MrNV);
 - 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 that 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 or *aquatic animal products* derived from 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.

Annex 16 (contd)

- 3) When considering the importation or transit of aquatic animals and of aquatic animal products derived from of a species not referred to covered in Article 9.8.2. but which could reasonably be expected to pose a *risk of transmission of spread of 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 analysis 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:
- there has been no ~~observed~~ occurrence of infection with MrNV 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
 - basic biosecurity conditions* have been continuously met for at least the last two years;

OR

- 3) the ~~disease~~ infection with MrNV status prior to *targeted surveillance* is unknown but the following conditions have been met:
- basic biosecurity conditions* have been continuously met for at least the last two years; and
 - targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of 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 MrNV WTD but the following conditions have been met:
- on detection of MRNV MrNV the disease, the affected area was declared an *infected zone* and a *protection zone* was established; and
 - infected populations ~~have been destroyed or removed from~~ within the infected zone have been killed and disposed of by means that minimise the *risk likelihood* of further transmission of spread of MrNV the disease, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and
 - previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with MrNV the disease; and
 - targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of MrNV WTD.

In the meantime, part or all of the unaffected 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 infection with MrNV ~~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 free from infection with MrNV* 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 MrNV ~~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 infection with MrNV ~~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~~ infection with MrNV 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 MrNV ~~WTD~~;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with MrNV ~~WTD~~ and subsequently lost its ~~disease-free~~ status due to the detection of MrNV ~~WTD~~ in the *zone* but the following conditions have been met:
 - a) on detection of MrNV ~~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 within the infected zone~~ have been killed and disposed of by means that minimise the ~~risk~~ likelihood of further transmission ~~spread~~ of MrNV ~~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 infection with MrNV ~~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 MrNV ~~WTD~~.

Annex 16 (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 free status ~~as free from 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* ~~should~~ ~~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~~ or aquatic animal products from a country, zone or compartment declared free from infection with MrNV ~~white tail disease~~

When importing *aquatic animals* of a species referred to in Article 9.8.2. ~~and~~ or *aquatic animal products* derived thereof, 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 *exporting country* or a *certifying official* approved by the *importing country*. ~~The *international aquatic animal health certificate* should state 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~~ or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with MrNV ~~WTD~~.

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

This Article does not apply to *commodities aquatic animal products* 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 a 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: in accordance with Chapter 2.1. and consider the risk mitigation measures in points~~ 2) 1 and 3) 2 below.

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

- a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a quarantine facility; 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 MrNV in accordance with Chapters 4.3, 4.7. and 5.5.

OR

~~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.

2) 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 infection with **MrNV**.
- b) In the importing country:
- i) import the F-0 population into a *quarantine* facility;
 - ii) test the F-0 population for MrNV in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in *quarantine*;
 - iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with MrNV (as described in **Chapter 2.2.8. of the Aquatic Manual**) and test for MrNV in accordance with Chapter 1.4.;
 - v) if MrNV is not detected in the F-1 population, it may be defined as free from infection with MrNV and may be released from *quarantine*;
 - vi) if MrNV is detected in the F-1 population, those animals should not be released from *quarantine* and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- 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 or~~ or 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* of a species referred to in Article 9.8.2. ~~and~~ or *aquatic animal products* derived thereof, 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 processed 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

Annex 16 (contd)

- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of MrNV or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species
- 3) all processing effluent and waste materials are treated to ensure inactivation of MrNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.8.10.

Importation of live aquatic animals or aquatic animal products intended for use uses other than human consumption including in animal feed, or for agricultural, industrial, research 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, research or pharmaceutical use, live aquatic animals of a species referred to in Article 9.8.2. and, or aquatic animal products derived thereof, 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 or containment facilities for slaughter and until processed processing into one of the products referred to in point 1 of Article 9.8.3. or other products authorised by the Competent Authority; and
- 2) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that to ensure ensures inactivation of MrNV WTDV or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and
- 3) all processing effluent and waste materials are treated to ensure inactivation of MrNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

This Article does not apply to commodities referred to in point 1 of Article 9.6.3.

Article 9.8.11.

Importation (or transit) of aquatic animals and or aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from regardless of the infection with MrNV white tail disease status of the exporting country, zone or compartment.

- 1) Competent Authorities should not require any conditions related to infection with MrNV WTD, regardless of the infection with MrNV WTD status of the exporting country, zone or compartment, when authorising the importation (or transit) of frozen peeled shrimp (shell off, head off) which that 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 aquatic animal products Member Countries may wish to consider introducing internal measures to address the risks associated with the commodity aquatic animal products 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 derived from a 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 apply appropriate risk mitigation measures.

— Text deleted.

CHAPTER 9.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

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}), of the Family Vibrionales, and *V. harveyi* that contain a ~70-kbp plasmid with genes that encode homologues of the *Photobacterium* insect-related (Pir) toxins, PirA and PirB, carrying one or more extrachromosomal plasmid(s) that encode for a toxin (Pir^{sp}) 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~~ is provided in the *Aquatic Manual*.

Article 9.X.2.

Scope

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

~~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 or 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* derived from the a species referred to in Article 9.X.2., which are intended for any purpose and which comply with Article 5.4.1.:
 - fa) 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 that has been demonstrated to inactivate Vp_{AHPND});
 - b) cooked crustacean products that have been subjected to heat treatment at 100°C for at least three one minutes (or any time/temperature equivalent which that has been demonstrated to inactivate Vp_{AHPND});
 - e) ~~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});~~
 - cd) crustacean oil;
 - de) crustacean *meal*;
 - ef) chemically extracted chitin.]
- 2) When authorising the importation or transit of aquatic animals and or *aquatic animal products* derived from 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 or *aquatic animal products* derived from of a species not referred to covered in Article 9.X.2. but which could reasonably be expected to pose a *risk of spread of transmission 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 analysis assessment.

Annex 17 (contd)

Article 9.X.4.

Country free from AHPND

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~~ AHPND 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~~ AHPND 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 AHPND ~~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 within the infected zone~~ have been killed and disposed of ~~destroyed or removed~~ by means that minimise the ~~risk~~ likelihood of further transmission spread of AHPND ~~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 AHPND ~~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 ~~unaffected 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 AHPND

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

Annex 17 (contd)

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 AHPND ~~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~~ AHPND 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 AHPND ~~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 within the infected zone~~ have been killed and disposed of ~~destroyed or removed~~ by means that minimise the *risk likelihood* of further transmission spread of AHPND ~~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 AHPND ~~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.

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 free 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* ~~should needs to~~ be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

Annex 17 (contd)

Article 9.X.7.

Importation of aquatic animals ~~and~~ or aquatic animal products from a country, zone or compartment declared free from AHPND

When importing *aquatic animals* of a species referred to in Article 9.X.2. ~~and~~ or *aquatic animal products* derived thereof, 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*. The *international aquatic animal health certificate* should state ~~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~~ or *aquatic animal products* is a country, zone or compartment declared free from AHPND.

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

This Article does not apply to *commodities aquatic animal products* 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 AHPND

~~4)~~ When importing, for *aquaculture*, ~~live~~ *aquatic animals* of a 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: in accordance with Chapter 2.1. and consider the risk mitigation measures in points~~ 2) 1 and 3) 2 below.

~~1)-2)~~ If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

- a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine facility*; 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 *V*_{AHPND} in accordance with Chapters 4.3, 4.7, and 5.5.

OR

~~4)~~ 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.

~~2) 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 AHPND.
- b) In the *importing country*:
 - i) import the F-0 population into a *quarantine facility*;
 - ii) test the F-0 population for *V*_{AHPND} 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 AHPND (as described in Chapter 2.2.X. of the *Aquatic Manual*) and test for *V*_{AHPND} in accordance with Chapter 1.4.;

Annex 17 (contd)

- v) if $V_{p_{AHPND}}$ is not detected in the F-1 population, it may be defined as free from AHPND and may be released from quarantine;
- vi) if $V_{p_{AHPND}}$ is detected in the F-1 population, those animals should not be released from quarantine and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- 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 $V_{p_{AHPND}}$, 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 $V_{p_{AHPND}}$ and perform general examinations for pests and general health/disease status;~~
- g) ~~if $V_{p_{AHPND}}$ 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 $V_{p_{AHPND}}$;~~
- 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~~ or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from AHPND

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.X.2. ~~and~~ or aquatic animal products derived thereof, 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 processed 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) all containers and water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures to ensure inactivation of $V_{p_{AHPND}}$ or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and that prevents contact of waste with susceptible species.
- 3) all processing effluent and waste materials are treated to ensure inactivation of $V_{p_{AHPND}}$ or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Annex 17 (contd)

Article 9.X.10.

Importation of ~~live~~ aquatic animals or aquatic animal products intended for ~~use~~ uses other than human consumption including in animal feed, or for agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from AHPND

When importing, for use in animal feed or for agricultural, industrial, research or pharmaceutical use, ~~live aquatic animals~~ of a species referred to in Article 9.X.2. ~~and, or aquatic animal products derived thereof,~~ 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 or containment facilities for slaughter and until processed ~~processing~~ into one of the products referred to in point 1 of Article 9.X.3. or other products authorised by the Competent Authority; and
- 2) all containers and water used in transport ~~and all effluent and waste materials from the processing~~ are treated in a manner that to ensure ~~ensures~~ inactivation of V_{pAHPND} or is disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and
- 3) all processing effluent and waste materials are treated to ensure inactivation of V_{pAHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

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

Article 9.X.11.

Importation (or transit) of ~~aquatic animals and or~~ aquatic animal products for retail trade for human consumption ~~from a country, zone or compartment not declared free from regardless of the AHPND status of the exporting country, zone or compartment~~

- 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 that 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~~ aquatic animal products Member Countries may wish to consider introducing internal measures to address the risks associated with the ~~commodity~~ aquatic animal products 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 derived from a~~ 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.

— Text deleted.

Model Article X.X.8. for all disease-specific chapters (or article 10.4.12. for infection with infectious salmon anaemia virus)

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

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

- 1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:
 - a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and
 - b) the treatment of all transport water, equipment, effluent and waste materials to inactivate 'pathogen X' in accordance with Chapters 4.3., 4.7. and 5.5.

OR

- 2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:
 - a) In the *exporting country*:
 - i) identify potential source populations and evaluate their *aquatic animal* health records;
 - ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 'infection with pathogen X'/'disease X'.
 - b) In the *importing country*:
 - i) import the F-0 population into a *quarantine* facility;
 - ii) test the F-0 population for 'pathogen X' in accordance with Chapter 1.4. to determine their suitability as broodstock;
 - iii) produce a first generation (F-1) population in *quarantine*;
 - iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of 'infection with pathogen X'/'disease X' (as described in Chapter X.X.X. of the *Aquatic Manual*) and test for 'pathogen X' in accordance with Chapter 1.4.;
 - v) if 'pathogen X' is not detected in the F-1 population, it may be defined as free from 'infection with pathogen X'/'disease X' and may be released from *quarantine*;
 - vi) if 'pathogen X' is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner.

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

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from ‘infection with pathogen X’/ ‘disease X’

- 4)** When importing live for aquaculture, aquatic animals of a species referred to in Article X.X.2. from a country, zone or compartment not declared free from ‘infection with pathogen X’/‘disease X’, 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 12) and 23) below.
- 12)** If the intention is to grow out and harvest the imported aquatic animals, consider applying the following:
- a) the direct delivery to and lifelong holding of the imported aquatic animals in a quarantine facility; 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 Chapters 4.3., 4.37. and 5.5. 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.~~

OR

- 23)** 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 ‘infection with pathogen X’/‘disease X’.
 - b) In the importing country:
 - i) import the F-0 population into a quarantine facility;
 - ii) test the F-0 population for ‘pathogen X’/‘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 ‘infection with pathogen X’/‘disease X’ (as described in Chapter X.X.X. of the Aquatic Manual); and test for ‘pathogen X’/‘disease X’ in accordance with Chapter 1.4.;
 - v) if ‘pathogen X’/‘disease X’ is not detected in the F-1 population, it may be defined as free from ‘infection with pathogen X’/‘disease X’ and may be released from quarantine;

Annex 18B (contd)

- vi) if 'pathogen X' 'disease X' is detected in the F-1 population, **they those animals** should not be released from *quarantine* and should be ~~destroyed~~ killed and disposed of in a biosecure manner.
- 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.~~
- 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 referred to in point 1 of Article 10.10.3.

— Text deleted

CHAPTER 2.2.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

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 *Photobacterium* insect-related (Pir) toxins, PirA and PirB. Although there are reports of the isolation of other *Vibrio* species from clinical cases of AHPND, only Vp_{AHPND} has been demonstrated to cause AHPND.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

AHPND has a bacterial aetiology (Kondo *et al.*, 2015; Kwai *et al.*, 2014; Liu *et al.*, 2014; Tran *et al.*, 2013a; 2013b;). 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 *Photobacterium* insect-related (Pir) binary toxin, PirA and PirB (Gomez-Gil *et al.*, 2014; Gomez-Jimenez *et al.*, 2014; Han *et al.*, 2015a; Kondo *et al.*, 2014; Lee *et al.*, 2015; Yang *et al.*, 2014). The plasmid within 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* Vp_{AHPND} strains. 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 Vp_{AHPND} bacteria, natural deletion of the Pir^{VP} operon region may occur in a few individuals (Lee *et al.*, 2015; Tinwongger *et al.*, 2014). This deletion is due to the instability caused by the repeat sequences or transposase that flank the Pir toxin operon. Although different strains exhibit different levels of stability, When the deletion occurs, it means that a virulent strain of *V. parahaemolyticus* Vp_{AHPND} strain will lose its ability to induce AHPND. However, if the Pir toxin sequence is used as a target for detection, then a colony that has this deletion will produce a negative result even though the colony was derived from an isolate of AHPND-causing Vp_{AHPND} 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 *pndA* 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 *pndA* 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 producing PirA^{VP} and PirB^{VP}.

2.1.2. Survival outside the host

AHPND-causing strains of *V. parahaemolyticus* (Vp_{AHPND}) would be is expected to possess similar properties to other strains of *V. parahaemolyticus* found in seafood that have been shown to survive up to 9 and 18 days in filtered estuarine water and filtered seawater at an ambient temperature of 28 ± 2°C (Karunasagar *et al.*, 1987).

Annex 19 (contd)**2.1.3. Stability of the agent (effective inactivation methods)**

Experimental studies have shown that *Vp*_{AHPND} AHPND could not be transmitted via frozen infected shrimp (Tran *et al.*, 2013a). ~~In addition~~ Similarly, other strains of *V. parahaemolyticus* are known to be sensitive to freezing, refrigeration, heating and common disinfectants (Andrews *et al.*, 2000; Muntada-Garriga *et al.*, 1995; Su & Liu, 2007; Thompson & Thacker, 1973).

2.1.4. Life cycle

Not applicable.

2.2. Host factors**2.2.1. Susceptible host species**

Species that fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Code* include: giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*P. vannamei*).

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; Leaño & Mohan, 2013; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b). ~~There is a report~~ (De la Pena *et al.* (2015) ~~of reported~~ disease outbreaks in the Philippines occurring as late as 46–96 days after pond-stocking.

2.2.4. Species or subpopulation predilection (probability of detection)

Not applicable.

2.2.5. Target organs and infected tissue

Gut-associated tissues and organs

2.2.6. Persistent infection

No data or not known.

2.2.7. Vectors

None is known, although as *Vibrio* spp. are ubiquitous in the marine environment, ~~the possibility presence of vector species would not be unexpected~~ vector species could be expected.

2.3. Disease pattern**2.3.1. Transmission mechanisms**

*Vp*_{AHPND} AHPND has been transmitted experimentally by immersion, ~~in feed feeding (per os)~~ and reverse gavage (Dabu *et al.*, 2015; 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 ~~has been~~ was reported initially in 2010 from China (People's Rep. of) (2010), 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) (Dabu *et al.*, 2015; de la Pena *et al.*, 2015).

2.3.4. Mortality and morbidity

AHPND is characterised by sudden, mass mortalities (up to 100%) usually within 30–35 days of stocking grow-out ponds with PLs or juveniles (FAO, 2013; Hong *et al.*, 2016; NACA, 2012) and can be reproduced experimentally (Joshi *et al.*, 2014a; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013b). Older juveniles may also be affected (de la Pena *et al.*, 2015).

2.3.5. Environmental factors

Water sources with low salinity (<20 ppt) seem to reduce the incidence of the disease. Peak occurrence seems to occur during the hot 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 occurrences of AHPND in endemic areas (FAO, 2013; NACA, 2012).

2.4. Control and prevention

2.4.1. Vaccination

Not applicable.

2.4.2. Chemotherapy

~~None available~~ Not applicable.

2.4.3. Immunostimulation

~~None known to be effective~~ Not applicable.

2.4.4. Resistance–Breeding for resistance

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~~ appropriate stocking density etc. are all well-established practices that reduce the impact of disease, including AHPND (NACA, 2012).

Annex 19 (contd)**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* *Vp_{AHPND}* 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 Davidson'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).

3.3. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples small life stages, especially PL or specimens up to 0.5 g, can may need to be pooled to obtain enough material for molecular testing. Larger shrimp should be processed individually as the effect of pooling on diagnostic sensitivity has not been evaluated.

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 suitable (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. Clinical 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 is known.

4.2.3. Microscopic pathology

The disease has two distinct phases:

Annex 19 (contd)

- 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; Leañó & Mohan, 2013; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections (for ISH)

ISH is not currently available (October 2015).

4.2.7. 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 species identification may be carried out using 16S rRNA PCR (Weisburg *et al.*, 1991) or *toxR*-targeted PCR (Kim *et al.*, 1999) and sequencing (Weisburg *et al.*, 1991), and their probable ability to cause AHPND using AHPND-specific PCR methods that target the *Vp*_{AHPND} toxin genes are described in section ~~4.3.1.2.3.~~ 4.3.1.2.3.1.~~

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).

Annex 19 (contd)

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 Vp_{AHPND} AHPND-toxin genes. The AP3 method is a single-step PCR that targets the 12.7 kDa PirA^{VP} gene (Sirikharin *et al.*, 2015). It was validated for 100% positive and negative predictive value by testing 104 isolates of Vp_{AHPND} 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 Vp_{AHPND} 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. VpPirA-284, VpPirB-392 (Han *et al.*, 2015a) and TUMSAT-Vp3 (Tinwongger *et al.*, 2014), have relatively low sensitivity when used for detection of Vp_{AHPND} 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 Vp_{AHPND} 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 Vp_{AHPND} AHPND-causing bacteria), allowing it to be used directly with tissue and environmental samples without an enrichment step.

In addition, real-time PCR methods, for example the Vp_{AHPND} AHPND-specific TaqMan real-time PCR developed by Han *et al.* (2015b), and an isothermal loop-mediated amplification protocol (LAMP) method developed by Koiwai *et al.* (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 Vp_{AHPND} 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

Vp_{AHPND} 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 of Vp_{AHPND} 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 One-step PCR detection of pVA1 plasmid

Two one-step PCR methods (AP1 and AP2) are described here for detection of the pVA1 plasmid in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.1.

Table 4.1. PCR primers for one-step PCR detection of AHPND-causing bacteria

Method name	Primers	Target gene	Expected amplicon size	Reference
AP1	AP1F: 5'-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3' AP1R: 5'-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3'	<i>pVA1</i>	700bp	Flegel & Lo (2014)
AP2	AP2F: 5'-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3' AP2R: 5'-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3'	<i>pVA1</i>	700bp	Flegel & Lo (2014)

4.3.1.2.3.1.5 Protocol for the AP1 and AP2 PCR methods

This protocol follows the method described by Flegel & Lo. (2014). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM MgCl₂, 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 0.01-1 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 25–30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds with a final extension step at 72°C for 10 minutes and then the reaction mixture can be held at 4°C (http://www.enaca.org/modules/library/publication.php?publication_id=1128).

4.3.1.2.3.1.45 PCR primers for One-step PCR detection of *PirA/PirB* toxin genes 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 in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.42.

Table 4.42. 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'	<i>pirA^{Vp}</i>	333bp	Sirikharin <i>et al.</i> , 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'	<i>pirA^{Vp}</i>	360bp	Tinwongger <i>et al.</i> , 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'	<i>pirA^{Vp}</i>	284bp	Han <i>et al.</i> , 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'	<i>pirB^{Vp}</i>	392bp	Han <i>et al.</i> , 2015a

4.3.1.2.3.1.56 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 MgCl₂, 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.

4.3.1.2.3.1.67 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 MgCl₂, 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.

Annex 19 (contd)

4.3.1.2.3.1.78 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.

4.3.1.2.3.1.89 AP4 nested PCR primers protocol for detection of *Vp_{AHPND}* AHPND bacteria

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) and Dangtip *et al.* (2015)~~. The first PCR reaction mixture consists of 2.5 µl 10× PCR mix, 1.5 µl 50 mM MgCl₂, 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 MgCl₂, 0.5 µl 10 mM dNTPs, 0.375 µl 10 µM AP4-F2, 0.375 µl 10 µM AP4-R2, 0.3 µl Taq DNA pol (5 units µl⁻¹) and 2 µl of the first PCR reaction in a total volume of 25 µl. The nested PCR protocol is 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds and hold at 4°C.

The nested PCR primers, designed using the China (People's Rep. of) isolate of AHPND bacteria (Yang *et al.*, 2014), are shown in Table 4.23. 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 4.23. Primers for the AP4, nested PCR method for detection of *Vp_{AHPND}* 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
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	<i>et al.</i> , 2015

4.3.1.2.3.1.910 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 4.12, 4.23 and 4.3) indicate a positive result. Positive results must be confirmed by sequence analysis.

4.3.1.2.3.1.1011 Protocol for the AHPND-specific real-time PCR 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 real-time PCR mixture containing 0.3 µM of each primer and 0.1 µM probe to a final volume of 10 µl. Real-time PCR conditions consist of 20 seconds at 95°C, followed by 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. At the completion of the TaqMan real-time PCR assay, the presence of PirA DNA is demonstrated by the presence of specific amplicons, identified by software-generated characteristic amplification curves. No-template controls must have no evidence of specific amplicons.

4.3.1.2.3.1.6 Primers and Probe for AHPND-specific real-time PCR

The primers and probe and target gene for the Vp_{AHPND}-AHPND-specific real-time PCR are listed in Table 4.34.

Table 4.34. Primers and probe for the real-time PCR method for detection of Vp_{AHPND}-AHPND-causing bacteria

Primer/ probe name	Sequence	Target gene	Reference
VpPirA-F	5'-TTG-GAC-TGT-CGA-ACC-AAA-CG-3'	pirA	Han <i>et al.</i> , 2015b
VpPirA-R	5'-GCA-CCC-CAT-TGG-TAT-TGA-ATG-3'		
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 MgCl₂, 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 MgCl₂, 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) and Dangtip *et al.* (2015). The first PCR reaction mixture consists of 2.5 µl 10× PCR mix, 1.5 µl 50 mM MgCl₂, 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 10× PCR mix, 1.5 µl 50 mM MgCl₂, 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 6× loading dye added, is loaded onto a 1.5% agarose gel and electrophoresis is carried out at 90 volts for 40 minutes. Amplicons are visualised with SYBR Safe gel stain (Invitrogen, Cat. No. 33102) according to the manufacturer's instructions. Amplicons of the expected size appropriate for the PCR methods used (Tables 4.1, 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 real-time PCR method

Annex 19 (contd)

~~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 real time PCR mixture containing 0.3 μM of each primer and 0.1 μM probe to a final volume of 10 μl . Real time PCR conditions consist of 20 seconds at 95°C, followed by 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. At the completion of the TaqMan real time PCR assay, the presence of PirA DNA is demonstrated by the presence of specific amplicons, identified by software generated characteristic amplification curves. No template controls must have no evidence of specific amplicons.~~

4.3.1.2.3.1. ~~4.1.2~~ Controls for all PCR methods

The following controls should be included in all *Vp*_{AHPND} AHPND PCR assays: a) negative extraction control i.e. DNA template extracted at the same time from a known negative sample; b) DNA template from a known positive sample, such as *Vp*_{AHPND} AHPND-affected shrimp tissue or DNA from an *Vp*_{AHPND} 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

*Vp*_{AHPND} 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 can be processed for *Vp*_{AHPND} AHPND PCR and sequence analysis. Moribund or surviving shrimp are processed for histology, and bacterial re-isolation, and AHPND-PCR and sequence analysis. A positive bioassay is indicated by the detection of characteristic histological lesions and *Vp*_{AHPND} by PCR and sequencing.

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	ed	ed	c	d
Bioassay	d	d	d	d	d	a
Histopathology	d	c	a	c	a	b
Real-time PCR	d	a	a	a	a	b
Nested PCR and sequence	d	b	b	b	a	a
1-step PCR and Sequence	d	ec	ec	ec	a	a

PL = postlarvae; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from AHPND

As indicated in Table 5.1, real-time PCR 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 is suspected if at least one of the following criteria is met:

- i) Mortality associated with and clinical signs consistent with of AHPND
- ii) Histopathology indicative of consistent with AHPND
- iii) Detection of Pir toxin genes in the pVA1 plasmid in *Vibrio parahaemolyticus* by PCR or real-time PCR.

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 consistent with AHPND
- ii) Detection of Pir toxin gene and in the pVA1 plasmid in *Vibrio parahaemolyticus* by PCR and sequence analysis
- iii) Positive results by bioassay (characteristic histological lesions and detection of clinical signs, mortality, histopathology, ~~VpAHPND by~~ PCR and sequencing)

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CHAPTER 2.2.1.

INFECTION WITH CRAYFISH PLAGUE (APHANOMYCES ASTACI (CRAYFISH PLAGUE))

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci* Schikora, a member of the of the Family Leptolegniaceae, Phylum 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 or oomycota, are now considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista. Chromista are a eukaryotic supergroup, probably polyphyletic, which may be treated as a separate kingdom or included among the Protista.

Four groups (A–D) of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) (Dieguez-Urbeondo *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 to Europe, *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-Urbeondo *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.

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.

Annex 20 (contd)**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-Urbeondo *et al.*, 1995).

2.2. Host factors**2.2.1. Susceptible host species**

~~Species that fulfil the criteria for listing as susceptible to infection with *A. 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 crayfish (*Procambarus clarkii*), *Austropotamobius torrentium*, *Austropotamobius pallipes*, *Orconectes limosus*, *O. immunis*, *Procambarus alleni* and *Potamon potamios*.~~

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 & Soyulu, 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; Schrimpf *et al.*, 2014).

2.2.2. Susceptible stages of the host

All live stages should be considered as susceptible to infection.

2.2.3. Species or subpopulation predilection (probability of detection)

The host species susceptible to infection with *A. astaci* fall largely into two categories: those highly susceptible to infection with development of clinical disease and mortalities, and those 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.

In 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 thorough~~ 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.

2.2.4. 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 uropods and telson) and soft abdominal cuticle appear to be frequently infected (Oidtmann *et al.*, 2006; Vrålstad *et al.*, 2011).

2.2.5. Persistent infection with lifelong carriers

A number of North American crayfish species have been investigated for their susceptibility to infection shown to be infected 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. Infected naturalised or aquaculture-reared North American crayfish populations usually do not develop clinical disease or increased mortalities (Oidtmann *et al.*, 2006; Strand *et al.* 2011; 2012).

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.

A recent report from Finland also suggests that noble crayfish populations in cold water environments may be chronically infected at low prevalence (Viljamaa-Dirks *et al.*, 2011).

2.2.6. Vectors

Finfish movement may facilitate the spread of *A. astaci* in a number of ways, such as through the presence of spores in the digestive tract of fish, transport water, co-transport of infected crayfish specimens, or a combination of both (Alderman *et al.*, 1987; Oidtmann *et al.*, 2002). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.) (Alderman *et al.*, 1987). There is good field and experimental evidence that movements of fish from areas in which crayfish plague is active can transmit infection from one watershed to another (Alderman *et al.*, 1987; Oidtmann *et al.*, 2006).

Fomites: The spread of *A. astaci* can also be linked to 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.

Annex 20 (contd)**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 of finfish, 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 the~~ 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~~ *A. astaci* in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms (Alderman, 1996; Dehus *et al.*, 1999). ~~Nowadays~~, Spread now 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 epizootic epidemic among the highly susceptible animals. The velocity-rate of spread will depend, among other factors, on the prevalence of infection in the population of North American crayfish.

Finfish 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 both all three (Alderman *et al.*, 1987; Oidtmann *et al.*, 2002). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.) (Alderman *et al.*, 1987).

2.3.2. Prevalence

In the highly susceptible European crayfish species, exposure to *A. astaci* spores usually is considered ~~to lead~~ to infection and eventually to death. The minimal minimum 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-rate 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 acute mortality event due to infection with *A. astaci* outbreak of crayfish plague in 2001 and that was followed in subsequent years suggest that in sparse noble crayfish populations, spread of disease throughout the host population may ~~be take prolonged over a time span~~ of several years (Viljamaa-Dirks *et al.*, 2011).

Prevalence in North American crayfish appears to vary greatly. Limited studies suggest prevalences ranging from between 0 and 100% are possible (Oidtmann *et al.*, 2006).

2.3.3. Geographical distribution

First in Europe the reports of large ~~crayfish~~ mortalities of crayfish 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 into

Europe until the 1890s, but the post-1960s extensions are largely linked to movements more recent introductions of North American crayfish introduced more recently for purposes of crayfish farming (Alderman, 1996). Escapes of such the introduced species were almost impossible to prevent occurred and *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

As North American crayfish serve as a reservoir of *A. astaci*, any areas where North American crayfish species are found should be considered as areas where *A. astaci* is present (unless shown otherwise).

Australia and New Zealand have never experienced any outbreaks of infection with *A. astaci* crayfish plague to date and are currently considered free from the infection with *A. astaci* (OIE WAHID website, accessed June 2016).

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 may affect 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 infection with *A. astaci* 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 or lake or in a part of the affected river/lake that was not reached by spores, or crayfish may have stayed in burrows during the epizootic 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 infection with *A. astaci* crayfish plague in the form of and 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-Urbeondo *et al.*, 1995).

Field observations show that crayfish plague outbreaks of infection with *A. astaci* occur over a wide temperature range, and at least in the temperature range from 4–20°C. The velocity-rate of spread within a population depends on several factors, including water temperature. In a temperature range between 4 and 16°C, the speed of an epizootic epidemic is enhanced by higher water temperatures. At low water temperatures, the epizootic epidemic curve can increase very slowly and the period during which mortalities are observed can be several months (B. Oidtmann, unpublished data).

Annex 20 (contd)

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 6.0–7.5, with a maximum range between pH 4.5 and 9.0 (Unestam, 1966).

Zoospore emergence is influenced by the presence of certain salts in the water. CaCl₂ stimulates zoospore emergence from primary cysts, whereas MgCl₂ 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. The following measures are necessary to prevent introduction via identified pathways:

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 of finfish are being planned, ~~whether~~ the water source should be assessed for the likelihood that it may harbour infected crayfish (including North American carrier crayfish) should be assessed.
3. Any ~~fish~~-movements of finfish from the site of a current epizootic epidemic of infection with *A. astaci* ~~crayfish plague~~ carry a high risk of spread and should be avoided.
4. If ~~fish~~-movements of finfish from a source containing North American crayfish are being planned, harvest methods at the source site should 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. If release into the wild is planned, then ~~Therefore~~ prior to any planned release, a risk assessment should be conducted to estimate ~~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, should careful consideration need to be given as to whether such facilities should be established. Biosecurity measures to ensure crayfish do not escape from aquaculture facilities are extremely difficult to implement. Therefore, a risk assessment needs to be conducted to determine whether these 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~~ not practical.

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 for resistance~~

In the 125 years since infection with *A. astaci* crayfish plague first occurred in Europe, there is little evidence of resistant populations of European crayfish. However, the fact that North American crayfish generally do not ~~are not very susceptible to~~ developing clinical disease suggests that selection for resistance may be possible and laboratory studies using attenuated strains of *A. astaci* might be successful. However, there are currently no published data from ~~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 is considered a susceptible species are potential hosts for *A. astaci*, restocking with North American crayfish may provide a reservoir would further the spread of *A. astaci*. This would minimise the chances for success of re-introduction of indigenous species. A risk assessment should be conducted to assist in decisions on restocking. ~~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 (1977) mention that they experimentally exposed *Astacus astacus* and *P. leniusculus* eggs to zoospore suspensions and were unable to induce infection. 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 endemic area established site, where the highly susceptible species are being farmed, the following biosecurity recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

- 1-6. General biosecurity should be in place (e.g. controlled access to premises; disinfection of boots when entering the site is entered; investigation of mortalities if they occur; introduction of live animals (crayfish, finfish) only from sources known to be free from infection with *A. astaci*.
- 2-1. 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.
- 3-2. If fish transfers of finfish or crayfish are being planned, these should not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of infection with *A. astaci* crayfish plague or North American carrier crayfish).
- 4-3. North American crayfish should not be brought onto the site.
- 5-4. Finfish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of infection with *A. astaci* 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 to kill *A. astaci* (see Section 2.1.3).

Annex 20 (contd)

- ~~5-5.~~ Any equipment that is brought onto site should be disinfected.
- ~~6.~~ ~~General biosecurity should be in place (e.g. controlled access to premises; disinfection of boots when site 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 infection with *A. astaci* ~~crayfish plague~~ in a population of highly susceptible crayfish species, the batch of crayfish selected ~~to identify for investigation for the presence of *A. astaci*~~ should ideally consist of: a) live crayfish showing signs of disease, and 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 ensure that temperatures are not deleterious to the animals. These can be attached at the inside bottom of the transport container. However, 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 or 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 infection with *A. astaci* crayfish plague. Clinical signs of infection with *A. astaci* 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 diagnostic laboratory (Oidtmann *et al.*, 1999). Molecular methods are now available that are less dependent on speed of sample 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 an epizootic crayfish plague mortality may be the presence appearance 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 falling over onto their backs and remaining unable to right themselves. Often, however, unless waters are carefully observed, the first sign that there is a problem will of an outbreak may 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 numbers are sufficient present to allow infection to spread rapidly disease spread, particularly at summer water temperatures, infection will spread quickly and all the highly susceptible native crayfish within stretches of over 50 km may die lose all their highly susceptible native crayfish within less than 21 days or less from the first observed mortality (D. Alderman, pers. comm.). Infection with *A. astaci* 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 infection with *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).

Annex 20 (contd)**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 pereopods (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. ~~A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used.~~ Additionally ~~However,~~ such material does not prove that any hyphae observed are those of *A. astaci*. ~~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 i) the presence of hyphae pervading the cuticle and ii) 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 culture 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 H₂O) clean disposable paper towel. Simple aseptic excision of infected tissues, which are then placed as small pieces (3–5 mm²) 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 pereopods (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).

Annex 20 (contd)

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. Parafilm¹) 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-water mould~~ 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-water mould~~ 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.

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).

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*.

Annex 20 (contd)

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 (≥310:1 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 H₂O) 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 assay (Vrålstad *et al.*, 2009). Negative controls should be run alongside the samples. Shrimp tissues may be used as negative controls.

Annex 20 (contd)

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 [NH₄]₂SO₄, 0.01% (v/v) Tween 20), 1.5 mM MgCl₂, 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 should 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 an ~~an crayfish plague~~ outbreak is investigated, the test sensitivity in animals that had died of ~~infection with *A. astaci* 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 outbreaks of infection with *A. astaci* 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 resulting from infection with *A. 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.

Annex 20 (contd)

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
Real-time PCR	a	b or a ¹
Sequencing of PCR products	n/a	a
Transmission EM	∅-n/a	∅-n/a
Antibody-based assays	n/a	n/a
<i>In-situ</i> DNA probes	n/a	n/a

PCR = polymerase chain reaction; 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
Histopathology	d	d
PCR	a	b, possibly a ¹
Real-time PCR	a	b, possibly a ¹
Sequencing of PCR products	n/a	a
Transmission EM	∅-n/a	∅-n/a
Antibody-based assays	n/a	n/a
<i>In-situ</i> DNA probes	n/a	n/a

PCR = polymerase chain reaction; 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
Real-time PCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	∅-n/a	∅-n/a
Antibody-based assays	n/a	n/a
<i>In-situ</i> DNA probes	n/a	n/a

/PCR = polymerase chain reaction; 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 should 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 both finfish and crayfish stocks from infected waters present a risk of disease transmission, monitoring the status of crayfish populations to confirm that they remain healthy, is necessary.

In a crayfish farm setting, an infection with ~~crayfish plague~~ *A. astaci* should ~~would~~ be noticed relatively quickly, due to the rapid ~~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 should 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

In highly susceptible crayfish species, infection with *A. astaci* shall be ~~is~~ suspected if at least one of the following criteria is met:

Annex 20 (contd)

- i) 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.
- ii) The presence of gross and microscopic signs consistent with infection with *A. astaci*.
- iii) Isolation and culture of a water mould consistent with *A. astaci*.
- iv) A positive result for *A. astaci* by PCR.
- v) A positive result for *A. astaci* ~~or by~~ real-time PCR.

North American crayfish species

~~In Any population of North American crayfish is generally to be regarded as potentially infected with *A. astaci* species, infection with *A. astaci* shall be is~~ suspected if at least one of the following criteria is met:

- i) A positive result for *A. astaci* by PCR.
- ii) A positive result for *A. astaci* by real-time PCR.

7.2. Definition of confirmed case*Highly susceptible crayfish species*

~~Confirmation of presence of *A. astaci* by PCR or real time PCR and sequencing.~~

In highly susceptible crayfish species, infection with *A. astaci* shall be is confirmed if at least two of the following criteria are met:

- i) Isolation and culture of a water mould consistent with *A. astaci*.
- ii) A positive result for *A. astaci* by PCR.
- iii) A positive result for *A. astaci* ~~or by~~ real-time PCR.
- iv) Sequenced PCR products that match known sequences of *A. astaci*.

~~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) If the investigated suspect case is not the first case of detection of *A. astaci* in the a country or region zone previously considered free from infection with *A. astaci*, sequencing of PCR products should be conducted for, the PCR result alone may be considered sufficient as a confirmation.~~

In North American crayfish species, infection with *A. astaci* shall be is confirmed if at least two of the following criteria are met:

- i) A positive result for *A. astaci* by PCR
- ii) A positive result for *A. astaci* ~~or by~~ real-time PCR
- iii) Sequenced PCR products that match known sequences of *A. astaci*

North American crayfish species

~~Confirmation of presence of *A. astaci* by PCR or real time PCR and sequencing~~

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* *

NB: There are OIE Reference Laboratories for Crayfish plague (infection with *Aphanomyces astaci*) (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 Crayfish plague (*Aphanomyces astaci*)

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2012

CHAPTER 2.2.3.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

~~Infection with~~ infectious hypodermal and haematopoietic necrosis ~~virus~~ (IHHN) ~~disease means is caused by~~ infection with ~~the pathogenic agent~~ infectious hypodermal and haematopoietic necrosis virus (IHHNV), ~~of the~~ Genus *Penstyldensovirus*, Family *Parvoviridae*, ~~g~~Genus *Penstyldensovirus* 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 the genus *Brevidensovirus*, Family *Parvoviridae* with the species name of PstDNV (for *Penaeus stylirostris* densovirus) *Decapod penstyldensovirus 1* (Fauquet *et al.*, 2005; King *et al.*, 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 homologous to part of the IHHNV genome are found embedded in the genome of penaeids. These were initially described as Type 3A from East Africa, India and Australia, and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al.*, 2007). ~~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). Tissues containing the putative IHHNV-homologous sequences in the *P. monodon* genome are not infectious to the susceptible 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.

Annex 21 (contd)

2.2. Host factors**2.2.1. Susceptible host species**

Species that fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: giant river prawn (*Macrobrachium rosenbergii*), yellowleg shrimp (*P. californiensis*), giant tiger prawn (*P. monodon*), northern white shrimp (*P. setiferus*), blue shrimp (*P. stylirostris*), and white leg shrimp (*P. vannamei*).

Most penaeid species can be infected with IHNV, including the principal cultured species, *P. monodon* (black tiger shrimp/prawn), *P. vannamei* (Pacific white shrimp), and *P. stylirostris* (Pacific blue shrimp).

IHNV 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).

IHNV 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; Castillo *et al.*, 1993; Kalagayan *et al.*, 1991; Lightner, 1996a; 1996b; Motte *et al.*, 2003). IHNV infection in *P. monodon* is usually subclinical, but RDS, reduced growth rates and reduced culture performance have been reported in IHNV infected stocks (Chayaburakul *et al.*, 2004; Primavera & Quinitio, 2000).

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 IHNV according to Chapter 1.5. of the *Aquatic Code* include: northern brown shrimp (*Penaeus aztecus*). Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHNV, 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. japonicus*), green tiger prawn (*P. semisulcatus*), *Hemigrapsus penicillatus*, Argentine stiletto shrimp (*Artemesia longinaris*), Cuata swimcrab (*Callinectes arcuatus*), Mazatlan sole (*Achirus mazatlanus*), yellowfin mojarra (*Gerres cinereus*), tilapias (*Oreochromis* sp.), Pacific piquitinga (*Lile stolifera*) and blackfin snook (*Centropomus medius*).

2.2.3. Susceptible stages of the host

IHNV has been ~~detected~~ demonstrated in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei*. Eggs, produced by IHNV-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 IHNV infection (Motte *et al.*, 2003).

2.2.4. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1 and 2.2.2.

2.2.5. Target organs and infected tissue

IHNV 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-IHNV and are usually negative for IHNV by ISH (Lightner, 1993; 1996a; 2011; Lightner *et al.*, 1992b).

2.2.56. Persistent infection with lifelong carriers

Some members of *P. stylirostris* and *P. vannamei* populations that survive infection with 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).

2.2.67. 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, IHHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although infection with 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). Infection with 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).

An IHHN-like virus sequence has been reported from Australia (Krabsetsve *et al.*, 2004; Owens *et al.*, 1992), and the presence of infection with IHHNV 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).

Annex 21 (contd)

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, runt-deformity syndrome (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 the disease 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, particularly 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 times 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 Breeding for resistance

Selected stocks of *P. stylirostris* that are resistant to ~~infection with IHHNV disease~~ 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 However~~ lines of *P. stylirostris* bred for IHHN disease resistance ~~to infection with IHHNV were found to be refractory to infection~~ (Tang et al., 2000). ~~However, such stocks do not have increased resistance to other diseases, such as white spot syndrome virus (WSSV), and, hence, so their use has been limited. In some stocks a genetic basis for IHHNV susceptibility in *P. vannamei* has been reported (Alcivar-Warren et al., 1997).~~

2.4.5. Restocking with resistant species

There has been some limited application and success with IHHNV-disease-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarin-Herzberg & Ascencio 2001). The relative resistance of *P. vannamei* to IHHN-disease-infection with IHHNV, 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

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 disease agents), the method is not effective for preventing transovarian transmission of IHHNV (Motte *et al.*, 2003).

2.4.8. General husbandry practices

Some husbandry practices have been successful ~~applied to~~ in preventing the spread ~~on~~ of IHHNV ~~infections and disease~~. Among these has been the application of PCR pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii, and discarding those that test positive for the virus (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 infection with IHHNV (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~~ are not suitable samples for IHHNV detection or certification of ~~for~~ IHHN-disease 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.~~

Annex 21 (contd)

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing.

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 or 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 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 disease is required.

4.1.2. Behavioural changes

In acute 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 disease in *Penaeus stylirostris*

Infection with IHHNV is often causes an acute disease with very high mortalities occurring in juveniles life stages 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 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 infection with 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 infection with 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 that occurred during the larval or early postlarval 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* free from infection with IHHNV (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 sections ~~histological methods~~ (see Section 4.2.6). Chronic infection with IHHNV ~~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 infection with IHHNV ~~IHHNV infection~~. These characteristic IHHNV inclusion bodies are eosinophilic and often haloed (with H&E stains of tissues preserved with fixatives that contain acetic acid, such as Davidson's AFA and Bouin's solution) (Bell & Lightner, 1988; Lightner, 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 infection with 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 infection with ~~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 infection with IHHNV~~ ~~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) is immersed in fixative for 24 to 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported (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).

Annex 21 (contd)

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 IHNV.

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 or artificial media

IHNV 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 IHNV are available in dot-blot and ISH formats. PCR tests for IHNV 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 ~~de more~~ traditional techniques for IHNV 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 IHNV:* 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™ 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 IHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHNV 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 IHNV DNA and the Roche PCR-DIG Probe Synthesis Kit™.~~

Dot-blot hybridisation procedure for IHNV: the dot-blot hybridisation method given below uses a digoxigenin-11-dUTP (DIG)-labelled DNA probe for IHNV and generally follows the methods outlined in Mari *et al.* (1993) and Lightner (1996a). Formulas for the required reagents are given after the protocols.

Annex 21 (contd)

- 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 40 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.~~
- 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.
- vi) Boil the DIG-labelled probe for 40-3-5 minutes and then 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.
- 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 | 4 × | 30 minutes at room temperature |
| Buffer I | 4 × | 5 minutes at room temperature |
| (Buffers are prepared ahead of time). | | |
- viii) React the membrane in bags with anti-DIG AP alkaline phosphatase conjugate (Roche Diagnostics² 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.
- ix) Wash membrane well with:
- | | | |
|------------|-----|--------------------------------|
| Buffer I | 2 × | 15 minutes at room temperature |
| Buffer III | 1 × | 5 minutes at room temperature |
- 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.
- xi) Photograph the results (colour fades over time).
- xii) Store dry membranes in heat-seal bags.

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*.

Annex 21 (contd)

In-situ hybridisation (ISH) procedure: the ISH method given below uses a DIG-labelled DNA probe for IHNV 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 $\mu\text{g ml}^{-1}$ 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 ~~40~~ 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 phosphatase 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).

Annex 21 (contd)

- 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 H₂O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.~~

~~ii) 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 H₂O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.~~

~~iii) 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) 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 H₂O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H₂O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H₂O. Filter solutions through a 0.45 µm filter; store at 4°C.~~

Annex 21 (contd)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 polyvinylpyrrolidone
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.

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.~~

xiv) ~~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-H₂O. Filter through a 0.45 µm filter; store at 4°C.~~

xv) ~~Buffer II (blocking buffer)~~

Blocking reagent	0.25 g Blocking reagent (Roche Diagnostics 1-096-476)
Buffer I	50 ml 1 × Buffer I

~~Store at 4°C for up to 2 weeks.~~

xvi) ~~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.~~

xvii) ~~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.~~

xviii) ~~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)

xviii) 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.	

xvix) 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 IHNV: several one-step PCR methods (Krabsetsve *et al.*, 2004; Nunan *et al.*, 2000; Shike *et al.*, 2000; Tang *et al.*, 2000; 2007; Tang & Lightner 2004), and a number of commercial PCR kits are available for IHNV detection. Nested methods are also available from commercial sources.

There are multiple geographical variants of IHNV, some of which are not detected by all of the available methods for IHNV. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHNV (Krabsetsve *et al.*, 2004; Tang & Lightner, 2002; Tang *et al.*, 2000; 2007). However these tests also detect IHNV-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 IHNV viral IHNV sequence but do not react with IHNV-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 IHNV types 1 and 2 (the infectious forms of IHNV), 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 or negative PCR results for IHNV 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 IHNV

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-CAA-GAC-CAA-AAT-ACG-AA-3'	45%/71°C	(Tang <i>et al.</i> , 2007)
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 <i>et al.</i> , 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 <i>et al.</i> , 2000)
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 <i>et al.</i> , 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 <i>et al.</i> , 2007)

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region (ORF 1) of the IHNV 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.

Annex 21 (contd)

General PCR method for IHNV: the PCR method described below for IHNV generally follows the methods outlined in Tang *et al.* (2007) and Nunan *et al.* (2000). However, recent minor modifications including the sources of the reagents and the use of an automated DNA extraction instrument are acceptable. The modifications include DNA extraction method, choice of primers (Table 4.1), and the volume of reaction. These slightly modified methods have been validated in accordance with Chapter 1.1.2 Principles and methods of validation of diagnostic assays for infectious diseases and do not affect the diagnostic performance of the assay. Cumulative experience with the technique has led to modifications with respect to template (DNA extraction of clinical specimens), choice of primers (Table 4.1), and volume of reaction.

- 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 tissues or haemolymph that was fixed-preserved in 95% ethanol and then dried. A control consisting of tissues or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods, 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 as a template per 50–25 µl reaction volume.
- ii) The following controls should be included in every PCR assay for IHNV: (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 IHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHNV-infected material. Prepare primers at 400 µg l⁻¹ 10 µM 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. If PuReTaq™ Ready-To-Go PCR Beads (GE Healthcare) are used, the PCR profile involves a 3–5 minutes at 95°C to denature DNA prior to the primers binding and activation of the enzyme. This programme is then linked to the cycling programme (followed by 35 cycles) and an of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension programme. The programme is set as follows: at 72°C for 5 minutes.~~

Hot start	Programme 1	5 minutes 95°C	
Linked to	Programme 2	30 seconds 95°C	
		30 seconds 55°C	35 cycles
		4 minute 72°C	
Linked to	Programme 3	7 minutes 72°C	
Linked to	Programme 4	4°C until off	

v) Prepare a 'Master Mix' consisting of water and primers.

- vi) For a 50–25 µl reaction mix, add 49–24 µl Master Mix to each tube and then add 1 µl of the sample DNA template 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 the PCR program.~~
- 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 After PCR, run 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~~

Annex 21 (contd)

~~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. 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 IHNV.~~

Real-time PCR method for IHNV: real-time PCR methods have been developed for the detection of IHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHNV genome (Dhar *et al.*, 2001; Tang & Lightner, 2001). ~~Using primers 309F/309R, it is possible to distinguish infectious forms of IHNV from non-infectious forms. Using MG831F/MG831R it is possible to distinguish the non-infectious forms.~~

The real-time PCR method using TaqMan chemistry described below for IHNV generally follows the method used in Tang & Lightner (2001).

- i) The PCR primers and TaqMan probe are selected from a region of the IHNV 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 (IHNV1608F) and downstream (IHNV1688R) 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 4632 to 4644,~~ is synthesised and labelled with fluorescent dyes 5-carboxyfluorescein (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 real-time PCR 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 IHNV-infected tissue.

Sequencing: PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHNV, 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 IHNV genome in host DNA (Tang & Lightner, 2002; 2006).

Annex 21 (contd)

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 IHHNV ~~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.

PCR commercial kits are available for detection of 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 detection of infection with IHHNV 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. Methods for surveillance, detection and diagnosis of IHHNV

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 <u>(wet mount)</u>	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
<u>In-situ DNA probes hybridisation</u>	d	<u>dc</u>	<u>bc</u>	b	a	a
<u>Dot-blot hybridisation</u>	<u>d</u>	<u>d</u>	<u>c</u>	<u>c</u>	<u>a</u>	<u>a</u>
PCR, Real-time PCR	a	a	a	a	a	a
Sequence	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 infectious hypodermal and hematopoietic necrosis virus

As indicated in Table 5.1, PCR or real-time PCR is-are the recommended methods 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 is 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~~
- or
- iii) Positive result by PCR

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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NB: There are OIE Reference Laboratories for **Infection with** infectious hypodermal and haematopoietic necrosis **virus** (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 **Infection with** infectious hypodermal and haematopoietic necrosis **virus**

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2015

CHAPTER 2.2.4.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

1. Scope

Infection with infectious myonecrosis virus means infection with the pathogenic agent infectious myonecrosis virus (IMNV), ~~which that 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 *Penaeus vannamei* stocks from Brazil) indicate that the disease was introduced from Brazil to Indonesia in 2006.

~~Infection with IMNV 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 similar to infection with IMNV IMN, but which are caused by two different types of virus: a nodavirus named *Penaeus vannamei* novavirus—PvNV (Tang *et al.*, 2007) and a nodavirus named *Macrobrachium rosenbergii* nodavirus—MnNV (see Chapter 2.2.8 White tail disease Infection with *Macrobrachium rosenbergii* nodavirus).~~

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 genotype 1 (YHV1), 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)

No data.

Annex 22 (contd)**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 as susceptible to infection with IMNV according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include: brown tiger prawn (*P. esculentus*), banana prawn (*P. merguensis*), and whiteleg shrimp (*P. 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 (*P. 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 possible 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 infection with 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, and sudden changes in water salinity or temperature. ~~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). 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).

2.3.1. 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 infection with IMNV is enzootic in farmed stocks of *P. vannamei*, its prevalence may reach 100% (Andrade *et al.*, 2007; Nunes *et al.*, 2004).

2.3.3. Geographical distribution

Infection with 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-Indonesia (Sutanto, 2011; Naim *et al.*, 2014). There are unofficial and anecdotal reports of infection with 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. Breeding for resistance-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).

Annex 22 (contd)**2.4.5. Restocking with resistant species**

While there are no published reports, some shrimp farms in Indonesia are believed to have stocked *P. monodon* and *P. stylirostris* because of data from a preliminary study ~~showing~~ suggesting that these species are 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 diseases from female spawners to their eggs or larvae, and the practice may reduce IMNV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry practices have been applied successfully to prevent infection with 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 or their spawned eggs or nauplii and discarding those that test PCR-positive (Andrade *et al.*, 2007). Following 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 virus diseases 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 infection with IMNV infection—using molecular methods (e.g. RT-PCR, nested RT-PCR, real-time RT-PCR, etc.) include postlarvae (PL), juveniles, subadults and adults. While IMNV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in larval stages, so these life stages may not be suitable for detecting IMNV or for 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.~~

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. Larger shrimp should be processed individually as the effect of pooling on diagnostic sensitivity has not been evaluated.

3.4. Best organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of infection with IMNV are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.5. Samples or 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. Clinical signs may have a sudden onset following stresses (e.g. capture by cast netting, feeding, and sudden changes in temperature or salinity). Affected shrimp present with visibly white tails. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Clinical signs may have a sudden onset following stresses (e.g. capture by cast-netting, feeding, and sudden changes in temperature or salinity).~~

4.1.2. Behavioural changes

~~Only shrimp in the 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.

Annex 22 (contd)**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 *P. vannamei* nodavirus (PvNV) 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).

Significant hypertrophy of the LO caused by accumulations of lymphoid organ spheroids (LOS) is a highly consistent lesion in shrimp with acute or chronic-phase infection with IMNV-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 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 detection of 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 (e.g. Qiagen RNA extraction kit, Promega and Roche RNA purification kit recommend using 25–50 mg of tissue³). 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)

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 22 (contd)

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

¹Template must be heated to >95°C boiled for 3 minutes and chilled on ice just prior to adding to reaction mix.

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

RT-PCR thermal cycling conditions:

PCR Primers	Temperature (°C)	Time	No. cycles	Amplicon length
	60, 95	30 minutes, 2 minutes	1	
4587F/4914R	95, 60 62	45 seconds, 45 seconds	39	328 bp
	60	7 minutes	1	

Nested PCR reaction (~~Amersham Biosciences pure Taq illustra™ PuReTaq™ Ready-To-Go PCR Beads #27 9558-04, GE Healthcare~~):

Reagent	25 µl reaction	Final concentration
DD H ₂ O	22.5 23 µl	–
Primer NF (100 ng µl ⁻¹ -10 µM)	4.0 0.5 µl	0.465 0.2 µM
Primer NR (100 ng µl ⁻¹ -10 µM)	4.0 0.5 µl	0.465 0.2 µM
Template ²	0.5 1.0 µl	–

²Template 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
	95	2 minutes	1	
4725 NF/ 4863 NR	95, 65, 72	30 seconds, 30 seconds, 30 seconds	39	139 bp
	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,
4914R	ACT-CGG-CTG-TTC-GAT-CAA-GT		2006
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 μ l 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-carboxyfluorescein (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 μ l RNA sample, ~~42.5 μ l~~ TaqMan Master mix (2 \times), ~~0.625 μ l~~ Multiscribe mix (40 \times), 300 nM each primer IMNV412F and IMNV545R, 200 nM. IMNVp1TaqMan probe in a ~~25–10–20 μ l~~ final volume. The ~~RT-qRT-PCR~~ thermal cycling conditions used are ~~48–50°C~~ for ~~30–3~~ minutes, ~~95°C~~ for ~~40–minutes–20~~ seconds followed by 40 cycles of ~~95°C~~ for ~~45–3~~ seconds and ~~60°C~~ for ~~4–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 QIAquick 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 μ l reaction containing 1 μ g plasmid DNA, followed by DNase I digestion at 37°C for 30 minutes for remove DNA. The length and integrity of the synthetic 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.

Annex 22 (contd)

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 infection with IMNV 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 (<u>wet mount</u>)	d	d	<u>ed</u>	<u>ed</u>	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	<u>ec</u>
DNA probes (ISH)	d	d	<u>ab</u>	<u>ab</u>	a	a
Nested RT-PCR or <u>real-time RT-PCR</u>	a	a	a	a	a	a
<u>real-time RT-PCR Sequencing</u>	<u>ed</u>	<u>ed</u>	<u>ad</u>	<u>ad</u>	<u>a</u>	<u>a</u>

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; ISH = *in-situ* hybridisation (ISH); RT-PCR = reverse-transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with infectious myonecrosis virus

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 infection with IMNV IMN from penaeid white tail disease caused by PvNV, in which the gross signs and histopathology mimics infection with IMNV IMN disease (Tang *et al.*, 2007).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with IMNV shall be is suspected if at least one of the following criteria is met:

i) Clinical clinical signs consistent with infection with IMNV

or

ii) histopathology consistent with infection with IMNV

or

iii) a positive result by nested RT-PCR or real-time RT-PCR.

~~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 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 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):~~

Infection with IMNV is considered to be confirmed if two or more of the following criteria are met:

i) histopathology consistent with infection with IMNV ~~Histological demonstration of diagnostic acute, transition or chronic phase IMNV lesions in the striated muscles or the LO.~~

ii) ISH positive result in target tissues ~~(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.~~

iii) One one ~~step or nested RT-PCR (followed by sequencing), or real-time RT-PCR with positive results for IMNV.~~

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* *

NB: There is an OIE Reference Laboratory for **infection with** infectious myonecrosis **virus** (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 **infection with** infectious myonecrosis **virus**

NB: FIRST ADOPTED IN 2009; MOST RECENT UPDATES ADOPTED IN 2012

CHAPTER 2.2.5.

INFECTION WITH *HEPATOBACTER PENA EI* (NECROTISING HEPATOPANCREATITIS)

1. Scope

Infection with *Hepatobacter penaei* means infection with the pathogenic agent **Candidatus Candidatus *Hepatobacter penaei***, an obligate 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 *Hepatobacter penaei*~~ is a pleomorphic, Gram-negative, intracytoplasmic bacterium (Nunan *et al.*, 2013). It is a member of the α -subclass of *Proteobacteria* (Frelier *et al.*, 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.*, 1996). The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.*, 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.*, 1996). Genetic analysis of ~~NHPB Candidatus *H. penaei*~~ associated with North and South American outbreaks of NHP suggests that the isolates are either identical or very closely related subspecies (Loy & Frelier, 1996; Loy *et al.*, 1996).

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 to –70°C and –80°C have been shown to retain infectivity in experimental transmission trials with *Penaeus vannamei* (Crabtree *et al.*, 2006; Frelier *et al.*, 1992).

2.1.4. Life cycle

Not applicable.

Annex 23 (contd)

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. penaei* according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: whiteleg shrimp (*P. 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: aloha prawn (*P. marginatus*), banana prawn (*P. merguensis*), blue shrimp (*P. stylirostris*), giant tiger prawn (*P. monodon*), northern brown shrimp (*P. aztecus*), northern pink shrimp (*P. duorarum*) and northern white shrimp (*P. setiferus*).

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

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* (Frelief *et al.*, 1995; Lightner, 1996). *Penaeus setiferus* is reportedly less susceptible to disease than *P. vannamei* (Frelief *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).

2.2.3.2. Susceptible stages of the host

NHPB infection with *H. penaei* has been demonstrated in juveniles, adults and broodstock of *P. vannamei*.

2.2.4.3. Species or sub-population predilection

See Sections 2.2.1 and 2.2.2.

2.2.5.4. Target organs and infected tissue

The target tissue is the hepatopancreas; with NHPB infection with *H. penaei* has been reported in all hepatopancreatic cell types.

2.2.6.5. 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 transmit it to other populations by horizontal transmission (Aranguren *et al.*, 2006; Lightner, 2005; Morales-Covarrubias, 2008; 2010; Vincent & Lotz, 2005).

Natural transmission of NHPB 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 via the water column may also play a role (Frelier *et al.*, 1993; 1995). NHPB 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 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).

2.2.7.6. 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

Horizontal transmission of NHPB *Candidatus-H. penaei* can be horizontal by through cannibalism; transmission or by contaminated water has also 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). *H. penaei* in faeces shed into pond water has also been suggested as a source of contamination (Aranguren *et al.*, 2006; Briñez *et al.*, 2003; Morales-Covarrubias *et al.*, 2006).

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). Lightner & Redman (1994) reported a prevalence of 0.77% in cultured *P. vannamei*, and 0.43% in cultured *P. stylirostris* collected from the Tumbes Region, Peru (Lightner & Redman, 1994).

Some Reported mean values for NHPB *Candidatus-H. penaei* prevalence in shrimp farms were 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-Gómez *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 occurrence of infection with replication rate of NHPB *Candidatus-H. penaei* in farms may increase during lengthy-long periods of high temperatures (>29°C) and high salinity changes (20–38% ppt) (Morales-Covarrubias, 2008). 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).

Annex 23 (contd)

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 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 disease.
- b) Shrimp starvation and cannibalism of infected shrimps NHPB infection with *H. penaei*, as well as and positive conditions for NHPB Candidatus *H. penaei* cultivation multiplication, are important factors for the spread of NHPB Candidatus *H. penaei* propagation in *P. vannamei*.
- c) The use of quick-hydrated lime (Ca(OH)₂) to treat the bottom of ponds during pond preparation before stocking can help reduce the incidence of NHPB infection with *H. penaei*.
- d) Preventive measures include raking, tilling, and removing sediments from the bottom of the ponds, prolonged sun-drying (through exposure to sunlight) of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, and drying and extensive liming of ponds.
- e) The use of specific pathogen-free (SPF) broodstock is an effective preventive measure.

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. penaei* is detected in the initial phase (Frelier *et al.*, 1995; Morales-Covarrubias *et al.*, 2012).

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~~ Breeding for resistance

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 disease agents).

2.4.8. General husbandry practices

The prevalence and severity of infection with *H. penaei* may be increased by rearing shrimp in relatively crowded or stressful conditions. Some husbandry practices have been successfully applied to the prevention of NHPB Candidatus infection with *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 the following 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 or age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. Larger shrimp should be processed individually as the effect of pooling on diagnostic sensitivity has not been evaluated.

3.4. Best organs or tissues

NHPB ~~Candidatus~~ *Hepatobacter penaei* infects most enteric tissue. The principal target tissue for NHPB ~~Candidatus~~ *H. penaei* is the 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; Frelieir *et al.*, 1993; Lightner, 1996; Morales-Covarrubias *et al.*, 2012).

3.5. Samples or tissues those are not suitable

NHPB ~~Candidatus~~ *H. penaei* does not replicate in the midgut, caeca, connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells.

4. Diagnostic methods

4.1. Field diagnostic methods

The prevalence and severity of NHPB 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, ablation, and marginal water quality (e.g. 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 epicomensal 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.

Annex 23 (contd)

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 disease 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* show 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, 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 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 ($1-5 \text{ field}^{-1} \text{ organism}^{-1}$) and cell detachment.

Acute phase: infiltration of haemocytes, increased numbers of deformed tubules ($6-10 \text{ field}^{-1} \text{ organism}^{-1}$), encapsulation present in different regions of the sample (i.e. atrophied tubules surrounded by multiple layers of haemocytes).

Transition phase: infiltration of haemocytes, increased numbers of deformed tubules ($11-15 \text{ field}^{-1} \text{ organism}^{-1}$), 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.

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4.3.1.2. Agent isolation and identification*4.3.1.2.1. Cell culture or artificial media*

~~NHPB Candidatus *Hepatobacter 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), are available for *H. penaei* detection.

4.3.1.2.3. Molecular techniques

ISH and PCR tests for ~~NHPB detection of *H. penaei*~~ have been developed, and PCR kits for ~~NHPB~~ are commercially available. ~~PCR tests for *H. penaei* have been developed and a number of methods and commercial products using these methods are available~~ (Loy & Frelief, 1996; Loy *et al.*, 1996). Gene probes and PCR methods provide greater diagnostic sensitivity than ~~de~~-classic histological approaches to ~~NHP~~ diagnose 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, digoxigenin-11-dUTP (DIG)-labelled probes for NHPB Candidatus *H. penaei* may be produced in the laboratory.~~ The ISH method of Loy & Frelief (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 & Frelief, 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 2.2.3 *Infection with infectious hypodermal and haematopoietic necrosis virus* for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

4.3.1.2.3.2. 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 fragment~~ 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. DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega)⁴.
- ii) The following controls should be included when performing 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.

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*.

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- 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 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).

- i) The PCR primers and TaqMan probe were selected from the 16S, rRNA gene of ~~NHPB Candidatus~~ *H. penaei* (GenBank U65509) (Loy & Frelie., 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,N-tetramethyl-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, and also to rule out reagent contamination with the specific target of the assay.~~ A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from ~~NHPB~~ *H. penaei*-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).

Annex 23 (contd)

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-*Hepatobacter penaei*~~ is unculturable using traditional bacteriological methods, thus ~~NHPB-infection with *H. penaei*~~ must be maintained through continual exposure of uninfected ~~*LP. vannamei*~~ stock to a population undergoing an epizootic of ~~NHPB-infection with *H. penaei*~~.

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.

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	ed	ed	bd	d
Bioassay	d	d	d	d	c	d
Direct LM (<u>wet mount</u>)	d	d	ed	d	ed	d
Histopathology	d	bd	bc	c	a	bc
<i>In-situ</i> DNA probes	a	a	a	a	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	bc	bc
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 a suspect case

Infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

i) histopathology consistent with infection with *H. penaei*

or

ii) ISH positive results in target tissues

or

ii) a positive result by PCR or real-time PCR.

~~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

Infection with *H. penaei* is considered to be confirmed if two or more of the following criteria are met:

i) histopathology consistent with infection with *H. penaei*

ii) ISH positive result in target tissues

iii) PCR (followed by sequencing), or real-time PCR with positive results for infection with *H. penaei*.

~~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 lesions suggestive of NHPB infection with *H. penaei*.~~
- ~~• PCR positive results for NHPB infection with *H. penaei*.~~

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* *

NB: At the time of publication (2015) there was not yet an OIE Reference Laboratory for infection with *Hepatobacter penaei* (necrotising hepatopancreatitis) (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/>).

NB: FIRST ADOPTED IN 2012; MOST RECENT UPDATES ADOPTED IN 2015

CHAPTER 2.2.6.

INFECTION WITH TAURA SYNDROME VIRUS**1. Scope**

Infection with Taura syndrome virus means infection with the pathogenic agent Taura syndrome virus (TSV), of the Genus *Aparavirus*, Family *Dicistroviridae*, Order *Picornavirales*. Genus *Aparavirus* 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 of Taura syndrome (TS) is TSV was described as the cause of the disease commonly known as Taura syndrome by Bonami *et al.* (1997) and Mari *et al.* (1998; 2002). At least four genotypes (strains) of TSV have been documented based on the gene sequence encoding VP1 the largest and presumably dominant of the three major structural proteins of the virus. Based on VP1 sequence variations, these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; 3) the Belize group; and 4) the Venezuelan group (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 in CsCl. The genome of TSV consists of a linear, positive-sense single-stranded RNA 10,205 nucleotides in length, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for 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: TS in Ecuador was initially linked to fungicide contamination of shrimp farms, a contention that was supported by litigation for ~ 16 years after the disease was scientifically shown to have a viral aetiology (Bonami *et al.*, 1997; Hasson *et al.*, 1995; Lightner, 2005). Hence, several papers in the literature propose a toxic aetiology for TS (Intriago *et al.*, 1997; Jimenez, 1992; Jimenez *et al.*, 2000).

Annex 24 (contd)**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 as susceptible to infection with TSV according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include: greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), giant tiger prawn (*P. monodon*), northern white shrimp (*P. setiferus*), blue shrimp (*P. stylirostris*), and whiteleg shrimp (*P. vannamei*).

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 according to Chapter 1.5. of the Aquatic Code include: fleshy prawn (*P. chinensis*), giant river prawn (*Macrobrachium rosenbergii*), the copepod *Erqasilus manicatus*, and the barnacles *Chelonibia patula* and *Octolasmis muelleri*.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: northern pink shrimp (*P. duorarum*), kuruma prawn (*P. japonicus*), southern white shrimp (*P. schmitti*), gulf killifish (*Fundulus grandis*), blue crab (*Callinectes sapidus*), the crabs *Uca vocans* and *Sesarma mederi*, and Indo-Pacific swamp crab (*Scylla serrata*).

2.2.3. Susceptible stages of the host

Infection with TSV has been documented in all life stages (i.e. post-larvae [PL], juveniles and adults) of *P. vannamei* (the most economically significant of the two principal host species) except eggs, zygotes and larvae (Lightner, 1996a).

2.2.4. Species or subpopulation predilection (probability of detection)

No data. All postlarval stages of *P. vannamei*, and populations of other known susceptible species.

2.2.5. 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 with 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 infection with 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.

2.2.7. ~~6.~~ Vectors

Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV-infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus ~~gallus domesticus~~*, used as a laboratory surrogate for all shrimp-eating birds) thus suggesting that the virus can retain infectivity when passed through the gastro-intestinal system of any bird species. These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (Garza *et al.*, 1997; Vanpatten *et al.*, 2004).

Aquatic insects: the water boatman (*Trichocorixa reticulata* [Corixidae], an aquatic insect that feeds on shrimp carcasses in shrimp farm ponds), has also been shown to serve as a ~~mechanical~~ vector of TSV (Brock, 1997; Lightner, 1995, 1996a, 1996b).

~~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

Infection with TSV is best known as a disease of nursery- or grow-out-phase *P. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with infection with TSV 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 through 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 enzootic in farmed stocks, the prevalence of infection with TSV has been found in various surveys to range from 0 to 100% (Brock, 1997; Jimenez *et al.*, 2000; 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).

Annex 24 (contd)

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, TS and/or Infection with TSV has 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 enzootic 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 these regions (Hasson *et al.*, 1999a; Lightner, 1996a; 2005; 2011; Lightner *et al.*, 2012).

Asia and the Middle East: Infection with 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* not selected for resistance (Chang *et al.*, 2004; Lightner, 2011; Nielsen *et al.*, 2005; Tang & Lightner, 2005). Recently In-During 2010 and 2011, infection with TSV has also been was associated with significant mortalities in farmed *P. indicus* being farmed in Saudi Arabia. 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 (Tang *et al.*, 2012; Wertheim *et al.*, 2009).

2.3.4. Mortality and morbidity

At a farm level, TS epizootics outbreaks of infection with TSV involving unselected (i.e. not selected for TSV resistance) stocks of *P. vannamei* not selected for resistance, the principal host species for infection with 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 infection with TSV 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 for resistance

After TSV emerged in Ecuador in 1992–1994, *P. stylirostris* were found that possessed resistance to infection with TSV (genotype 1, MAb 1A1 Type A). Following from this discovery and due to the disease occurrence in 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 infection with TSV in Central America, improved TSV resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region (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 infection with TSV 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 that TSV might be transmitted vertically (transovarian transmission), despite ~~no~~ the lack of published reports documenting this route of transmission. Disinfection of eggs and larvae (Chen *et al.*, 1992) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry and disease control and management practices have been used successfully to reduce the risks of infection with TSV ~~infections and disease~~ occurring during farm grow-out. These include the application of PCR assays for prescreening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; 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 infection with TSV. ~~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).~~

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection with TSV include PL, juveniles and adults. While TSV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for TSV detection or certification of freedom from infection with TSV.

3.2. 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.~~

Annex 24 (contd)

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. Larger shrimp should be processed individually as the effect of pooling on diagnostic sensitivity has not been evaluated.

3.4. Best organs and tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of infection with TSV is the cuticular epithelium. In chronic infections the 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 or 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 with TSV.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only acute-phase ~~TS clinical infection with TSV-disease~~ TSV-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 clinical infection with TSV-disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase clinical infection with TSV ~~TS-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 ~~TS-disease~~ outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicates the presence of a serious disease outbreak (which is often either infection with TSV or ~~infection with white spot syndrome virus when sea birds are observed~~ to the farm manager).

4.2. Clinical methods

4.2.1. Gross pathology

Infection with the TSV 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 infection with TSV are unique and provide a presumptive diagnosis of the disease.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase infection with TSV include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish 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 infection with TSV ~~TS~~ 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 outbreaks of infection with TSV, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of infection with TSV. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites of resolving TSV lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (Brock, 1997; Hasson *et al.*, 1999b; Lightner, 1996a; 2011).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of infection with TSV in which persistently infected shrimp show no obvious signs of disease (Brock, 1997; Hasson *et al.*, 1999b; Lightner, 1996a; 1996b; 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 infections with TSV, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.*, 1999b; 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 infection with TSV 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 infection with TSV.

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 infection with TSV, the antennal gland tubule epithelium is also destroyed. Prominent in the multifocal cuticular lesions are conspicuous foci of affected cells that display an increased eosinophilia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often extremely abundant in these infections with TSV acute-phase lesions and these are generally presented as spherical bodies (1–20 µm in diameter) that range in staining from eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give acute-phase TS lesions a characteristic 'peppered' or 'buckshot-riddled' appearance, which is considered to be pathognomonic for the infection when there is no concurrent necrosis of the parenchymal cells of the LO tubules. The absence of necrosis of the LO in acute-phase infection with TSV ~~infections~~ distinguishes it from acute-phase infection with yellowhead virus genotype 1 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 infection with TSV from the transitional phase of the disease (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).

Annex 24 (contd)**4.2.3.2. Transition (recovery) phase of infection with Taura syndrome virus**

In the transitional phase of infection with TSV, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by *Vibrio* spp. (Hasson *et al.*, 1999b; Lightner, 1996a; 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 infection with Taura syndrome virus

Shrimp in the chronic phase of infection with TSV display no gross signs of infection, and histologically the only sign of infection is the presence of numerous prominent LOS, which may remain associated with the main body of the paired LO, or which may detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connective tissues, etc.). Such LOS are spherical accumulations of LO cells and haemocytes and may be distinguished from normal LO tissues by their spherical nature and the lack of the central vessel that is typical of normal LO tubules. When assayed by ISH with a cDNA probe for TSV (or with MAb 1A1 using ISH) some cells in the LOS give positive reactions to the virus, while no other target tissues react (Hasson *et al.*, 1999b; Lightner, 1996a; 1996b; 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 infection with TSV-~~infection~~) focal lesions of acute-phase infection with TSV-~~infection~~ in cuticular epithelial cells. Preparations presenting acute-phase infection with TSV-~~infection~~ 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~~ Although one publication ~~that~~ incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle et al., 2003), two other laboratories ~~that~~ repeated the study ~~and~~ both found that TSV does not infect or replicate in primate or human cell lines with that are known to have 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 infection with TSV 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 an outbreak of infection with 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).

Annex 24 (contd)

- 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 **infection with** 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 **infection with** 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 (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 **infection with** 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~~)⁵.
- 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.

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*.

- 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 detection of 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 detection of 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.

4.3.1.2.7.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled cDNA probes for detection of TSV may be produced in the laboratory. The ISH method provides greater diagnostic sensitivity than do more traditional methods for TSV detection and diagnosis that employ classic histological methods (Hasson *et al.*, 1999a; Lightner, 1996a; 1999; Lightner & Redman 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 infection with 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 2.2.3 Infection with infectious hypodermal and haematopoietic necrosis virus for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

False-negative ISH results may occur with Davidson's fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson's fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This hydrolysis can be 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.~~

Annex 24 (contd)

Primer	Product	Sequence	Temperature	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%

The RT-PCR method outlined below for detection of 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. 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.
- ii) The RT-PCR assay is carried out in solution, using 40–5 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.

<u>Reagent</u>	<u>Volume</u>	<u>Final concentration</u>
<u>dH₂O</u>	<u>5.5 µl</u>	
<u>2× Reaction Mix</u>	<u>12.5 µl</u>	<u>1×</u>
<u>Primer Forward/Reverse (10 M each)</u>	<u>1.0 µl</u>	<u>0.4 µM</u>
<u>RT/Taq enzyme Mix</u>	<u>1.0 µl</u>	
<u>RNA template*</u>	<u>5.0 µl</u>	<u>1–50 ng</u>

- 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.

Annex 24 (contd)

~~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 IHNN.~~

4.3.1.2.7.3. Real-time RT-PCR method for TSV

Real-time RT-PCR methods have been developed for the detection of TSV. These methods have the advantages of speed, specificity and sensitivity. The sensitivity of ~~real~~ real-time RT-PCR is ~100 copies of the target sequence from the TSV genome (~~Dahr~~ Dhar et al., 2002; Tang et al., 2004).

The real-time RT-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 AF4277675) 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-carboxyfluorescein (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 real-time RT-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 Biosystems; 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~~ 50°C for 30 minutes and initial denaturation at 95°C for ~~40 minutes-20 seconds~~, followed by 40 cycles of denaturation at 95°C for ~~15 3~~ 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.~~

Annex 24 (contd)

- 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).

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 ~~infection with~~ 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 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. Methods ~~for~~ surveillance, detection and diagnosis

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 (wet mount)	d	d	c	dc	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
<i>In-situ</i> DNA probes	d	c	b	b	a	a
RT-PCR, Real-time RT-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-~~transcription transcriptase~~-polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from **infection with Taura syndrome virus**

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.7.2) **or real-time RT-PCR (Section 4.3.1.2.7.3) are** the recommended methods 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

Infection with TSV shall be suspected if at least one of the following criteria is met:

i) histopathology consistent with infection with TSV

or

ii) a positive result by RT-PCR or real-time RT-PCR.

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 TS, 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

Infection with TSV is considered to be confirmed if two or more of the following criteria are met:

i) histopathology consistent with infection with TSV

ii) ISH positive result in target tissues

iii) RT-PCR (followed by sequencing), or real-time RT-PCR with positive results for **infection with TSV**.

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 lesions of infection with TSV 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 lesions are pathognomonic for infection with 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).

Annex 24 (contd)

- ~~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 infection with 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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NB: There is an OIE Reference Laboratory for Infection with Taura syndrome virus
(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 Infection with Taura syndrome virus

NB: FIRST ADOPTED IN 2000; MOST RECENT UPDATES ADOPTED IN 2015

CHAPTER 2.2.8.

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

1. Scope

Infection with *Macrobrachium rosenbergii* nodavirus means infection with the pathogenic agent *Macrobrachium rosenbergii* nodavirus (MrNV), (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 (MrNV) 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 MrNV (primary) and extra small virus (XSV) (associate) (Qian *et al.*, 2003; Romestand & Bonami, 2003). MrNV is important in WTD disease outbreaks in prawns, but the role of XSV in pathogenicity remains unclear. Strains are not yet known. MrNV belongs in the family Nodaviridae (Bonami *et al.*, 2005; King *et al.*, 2012 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 post-larvae (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 at 65°C for 2 hours destroyed infectivity of MrNV 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 as susceptible to infection with MrNV 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.~~

Annex 25 (contd)**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 (*Penaeus vannamei*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results (but not active infection) have been reported in the following species: kuruma prawn (*P. japonicus*), Indian white prawn (*P. indicus*), giant tiger prawn (*P. monodon*), dragonfly (*Aeshna* sp.), giant water bug (*Belostoma* sp.), beetle (*Cybister* sp.), backswimmer (*Notonecta* sp.), hairy river prawn (*Macrobrachium rude*), monsoon river prawn (*Macrobrachium malcolmsonii*), brine shrimps (*Artemia* sp.) and red claw crayfish (*Cherax quadricarinatus*).

2.2.32. 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).

2.2.34. Species or subpopulation predilection (probability of detection)

No mortality was observed either in naturally or experimentally (MrNV/XSV) infected subadult and adult prawns. Experimental studies confirmed vertical transmission from infected broodstock to PL (Sudhakaran *et al.*, 2006a-2007a).

2.2.45. Target organs and infected tissue

MrNV 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 MrNV WTD from broodstock to larvae and PL. Experiments proved that Pleopods are would be a convenient source of RNA for non-destructive screening of MrNV and XSV without stress to the prawns (Sahul Hameed *et al.*, 2004a).

2.2.56. Persistent infection with lifelong carriers

Challenge experiments indicate long-term persistent infection in adults and also the possibility of transmitting MrNV WTD from broodstock to larvae and PL (Sahul Hameed *et al.*, 2004a; Sudhakaran *et al.*, 2006a-2007a).

2.2.67. 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., *Aeshna* 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 MrNV-WTD infection has been reported in hatchery-reared larvae and PL of *M. rosenbergii*. The disease WTD 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-2007a).

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; 2004b).

2.3.3. Geographical distribution

The disease 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; Wang *et al.*, 2008), 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 MrNV-WTD, 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 infection with MrNV-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 infection with MrNV-WTD, but act as carriers (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 MrNV-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~~ Information on control and prevention of infection with MrNV is limited-WTD. However, proper preventive measures, such as screening of brood stock and PL, and good management practices may help to prevent infection with MrNV-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 MrNV-WTD.

2.4.3. Immunostimulation

No reports available concerning the use of immunostimulants infection with MrNV-WTD.

2.4.4. ~~Resistance breeding~~ Breeding for resistance

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).

Annex 25 (contd)**2.4.8. General husbandry practices**

Experimental infection confirmed the possibility of horizontal and vertical transmission of MrNV-WTD in culture systems (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a; Sudhakaran *et al.*, 2006a-2007a). 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 infection with MrNV-WTD in culture systems (Chen *et al.*, 1992; Sri Widada *et al.*, 2003; Sudhakaran *et al.*, 2008-2008a). There is no evidence of WTD prevention that crop rotation either with rice or polyculture with fish prevents infection with MrNV. 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-2006a) and Ravi *et al.* (2009) in their studies. Based on their results, it would seem that mixed culture of *M. rosenbergii* with *P. monodon* should be avoided before adopting any preventive measures in the management of infection with MrNV.

3. Sampling**3.1. Selection of individual specimens**

Infection with MrNV WTD of freshwater prawns is mainly diagnosed ~~indicated~~ 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 MrNV WTD and diagnosis is not easy, particularly in the earlier stages of infection. ~~WTD affected-PL~~ affected by infection with MrNV 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 or PL with prominent signs of whitish muscle in the abdominal region are collected from disease outbreak areas. Samples are washed in sterile saline, transferred to sterile tubes, transported to the laboratory on dry ice and stored at -70°C until further processed (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

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. Larger shrimp should be processed individually as the effect of pooling on diagnostic sensitivity has not been evaluated. 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) are a convenient source of RNA for non-destructive screening of MrNV and XSV ~~without stress to the broodstock~~ (Sahul Hameed *et al.*, 2004a).

3.5. Samples or 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 discolouration 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 MrNV ~~WTD~~ and mortality reaches a maximum in about 5 days after the appearance of whitish discolouration. Floating exuviae (moult) 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

~~Infection with MrNV~~ ~~WTD of *M. rosenbergii*~~, resulting from MrNV and XSV infection, is mainly ~~diagnosed~~ ~~indicated~~ by whitish coloration of abdominal muscle; however, this clinical sign is not ~~pathognomonic~~ ~~specific to WTD~~, but it is ~~associated with high mortality rates~~.

4.2.2. ~~Clinical chemistry~~

~~The prophenol oxidase activity significantly increased in MrNV 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 MrNV and XSV injected prawns on day 1 and 3 p.i. and there was no significant change in the level of hemocyanin in MrNV 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 MrNV in infected cells can be demonstrated in histological sections using a DIG-labelled DNA *in-situ* hybridisation probe specific for MrNV (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 par-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 MrNV. 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).

Annex 25 (contd)**4.3. Agent detection and identification methods****4.3.1. Direct detection methods**

Genome and antibody-based diagnostic methods are available to detect MrNV/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*

MrNV/XSV can be easily propagated in the C6/36 mosquito *Aedes albopictus* cell line (Sudhakaran *et al.*, 2007a 2007b) 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 2007b). The C6/36 cell line was found to be useful for propagation of these viruses, and viral replication was confirmed by RT-PCR, acridine orange staining, infectivity studies and electron microscopy. A specific cytopathic effect was not observed in MrNV-infected cell lines, but multiple vacuolations were observed. 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 MrNV include the ELISA described by Romestand & Bonami (2003) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (Qian *et al.*, 2006).

4.3.1.2.2.1. 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-MrNV 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, H₂O₂ at a 0.33% final concentration).
- vii) Stop the reaction after 15 minutes by adding 25 µl of H₂SO₄ 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.

Annex 25 (contd)4.3.1.2.2.2. *TAS-ELISA protocol (Qian et al., 2006)*

- i) Coat ELISA plates with rabbit polyclonal antibody raised against MrNV 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 MrNV 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, H₂O₂ at a 0.33% final concentration).
- viii) Stop the reaction after 15 minutes by adding 25 µl H₂SO₄ 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 MrNV/XSV developed by Sri Widada *et al.* (2003) and Sahul Hameed *et al.* (2004a; 2004b) is recommended for all situations. MrNV 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 (RT-nPCR) is also available and recommended for screening broodstock and seed (Sudhakaran *et al.*, [2006a](#) [2007a](#)).

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}.

Annex 25 (contd)*RT-PCR protocol*

Three RT-PCR methods are described to detect MrNV 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 MrNV and XSV in PL of prawns collected from suspected WTD outbreaks. The second protocol is a sensitive RT-nPCR protocol described by Sudhakaran *et al.* (2006a 2007a). 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 MrNV 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 MrNV or XSV in infected prawn PL or juveniles (Sahul Hameed *et al.*, 2004b; Sri Widada *et al.*, 2003; Sudhakaran *et al.*, 2007b 2008b):

The following controls should be included in every RT-PCR assay for MrNV or XSV: a) a known MrNV/XSV-negative tissue sample; b) a known MrNV/XSV-positive sample (tissue or purified virus); and c) a 'no-template' control.

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 MrNV 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 MrNV and a 546 bp product for XSV. The sensitivity of the assay is approximately 2.5 fg of total RNA.

PCR primer sequences for MrNV (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 RT-nPCR for detection of MrNV and XSV (Sudhakaran *et al.*, 2007a)

The RT-nPCR is more sensitive and useful for screening seed and broodstock (Sudhakaran *et al.*, 2006a 2007a):

For the RT-nPCR, 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 RT-nPCR, add 2 µl 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 MgCl₂, 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 RT-nPCR 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 RT-nPCR 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-product** will be amplified for MrNV and 546 bp **DNA-product** for XSV in the first PCR step. In the nPCR step, a 205 bp product indicates detection of MrNV and a 236 bp product indicates detection of XSV. The detection sensitivity of the RT-nPCR is ~1000-fold greater than the one-step RT-PCR.

Annex 25 (contd)

The sequence of external primers for MrNV and XSV is given in protocol 1 and the sequence of internal primers is given below:

The sequence of internal primers for MrNV (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 MrNV 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 MrNV and XSV in a single-tube, one-step multiplex RT-PCR assay can be performed. The reaction is performed in 50 µl RT-PCR buffer containing 20 pmol of each primer specific to MrNV 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 MrNV and XSV are present in the sample, a 681 bp **DNA-product** for MrNV and 500 bp **DNA-product** for XSV will be amplified. The presence of both 681 bp and 500 bp products indicates the presence of MrNV and XSV. The detection sensitivity of the multiplex RT-PCR assay is approximately 25 fg of total RNA.

PCR primer sequences for MrNV (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: Real-time RT-PCR assay

Real-time RT-PCR assay can be performed to quantify the MrNV/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 real-time RT-PCR using real-time PCR mixture (1 µl of cDNA [10 ng], 6 µl of sterile water, 0.5 µl of each primer specific to MrNV and XSV [25 µM concentration] and 2 µl of reaction mixture containing Fast Start *Taq* polymerase, dNTP mix, SYBR Green, 10 mM MgCl₂ 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.

Annex 25 (contd)

- v) The number of viral cDNA copies in the sample will be determined using Light Cycler fit point method.

PCR primer sequences for MrNV (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.
- 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 MrNV.
- 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 MgCl₂).
- 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 MrNV and XSV in the freshwater prawn. A set of four primers, two outer and two inner, have been designed separately for detection of MrNV and XSV. In addition, a pair of loop primers specific to MrNV and XSV has been used to accelerate LAMP reaction.

Annex 25 (contd)

- 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 MgSO₄, 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 MrNV/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*

MrNV 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~~ **homogenise** 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.

Annex 25 (contd)

Table 5.1. Methods for targeted MrNV-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
<i>In-situ</i> DNA probes	c	b	b	c	a	a
PCR, Real-time RT-PCR, RT-PCR	a	a	a	a	a	a
Sequence	d	d	d	a	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; RT-PCR = reverse transcription 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 RT-nPCR.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with MrNV is considered to be confirmed or suspected if at least two or more one of the following criteria are met:

i) clinical signs consistent with infection with MrNV

or

ii) histopathology consistent with infection with MrNV

or

iii) a positive result by RT-PCR.

or

iv) a positive result by real-time RT-PCR.

Appearance of whitish muscle associated with mortality is a suspected case of infection with MrNV 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 colouration. Corroborative diagnostic criteria are summarised in Section 4.2 above.

7.2. Definition of confirmed case

Infection with MrNV is considered to be confirmed if two or more of the following criteria are met:

- i) histopathology consistent with infection with MrNV
- ii) ISH positive result in target tissues.
- iii) RT-PCR (followed by sequencing).
- iv) **Real-time RT-PCR.**

~~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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Annex 25 (contd)

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NB: There is an OIE Reference Laboratory for Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease) (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 Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

NB: FIRST ADOPTED IN 2009; MOST RECENT UPDATES ADOPTED IN 2012

CHAPTER 1.5.

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

Article 1.5.1.

The purpose of this chapter is to provide criteria for determining which species are listed as susceptible in Article ~~1.5.2- X.X.2~~ of each *disease-specific* chapter in the *Aquatic Code*.

Article 1.5.2.

Scope

Susceptibility may include clinical or non-clinical *infection* but does not include species that may carry the *pathogenic agent* without replication.

The decision to list an individual a species as susceptible in disease-specific chapters should be based on a finding that the evidence is definite in accordance with Article 1.5.3. All species in a taxonomic group may be listed as susceptible when certain criteria are met in accordance with Article 1.5.9.

~~However, possible~~ Possible susceptibility of a species is also important information and this should also be included in Section ~~2.2.4, 2.2.2. Species with incomplete evidence for susceptibility~~ entitled «Susceptible host species» of the relevant *disease-specific* chapter of the *Aquatic Manual*.

Article 1.5.3.

Approach

A three-stage approach is outlined in this chapter to assess susceptibility of a species to *infection* with a specified *pathogenic agent* and is based on:

- 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.).

Article 1.5.4.

Stage 1: criteria to determine whether the route of transmission is consistent with natural pathways for the infection

The evidence should be classified as transmission through:

- 1) natural occurrence; includes situations where *infection* has occurred without experimental intervention e.g. *infection* in wild or farmed populations; or
- 2) non-invasive experimental procedures; includes cohabitation with infected hosts, *infection* by immersion or ingestion; or
- 3) invasive experimental procedure; includes injection, exposure to unnaturally high loads of pathogen, or exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment.

Annex 26 (contd)

Consideration needs to be given to whether experimental procedures (e.g. inoculation, infectivity load) mimic natural pathways for *disease* transmission. Consideration should also be given to environmental factors as these may affect host resistance or transmission of the pathogen.

Article 1.5.5.

Stage 2: criteria to determine whether the pathogenic agent has been adequately identified

The *pathogenic agent* should be identified and confirmed in accordance with the methods described in Section 7 (corroborative diagnostic criteria) of the relevant disease chapter in the *Aquatic Manual*, or other methods that have been demonstrated to be equivalent.

Article 1.5.6.

Stage 3: criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection

A combination of the following criteria should be used to determine *infection* (see Article 1.5.7.):

- A. the *pathogenic agent* is multiplying in the host, or developing stages of the *pathogenic agent* are present in or on the host;
- B. viable *pathogenic agent* is isolated from the proposed *susceptible species*, or infectivity is demonstrated by way of transmission to naive individuals;
- C. clinical or pathological changes are associated with the *infection*;
- D. the specific location of the pathogen corresponds with the expected target tissues.

The type of evidence to demonstrate *infection* will depend on the *pathogenic agent* and potential host species under consideration.

Article 1.5.7.

Outcomes of the assessment

The decision to list a species as susceptible should be based on a finding of definite evidence. Evidence should be provided for the following:

- 1) transmission has been obtained naturally or by experimental procedures that mimic natural pathways for the *infection* in accordance with Article 1.5.4.;

AND

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

AND

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

Article 1.5.8.

Species for which there is incomplete evidence for susceptibility

The decision to list a species as susceptible in Article 1.5.2. of each *disease*-specific chapter should be based on a finding that the evidence is definite.

However, where there is insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3. because transmission does not mimic natural pathways of *infection*, or the identity of the *pathogenic agent* has not been confirmed, or *infection* is only partially supported, information will be included in the relevant *disease-specific* chapter in the *Aquatic Manual*.

If there is insufficient evidence to demonstrate susceptibility of a species, the *Competent Authority* should assess the risk of spread of the pathogen under consideration, in accordance with the recommendations in Chapter 2.1., prior to the implementation of import health measures.

Article 1.5.9.

Listing susceptible species at a taxonomic ranking higher than species Pathogenic agents with a broad host range

For pathogenic agents with a broad host range, it may be appropriate for the outcome of the assessment to be made at a taxonomic ranking higher than species (e.g. genus, family). It may be appropriate for the outcome of the assessment to be made at a taxonomic classification higher than species for a pathogenic agent that has a broad host range. A pathogenic agent will be considered to have a broad host range when it has been demonstrated as susceptible in at least three families.

1) A decision to conclude susceptibility for a taxonomic level above species should only be made where:

A. susceptibility has been demonstrated in at least one species from within each of three or more families;

AND

AB. more than one species within the family taxonomic ranking has been found to be susceptible in accordance with the criteria above;

AND

BC. no species within the taxonomic group ranking has been found to be refractory to infection.

The taxa taxonomic ranking chosen should be the lowest level supported by this evidence.

2) Evidence that a species is refractory to infection may include includes:

A. absence of infection in a species exposed to the pathogenic agent in natural settings where the pathogen is known to be present and it has causes caused disease in susceptible species;

B. absence of infection in species exposed to the pathogenic agent through a controlled challenges using experimental procedures.

 — Text deleted.

CHAPTER 5.4.

CRITERIA TO ASSESS THE SAFETY OF AQUATIC ANIMAL PRODUCTS COMMODITIES

In the context of this chapter the word 'safety' is applied only to animal health considerations for *listed diseases*.

Article 5.4.1.

Criteria to assess the safety of ~~aquatic animals and aquatic animal products~~ imported (or transited) for any purpose regardless of the disease X status of the exporting ~~for any purpose from a country, zone or compartment not declared free from disease X~~

~~In all disease chapters, point Point 1 of Article X.X.3. of all disease-specific chapters (Sections 8-11), lists aquatic animals and aquatic animal products that can be imported (or transited) for any purpose regardless of the disease X status of the exporting~~ traded for any purpose from a country, zone or compartment not declared free from disease X. The criteria for inclusion of ~~aquatic animals and aquatic animal products~~ in point 1 of Article X.X.3. are based on the absence of the *pathogenic agent* in the ~~traded aquatic animals and aquatic animal products~~ or inactivation of the *pathogenic agent* by treatment or processing.

The assessment of the safety of the ~~aquatic animals and aquatic animal products~~ using the criteria relating to treatment or processing can only be undertaken where treatments or processing are well defined. It may not be necessary to provide details of the entire treatment or process undertaken. However, the steps considered critical in the inactivation of the *pathogenic agent* of concern should be detailed.

It is assumed that treatment or processing (i) uses standardised protocols, which include the steps considered critical in the inactivation of the *pathogenic agent* of concern; (ii) is conducted in accordance with good manufacturing practices ~~Good Manufacturing Practices~~; and (iii) that any other steps in the treatment, processing and subsequent handling of the *aquatic animal product* do not jeopardise the safety of the traded *aquatic animal product*.

Criteria

For an ~~aquatic animal or aquatic animal product~~ to be considered safe for *international trade* under the provisions of Article X.X.3., it should comply with the following criteria:

- 1) Absence of pathogenic agent in the traded ~~aquatic animal or aquatic animal product~~:
 - a) There is strong evidence that the *pathogenic agent* is not present in the tissues from which the ~~aquatic animal or aquatic animal product~~ is derived.

AND

- b) The water (including ice) used to process or transport the ~~aquatic animal or aquatic animal product~~ is not contaminated with the *pathogenic agent* and the processing prevents cross contamination of the ~~aquatic animal or aquatic animal product to be traded~~.

OR

- 2) Even if the *pathogenic agent* is present in, or contaminates the tissues from which the ~~aquatic animal or aquatic animal product~~ is derived, the treatment or processing to produce the ~~aquatic animal or aquatic animal product to be traded~~ inactivates the *pathogenic agent*:

- a) physical (e.g. temperature, drying, smoking);

AND/OR

- b) chemical (e.g. iodine, pH, salt, smoke);

AND/OR

- c) biological (e.g. fermentation).

Annex 27 (contd)

Article 5.4.2.

Criteria to assess the safety of ~~aquatic animals or~~ aquatic animal products imported (or transited) for retail trade for human consumption regardless of the disease X status of the exporting ~~from a country, zone or compartment not declared free from disease X~~

~~In all disease chapters, point~~ Point 1 of Article X.X.12. (amphibian and fish disease-specific chapters) and Article X.X.11. (crustacean and mollusc disease specific chapters) lists ~~aquatic animals or aquatic animal products~~ for retail trade for human consumption. The criteria for inclusion of ~~aquatic animals or aquatic animal products~~ in point 1 of Article X.X.12. (amphibian and fish disease-specific chapters) and Article X.X.11. (crustacean and mollusc disease-specific chapters) include consideration of the form and presentation of the product, the expected volume of waste tissues generated by the consumer and the likely presence of viable *pathogenic agent* in the waste.

For the purpose of this criterion retail means the selling or provision of ~~the aquatic animals or aquatic animal products~~ directly to the consumer with the intended purpose of human consumption. The retail pathway may also include wholesale distribution of the products provided they are not further processed by the wholesale distributor or the retailer, i.e. are not subjected to actions such as gutting, cleaning, filleting, freezing, thawing, cooking, unpacking, packing or repackaging.

It is assumed that: (i) the ~~aquatic animals or aquatic animal products~~ are used for human consumption only; (ii) waste may not always be handled in an appropriate manner that mitigates the introduction of the *pathogenic agent*; the level of risk is related to the waste disposal practices in each Member's country or territory; (iii) treatment or processing prior to importation is conducted in accordance with good manufacturing practices ~~Good Manufacturing Practices~~, and (iv) any other steps in the treatment, processing and subsequent handling of the ~~aquatic animals or aquatic animal products~~ prior to importation do not jeopardise the safety of the ~~traded aquatic animals or aquatic animal products~~.

Criteria

For ~~aquatic animals or~~ aquatic animal products to be considered for *international trade* under the provisions of point 1 of Article X.X.12. (amphibian and fish disease specific chapters) and Article X.X.11. (crustacean and mollusc disease specific chapters), it should comply with the following criteria:

- 1) the ~~aquatic animal or~~ aquatic animal product is prepared and packaged for retail trade for human consumption; AND

EITHER

- 2) it includes only a small amount of raw waste tissues generated by the consumer;

OR

- 3) the *pathogenic agent* is not normally found in the waste tissues generated by the consumer.

 — Text deleted.

CHAPTER 2.2.7.

INFECTION WITH WHITE SPOT SYNDROME VIRUS DISEASE

1. Scope

For the purpose of this chapter, Infection with disease (WSD) is considered to be infection with white spot syndrome virus (WSSV) means infection with the pathogenic agent white spot syndrome virus (WSSV), Family Nimaviridae, Genus Whispovirus.

2. Disease information

2.1. Agent factors

Various WSSV isolates with small genetic polymorphisms have been identified (variants). It should be realised, however, that as the *Nimaviridae* is a newly recognised family, the species concept will be subject to change after existing and new isolates have been studied in more detail.

2.1.1. Aetiological agent, agent strains

WSSV was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus *Whispovirus* within the *Nimaviridae* family. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 80–120 nm in diameter and 250–380 nm in length. Most notable is the thread- or flagella-like extension (appendage) at one end of the virion. Today, although various geographical isolates with genotypic variability have been identified, they are all classified as a single species (white spot syndrome virus) within the genus *Whispovirus* (Lo *et al.*, 2012).

2.1.2. Survival outside the host

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al.*, 1998); and is viable in ponds for at least 3–4 days (Nakano *et al.*, 1998).

2.1.3. Stability of the agent (effective inactivation methods)

The agent is inactivated in <120 minutes at 50°C and <1 minute at 60°C (Nakano *et al.*, 1998).

2.1.4. Life cycle

In-vitro studies with primary cell culture and *in-vivo* studies with postlarvae (PL) show that the replication cycle is approximately 20 hours at 25°C (Chang *et al.*, 1996; Chen *et al.*, 2011; Wang *et al.*, 2000).

2.2. Host factors

WSSV has an extremely wide host range. The virus can infect a wide range of aquatic crustaceans especially decapod, including marine, brackish and freshwater prawns, crabs, crayfish and lobsters (Maeda *et al.*, 2000).

2.2.1. Susceptible host species

To date, no decapod (order Decapoda) crustacean from marine and brackish or freshwater sources has been reported to be resistant to infection with WSSV (Flegel, 1997; Lightner, 1996; Lo & Kou, 1998; Maeda *et al.*, 2000; Stentiford *et al.*, 2009).

2.2.2. Susceptible stages of the host

All life stages are potentially susceptible, from eggs to broodstock (Lightner, 1996; Venegas *et al.*, 1999).

Annex 28 (contd)**2.2.3. Species or subpopulation predilection (probability of detection)**

The best life stages of crustaceans for detection **of infection with WSSV** are late PL stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms).

2.2.4. Target organs and infected tissue

The major targets of **infection with WSSV infection** are tissues of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama *et al.*, 1994; Wongteerasupaya *et al.*, 1995). Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

2.2.5. Persistent infection with lifelong carriers

Persistent infection occurs commonly and lifelong infection has been shown (Lo & Kou, 1998). Viral loads during persistent infection can be extremely low and are very hard to detect even by sensitive methods such as real-time and nested PCR.

2.2.6. Vectors

The virus can transmit from host to host and does not need a biological vector.

2.2.7. Known or suspected wild aquatic animal carriers

Wild decapods include *Mysis* sp. (Huang *et al.*, 1995a), *Acetes* sp., *Alpheus* sp., *Callinassa* sp., *Exopalaemon* sp., *Helice* sp., *Hemigrapsus* sp., *Macrophthalmus* sp., *Macrophthel* sp., *Metaplex* sp., *Orithyia* sp., *Palaemonoidea* sp., *Scylla* sp., *Sesarma* sp., *Stomatopoda* sp. and (He & Zhou, 1996; Lei *et al.*, 2002), can be easily infected by WSSV and may express the disease under suitable environmental conditions. However, non-decapodal crustaceans, such as copepods (Huang *et al.*, 1995a), rotifers (Yan *et al.*, 2004), *Artemia salina* (Chang *et al.*, 2002), *Balanus* sp. (Lei *et al.*, 2002), and *Tachypleidue* sp. (He & Zhou, 1996) may become wild aquatic animal carriers by latent infection without disease. Other marine molluscs, polychaete worms (Vijayan *et al.*, 2005), as well as non-crustacean aquatic arthropods such as sea slaters (*Isopoda*) and Euphydradae insect larvae can mechanically carry the virus without evidence of infection (Lo & Kou, 1998).

2.3. Disease pattern

Infection **with WSSV** sometimes causes disease and sometimes not (Tsai *et al.*, 1999), depending on factors as yet poorly understood but related to species tolerance and environmental triggers. With an appropriate infection dose to allow sufficient time before mortality, animals susceptible to disease show large numbers of virions circulating in the haemolymph (Lo *et al.*, 1997), but this may also occur for tolerant species that show no mortality. Thus, high viral loads *per se* do not cause disease or mortality for all susceptible species.

2.3.1. Transmission mechanisms

The infection **with WSSV** can be transmitted vertically (trans-ovum), horizontally by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water-borne routes. Transmission of infection **with WSSV** can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (Lo & Kou, 1998).

2.3.2. Prevalence

Prevalence of **infection with WSSV** is highly variable, from <1% in infected wild populations to up to 100% in captive populations (Lo & Kou, 1998).

2.3.3. Geographical distribution

WSD Infection with WSSV has been identified from crustaceans in China (People's Rep. of), Japan, Korea (Rep. of), South-East Asia, South Asia, the Indian Continent, the Mediterranean (Stentiford & Lightner, 2011), the Middle East, and the Americas. **WSD free Zones** and compartments **free from infection with WSSV** are known within these regions (Lo *et al.*, 2012).

2.3.4. Mortality and morbidity

All penaeid shrimp species are highly susceptible to infection with WSSV, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection with WSSV, but morbidity and mortality as a consequence of infection is highly variable (Lo & Kou, 1998). High level infections with WSSV are known in some decapods in the absence of clinical disease.

2.3.5. Environmental factors

Disease outbreaks may be induced by stressors, such as rapid changes in salinity. Water temperature has a profound effect on disease expression, with average water temperatures of between 18 and 30°C being conducive to WSD WSSV outbreaks (Song *et al.*, 1996; Vidal *et al.*, 2001).

2.4. Control and prevention

Although the underlying mechanism remains unknown, laboratory experiments have shown that 'vaccinated' shrimp and crayfish have better survival rates after WSSV challenge. It was first shown that *Penaeus japonicus* shrimp that survived natural and experimental WSSV infections displayed resistance to subsequent challenge with WSSV (Venegas *et al.*, 2000). Later studies showed that intramuscular injection of inactivated WSSV virions or recombinant structural protein, (VP28), provided shrimp with some protection against experimental WSSV infection. Furthermore, shrimp fed with food pellets coated with inactivated bacteria over expressing VP28 showed better survival rates after WSSV challenge (Witteveldt *et al.*, 2004). However, although these results seemed promising, the protection was effective only when the shrimp were infected with a low dosage of WSSV. Also, the effect usually lasted for only a few days, or in the case of crayfish, for about 20 days. Another potential means of protecting shrimp against infection with WSSV infection is to use RNA interference (RNAi). WSSV gene-specific double-stranded (ds) RNAs produced strong anti-WSSV activity, protecting the shrimp against infection with WSSV infection, but the same study showed that long dsRNA induced both sequence-dependent and independent anti-viral responses in shrimp (Robalino *et al.*, 2005). A more recent study even showed that oral administration of bacterially expressed VP28 dsRNA could protect shrimp against infection with WSSV infection (Sarathi *et al.*, 2008). To date, however, there are still no field trial data for either the vaccination or the RNAi approach.

2.4.1. Vaccination

No consistently effective vaccination methods have been developed for infection with WSSV.

2.4.2. Chemotherapy

No scientifically confirmed reports for infection with WSSV.

2.4.3. Immunostimulation

Several reports have shown that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to infection with WSSV-WSD (Chang *et al.*, 2003; Chotigeat *et al.*, 2004).

2.4.4. Resistance breeding

No significant improvements have been reported for infections with WSSV.

2.4.5. Restocking with resistant species

Not applicable for infection with WSSV-WSD.

2.4.6. Blocking agents

There are no efficient blocking agents that can be recommended at this time. rVP28 has an effect, but it cannot yet be used as a practical blocking agent.

2.4.7. Disinfection of eggs and larvae

For transovum transmission, disinfection of egg is likely to be effective (Lo & Kou, 1998), but this has not yet been confirmed in formal scientific trials.

Annex 28 (contd)**2.4.8. General husbandry practices**

A number of husbandry practices have been used successfully to manage **infection with WSSV-WSD**, such as avoiding stocking in the cold season, use of specific pathogen free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, and use of biosecure water and culture systems (Withyachumnarkul, 1999) polyculture of shrimp and fish (He *et al.*, unpublished data).

3. Sampling**3.1. Selection of individual specimens**

Samples of moribund shrimp or shrimp that show clinical signs (see Section 4.1.1) or exhibit behavioural changes (Section 4.1.2) should be selected for **WSSV-detection of infection with WSSV**.

3.2. Preservation of samples for submission

See Chapter 2.2.0 *General information* (for diseases of crustaceans) for guidance on preservation of samples for the intended test method.

3.3. Pooling of samples

~~Samples taken for molecular or antibody based test methods for **infection with WSSV-WSD** may be combined as pooled samples of no more than five specimens per pooled sample of juveniles or subadults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50 to 150 PL depending on their size/age) may be necessary to obtain sufficient sample material. See also chapter 2.2.0.~~

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger life stages should be processed and tested individually. However, small life stages, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing.

3.4. Best organs or tissues

Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular connective tissues, as well as other target tissues (e.g. antennal gland, haematopoietic organ, etc.), are the main target tissues for **infection with** WSSV. Samples of or from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission (Lo *et al.*, 1997).

For non-destructive screening by PCR, it is recommended to submit (a small piece of) gill, (a small aliquot of) haemolymph or (a small piece of) pleopod. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed prior to submission.

Please see section 4.3.1.2.4.1 for details of the sample procedure.

3.5. Samples/tissues that are not suitable

Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the columnar epithelial cells of these two organs are of endodermal embryonic origin (Lo *et al.*, 1997), and they are not appropriate tissues for detection. The compound eye may contain a PCR inhibitor (Lo *et al.*, 1997) and it is therefore not suitable for PCR-based diagnosis.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

White spots embedded within the exoskeleton are the most commonly observed clinical sign. In most shrimp, these spots range from barely visible to 3 mm in diameter, and they sometimes coalesce into larger plates. However, it should be noted that environmental stress factors, such as high alkalinity, or bacterial disease can also cause white spots on the carapace of shrimp, and that moribund shrimp with **infection with WSSV-WSD** may in fact have few, if any, white spots. Therefore, the appearance of white spots is absolutely not a good diagnostic sign of **infection with WSSV-infection**. Furthermore, other crustaceans, such as most crayfish, are often reported to show no sign of white spots when infected with WSSV.

High degrees of colour variation with a predominance of reddish or pinkish discoloured shrimp are seen in diseased populations.

4.1.2. Behavioural changes

The presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. However, if the shrimp also appear lethargic, if their colour changes to pink or reddish-brown, if they gather around the edges of ponds/tanks at the water surface, or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of these signs.

4.2. Clinical methods

4.2.1. Gross pathology

See Section 4.1.1 and 4.1.2 above.

4.2.2. Clinical chemistry

Haemolymph withdrawn from WSSV-infected shrimp always has a delayed (or sometimes completely absent) clotting reaction.

4.2.3. Microscopic pathology

4.2.3.1. Wet mounts

Demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained.

4.2.3.1.1 T-E staining

A T-E staining solution may be prepared from Trypan blue 0.6%, Eosin Y 0.2%, NaCl 0.5%, phenol 0.5%, and glycerol 20% (Huang & Yu, 1995).and used as follows:

- i) Place a piece of lesion tissue (e.g. a piece of gill or stomach epithelium without the cuticle) on a slide and mince with a scalpel.
- ii) Add 1–2 drops of the T-E staining solution to the minced tissue, mix and allow to stain for 3–5 minutes.
- iii) Lay a cover glass over the stained tissue and cover with several pieces of absorbent paper. Use a thumb to squash the mince into a single layer of cells.

If the sample was taken from a heavily infected shrimp, it should be easy to see the hypertrophied nuclei and intranuclear eosinophilic or vacuolation-like inclusion bodies under a 400– 000× light microscope.

Annex 28 (contd)**4.2.3.2. Smears**

Demonstration of aggregates of WSSV virions in unstained smear preparations of haemolymph by dark-field microscopy.

NOTE: This is the simplest of the microscopic techniques and is recommended for people with limited expertise in diagnosing infection with WSSV. The aggregates appear as small reflective spots of 0.5 µm in diameter (Momoyama *et al.*, 1995).

4.2.3.3. Fixed sections

Histological demonstration of pathognomonic inclusion bodies in target tissues.

4.2.3.4. *In situ* hybridisation

Use of WSSV-specific DNA probes with histological sections to demonstrate the presence of WSSV nuclei acid in infected cells.

4.2.3.5. Immunohistochemistry

Use of WSSV-specific antibodies with histological sections or wet mounts to demonstrate the presence of WSSV antigen in infected cells.

4.2.4. Electron microscopy/cytopathology

Demonstration of the virus in tissue sections or in semi-purified negatively stained virus preparations (e.g. from haemolymph). See Section 2.1.1 for virion morphology.

4.3. Agent detection and identification methods**4.3.1. Direct detection methods**

Not reported.

4.3.1.1. Microscopic methods

See Section 4.2.3 above.

4.3.1.1.1. Wet mounts

See Section 4.2.4 above.

4.3.1.1.2. Smears

See Section 4.2.5 above.

4.3.1.1.3. Fixed sections

See Section 4.2.3 above.

4.3.1.2. Agent isolation and identification*4.3.1.2.1. Bioassay method*

If SPF shrimp are available, the following bioassay method is based on Nunan *et al.* (1998) and Durand *et al.* (2000), is suitable for WSSV diagnosis.

- i) For bioassay, remove the pleopods from shrimp suspected of being infected with WSSV infection and homogenise in TN buffer (0.02 M Tris/HCl, 0.4 M NaCl, pH 7.4).
- ii) Following centrifugation at 1000 **g** for 10 minutes, dilute the supernatant fluid 1/10 with 2% NaCl and filter (0.2 µm filter).

Annex 28 (contd)

- iii) Inject 0.2 ml of inoculum into the dorso-lateral aspect of the fourth abdominal segment of indicator shrimp (e.g. SPF *P. vannamei* at the juvenile stage), injecting between the tergal plates into the muscle of the third abdominal segment.
- iv) Examine moribund shrimp grossly or by using the methods described above. If at 3–5 days after inoculation there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

4.3.1.2.2. Cell culture/artificial media

WSSV can be isolated from primary cultures of lymphoid or ovary cells. However, it is NOT recommended to use cell culture as a routine isolation method because of: 1) the high risk of contamination, and, 2) the composition of the medium varies depending on the tissue type, host species and experimental purpose; that is, to date there is no standard or recognised medium that can be recommended. As primary cell culture is so difficult to initiate and maintain for virus isolation purposes, bioassay should be the primary means for virus propagation.

4.3.1.2.3. Antibody-based antigen detection methods

Both polyclonal and monoclonal antibodies raised against either the virus or a recombinant viral structural protein have been used in various immunological assays including western blot analysis, immunodot assay, indirect fluorescent antibody test (IFAT), immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA) to detect WSSV (Huang *et al.*, 1995a; Poulos *et al.*, 2001; Sithigorngul *et al.*, 2006; Yoganandhan *et al.*, 2004). Antibody-based methods can be fast, convenient and applicable to field use, but as they have only about the same sensitivity as 1-step PCR, they are recommended only to confirm acute **infection with WSSV-WSD**.

4.3.1.2.4. Molecular techniques

4.3.1.2.4.1 Polymerase chain reaction (PCR)

The PCR protocol described here is from Lo *et al.* 1996a and b, and uses sampling methods from Lo *et al.* 1997). It is recommended for all situations where **infection with WSSV** diagnosis is required. A positive result in the first step of this standard protocol implies a serious **infection with WSSV-infection**, whereas, when a positive result is obtained in the second amplification step only, a latent or carrier-state infection is indicated. Alternative PCR assays have also been developed (e.g. Numan & Lightner, 2011), but before use they should first be compared with the protocol described here.

PCR commercial kits are available for WSSV **detection diagnosis** and are acceptable provided they have been validated as fit for such purpose. Please consult the OIE Register for kits that have been certified by the OIE (<http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/the-register-of-diagnostic-tests/>).

DNA extraction

- i) Collect 100–200 mg shrimp tissue (pleopod of live juvenile to subadult shrimp, postlarvae 11 upwards [PL11 up] with removed heads, or whole PL10, or use 100 µl haemolymph) in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml⁻¹ proteinase K added just before use). For non-destructive screening, pleopods can be removed using red-hot forceps. For this procedure, the animal should be wrapped in a wet towel such that only the organ to be excised is left exposed.
- ii) Using a disposable stick, homogenise the tissue in the tube thoroughly.
- iii) After homogenisation, incubate at 65°C for 1 hour.
- iv) Add 5 M NaCl to a final concentration of 0.7 M. Next, slowly add 1/10 volume of N-cetyl N,N,N-trimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly.

NOTE: In addition to the CTAB extraction method described here, commercial extraction kits are often used as part of normal surveillance activities.

Annex 28 (contd)

- v) Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 *g* for 5 minutes and then transfer the aqueous solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol.
- vi) Mix gently and centrifuge at 13,000 *g* for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice.
- vii) Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24/1) and centrifuge at 13,000 *g* for 5 minutes.
- viii) Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80°C for 15 minutes.
- ix) Centrifuge at 13,000 *g* for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilised double-distilled water at 65°C for 15 minutes.
- x) Use 1 µl of this DNA solution for one PCR.

Note: the following nested PCR procedures are well established and provide reliable diagnostic results under the specified conditions. Care should be taken, however, to ensure that DNA samples are prepared from the recommended organs, and that the PCR temperature is accurately applied (particularly for annealing, the recommended temperature is 62°C). To prevent the possibility of false positive results, it is important to adhere to the specified procedures, especially when they are used to test new candidate hosts such as *Cherax quadricarinatus* (Claydon *et al.*, 2004), as well as *Procambarus clarkii* (red swamp crayfish) and *Procambarus zonangulus* (Southern white river crayfish). For diagnosed incidences of **infection with** WSSV in a new host or in a previously free zone, DNA sequencing should be used to confirm the positive results.

First-step PCR

- i) Add 1 µl DNA template solution (containing about 0.1–0.3 µg DNA) to a PCR tube containing 100 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM of each dNTP, 100 pmol of each primer, 2 units of heat-stable DNA polymerase).
- ii) The outer primer sequences are 146F1, 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3' and 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'.
- iii) The PCR profile is one cycle of 94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C. The WSSV-specific amplicon from this reaction is 1447 bp. The sensitivity is approximately 20,000 copies of a plasmid template.

Second step of the (nested) PCR

This second step is necessary for the detection of **infection with** WSSV in shrimp at the carrier stage.

- i) Add 10 µl of the first-step PCR product to 90 µl of a PCR cocktail with the same composition as above except that it contains the second (inner) primer pair: 146F2 (5'-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3') and 146R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3').
- ii) Use the same PCR amplification protocol as above. The WSSV-specific amplicon from this reaction is 941 bp. The overall sensitivity of both steps is approximately 20 copies of a WSSV plasmid template.
- iii) To visualise, electrophorese 10 µl PCR products on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml⁻¹.
- iv) Decapod-specific primers (143F 5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3' and 145R 5'-TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3' yielding an 848 bp amplicon; N represents G, A, T, or C) should be used in control reactions to verify the quality of the extracted DNA and the integrity of the PCR. In the penaeid shrimp *P. aztecus*, the PCR product generated by this decapod-specific primer pair corresponds to nucleotide sequence 352–1200 of the 18s rRNA. The decapod 18s RNA sequence is highly conserved and produces a similar sized PCR product in almost all decapods. A positive control (WSSV DNA template) and negative controls (no template and shrimp DNA template) should be included in every assay.

4.3.1.2.4.2 DNA sequencing of PCR products

For confirmation of suspected new hosts of **infection with** WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The cloning and sequencing protocols described here are according to Claydon *et al.* (2004).

Note: to save time and money, it is acceptable to sequence the PCR amplicon directly. If a positive result is obtained, then go to step iv below. In the event that only band[s] of unexpected size are obtained, then the sample should be tested again using the cloning and sequencing procedures described below.

- i) Excise the DNA fragments selected for further analysis from the agarose gels and purify them using any of the commercially available PCR clean up kits.
- ii) Ligate amplicons into vector plasmid and clone the construct.
- iii) Use suitable primers to amplify the inserted amplicon, and then subject the amplified product to DNA sequencing.
- iv) Compare the sequences obtained with available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

4.3.1.2.4.3 Taqman real-time PCR method

The protocol described here is from Durand & Lightner (2002). This detection method is highly specific to WSSV, is extremely sensitive (four copies) and has a wide dynamic range (seven logs).

Construction of positive control vector and preparation of standard curve

The DNA fragment of 69 bp amplified by the forward and reverse primers (indicated below) is cloned in pGEM-T easy or other suitable vectors, and then confirmed by sequencing. The plasmid DNA is purified by any commercial plasmid extraction kits and the concentration is determined by using a spectrophotometer or other methods. The gene copy number is determined according to the molar mass derived from the plasmid DNA containing the 69 bp insert. The plasmid DNAs are then serially diluted tenfold to generate standard curves ranging from 10^2 to 10^7 copies.

DNA extraction

DNA extraction should be performed according to the above protocol described for PCR (4.3.1.2.4.1) or by using a commercial kit. The concentration of purified DNA can be determined by spectrophotometer or by other methods.

Real-time PCR

The TaqMan assay is carried out using the TaqMan Universal PCR Master Mix, which contains AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP and optimised buffer components (PE Applied Biosystems, Foster City, CA, USA⁶). Primer sequences are WSS1011F: 5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3', WSS1079R: 5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3', Taqman Probe: 5'-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-3'.

- i) Add a sample of 10–50 ng of DNA to set up a 25 µl reaction mixture containing 0.3 µM of each primer and 0.15 µM of TaqMan probe.
- ii) The PCR profile is one cycle of 50°C for 2 minutes for AmpErase uracil-N-glycosylase (UNG) and 95°C for 10 minutes for activation of AmpliTaq, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

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*.

Annex 28 (contd)

- iii) To determine the WSSV copy number of the extracted DNA samples, the samples are subjected to PCR reaction alongside the serially diluted plasmid DNA standard. After reaction, the software accompanying the PCR system automatically determines the Ct value for each PCR sample. Based on the Ct values, the software calculates the standard curve for standard dilution and determines the WSSV copy number for the DNA samples by extrapolating values from the standard curve.

4.3.1.2.4.4. *In-situ hybridisation (ISH) method*

The protocol described here is based on that developed by Nunan & Lightner (1997).

- i) Fix moribund shrimp with Davidson's AFA fixative for 24–48 hours.
- ii) Embed the tissues in paraffin and cut into 5 µm sections. Place sections on to positively charged microscope slides.
- iii) Heat the slide on a hot plate at 65°C for 30 minutes.
- iv) Deparaffinise, rehydrate and then treat for 2–30 minutes (depending on tissue type) with 100 µg ml⁻¹ proteinase K in Tris/NaCl/EDTA (TNE) buffer at 37°C.
- v) Post-fix the slides by chilling in pre-cooled 0.4% formaldehyde for 5 minutes at 4°C and wash the slides in 2 × standard saline citrate (SSC; 1 × SSC = 150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0) at room temperature.
- vi) Pre-hybridise the slides with pre-hybridisation solution (50% formamide, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 5 × SSC, 1 mM EDTA, 50 mM Tris/HCl, pH 8) for 30 minutes at 42°C.
- vii) Follow with hybridisation with the 1447 bp WSSV-specific PCR amplicon (or with any other WSSV-specific PCR amplicon; see Section 4.3.1.2.3.1 "First-step PCR" above) that has been labelled with digoxigenin. It is recommended that the probe be labelled by incorporating DIG-dNTP by the PCR method. Optimum concentration should be determined by testing and adjusting until a high specific signal is obtained against a low background.
- viii) For hybridisation, boil the probe for 10 minutes and immediately place on ice. Dilute the probe to 30–50 ng ml⁻¹ in pre-hybridisation solution and apply 500 µl to each slide.
- ix) Put the slide on a hotplate at 85–95°C for 6–10 minutes (make sure that it does not reach boiling point), quench slides on ice for 5 minutes and then transfer to a humid chamber for 16–20 hours at 42°C.
- x) After hybridisation, wash the slides twice for 15 minutes each time with 2 × SSC at room temperature, twice for 5 minutes with 1 × SSC at 37°C, and twice for 5 minutes with 0.5 × SSC at 37°C.
- xi) For hybridisation detection, wash slides with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 minutes at room temperature.
- xii) Block the slides with blocking solution (2% normal goat serum and 0.3% Triton X-100 in maleic acid buffer) for 30 minutes at 37°C.
- xiii) Add 250 µl anti-DIG alkaline phosphatase (AP)-conjugated antibody solution (1 µl ml⁻¹ anti-DIG/AP-Fab fragment in maleic acid buffer containing 1% normal goat serum and 0.3% Triton X-100) to each slide, and incubate at 37°C for 30 minutes.
- xiv) Wash the slides twice with maleic acid buffer for 10 minutes each and once with detection buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) at room temperature.
- xv) Add 500 µl development solution (prepare immediately before use by adding 45 µl NBT salt solution [75 mg ml⁻¹ in 70% dimethylformamide], 35 µl 5-bromo-4-chloro-3-indoyl phosphate, toluidinum salt [X-phosphate] solution [50 mg ml⁻¹ in dimethylformamide] and 1 ml 10% PVA to 9 ml of detection buffer) to each slide and incubate in the dark in a humid chamber for 1–3 hours.

Annex 28 (contd)

- xvi) Stop the reaction by washing the slides in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) for 15 minutes at room temperature. Wash the slides in distilled water for ten dips, counterstain the slides in 0.5% aqueous Bismarck Brown Y for approximately 5 minutes and then rinse with water. Wet mount using aqueous mounting media for observation immediately or dehydrate the slides and mount with mounting media for long-term preservation.
- xvii) Mount the slides with cover-slips and examine with a bright field microscope. Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

4.3.1.2.4.5. Loop-mediated isothermal amplification (LAMP) method

The protocol described here is from Kono *et al.* (2004). The LAMP method is sensitive and rapid, and it amplifies the target nucleic acids under isothermal conditions, therefore needing no sophisticated machine for thermal cycling.

DNA extraction

DNA extraction could be performed according to the above protocol described for PCR (4.3.1.2.4.1) or by other suitable methods or by commercial kits.

LAMP reaction

- i) Add DNA to a tube to set up a 25 μ l reaction mixture (20 mM Tris/HCl, pH 8.8, 10 mM KCl, 8 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1% Tween 20, 0.8M Betaine, 1.4 mM of each dNTP, 40 pmol of WSSV-FIP and -BIP primers, 5 pmol of WSSV-F3 and -B3 primers).
- ii) The primer sequences are WSSV-FIP: 5'-GGG-TCG-TCG-AAT-GTT-GCC-CAT-TTT-GCC-TAC- GCA-CCA-ATC-TGT-G-3', WSSV-BIP: 5'-AAA-GGA-CAA-TCC-CTC-TCC-TGC-GTT-TTA-GAA-CGG-AAG-AAA-CTG-CC-TT-3', WSSV-F3: ACG-GAC-GGA-GGA-CCC-AAA-TCG-A-3', WSSV-B3: 5'-GCC-TCT-GCA-ACA-TCC-TTT-CC-3'.
- iii) Heat the mixture at 50°C for 5 minutes and at 95°C for 5 minutes, then chill on ice, and add 1 μ l (8 U) of *Bst* DNA polymerase.
- iv) Incubate the mixture at 65°C for 60 minutes, and then terminate the reaction at 80°C for 10 minutes.
- v) To visualise, electrophorese 2 μ l LAMP reaction products on 2% agarose gels containing ethidium bromide at a concentration of 0.5 μ g ml⁻¹. This reaction produces WSSV-specific LAMP products with multiple bands of various sizes from approximately 200 bp to the loading well.

Reliable LAMP commercial kits may be alternative for WSSV diagnosis.

4.3.1.2.5. Agent purification

The WSSV virion can be purified as described previously with slight modifications (Xie *et al.*, 2005). Briefly, collect five or six moribund crayfish or shrimp (20–25 g each) at 3 days to 1 week post-infection. Homogenise all tissues excluding the hepatopancreas for 2 minutes using a mechanical homogeniser in 1200 ml TNE buffer (50 mM Tris/HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM Na₂S₂O₅). Centrifuge at 3500 *g* for 5 minutes. Save the supernatant and rehomogenise the pellet in 1200 ml TNE buffer. Filter the pooled supernatant through a nylon net (400 mesh) and centrifuge at 30,000 *g* for 30 minutes. Discard the supernatant and carefully rinse out the upper loose layer (pink) of the pellet using a Pasteur pipette. Resuspend the lower compact layer (grey) in 10 ml TM buffer (50 mM Tris/HCl, 10 mM MgCl₂, pH 7.5). Pool the crude virus suspension and centrifuge at 3000 *g* for 5 minutes. Centrifuge the supernatant again at 30,000 *g* for 20 minutes. Remove the supernatant and pink loose layer and resuspend the white pellet in 1.2 ml TM buffer containing 0.1% NaN₃. Transfer to a 1.5-ml Eppendorf tube. Centrifuge the suspension three to five times at 650 *g* for 5 minutes each time to remove pink impurities. Finally, store the milk-like pure virus suspension at 4°C until use.

Annex 28 (contd)**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** WSSV 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	c	c	c	c
Histopathology	d	c	c	c	a	c
Transmission EM	d	d	d	d	d	a
Antibody-based assays	d	d	c	c	a	b
<i>In-situ</i> DNA probes	d	d	c	c	a	a
PCR	d	b	a	a	a	a
LAMP	d	d	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy;
 PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification.

6. Test(s) recommended for targeted surveillance to declare freedom from **infection with white spot **syndrome virus** disease**

~~Two step PCR and sequencing are the recommended methods for declaring freedom, only for juveniles and adults and possibly PLs. Two step PCR negative results are required. Where a two step PCR positive result cannot be confirmed as **infection with** WSSV by sequencing, this also counts as a negative result.~~

Real-time PCR is the recommended test for targeted surveillance to declare freedom from white spot disease.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

For juvenile and adult shrimp: gross signs of WSD (See Sections 4.1.1 and 4.1.2 above).

For shrimp at any life stage (larva to adult): mortality.

For shrimp and crab at any life stage (larva to adult): hypertrophied nuclei in squash preparations of gill and/or cuticular epithelium; unusual aggregates in haemolymph by dark field microscopy; inclusion bodies in histological sections in target tissues.

Infection with WSSV is suspected if at least one of the following criteria is met:

1. Histopathology consistent with WSSV
2. Positive conventional PCR result
3. Positive real-time PCR result

Positive LAMP result

7.2. Definition of confirmed case

Suspect cases should first be checked by PCR or LAMP. If in a previously WSSV free country/zone/compartiment, where PCR results are positive, they should be confirmed by sequencing. Histopathology, probes and electron microscopy also can be used to confirm the case.

Infection with WSSV is considered to be confirmed if one or more of the following criteria are met:

1. Histopathology consistent with WSSV and positive *in-situ* hybridisation test
2. Positive conventional PCR and conventional PCR targeting a different region of the WSSV genome
3. Positive real-time PCR and conventional PCR targeting a different region of the WSSV genome
4. Positive LAMP and conventional PCR targeting a different region of the WSSV genome

For confirmation of an index case in a previously free zone or country, sequence analysis of conventional PCR amplicons is required.

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* *

NB: There is an OIE Reference Laboratory for infection with white spot syndrome virus disease (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 for infection with white spot syndrome virus disease

NB: FIRST ADOPTED IN 1997; MOST RECENT UPDATES ADOPTED IN 2012

Circulated for Member Country information in the report of the September 2016 meeting

Amended by the Aquatic Animals Commission at its February 2017 meeting

Revised assessment for listing of *Batrachochytrium salamandrivorans* in the Aquatic Code

Overall assessment

The OIE 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	
<i>Batrachochytrium salamandrivorans</i>	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 salamanders (*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) concluded 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

Annex 29 (contd)

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.*

Assessment:

Investigations by Martel *et al.* (2013) provide 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 seven 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 Palearctic salamander species are susceptible to *Bsal*, and it is lethal to at least some New World salamandrid 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 concluded that it is a serious threat to biodiversity there. This conclusion was further supported by a review by the US Fish and Wildlife Service report which concluded that 'introduction of *Bsal* into the United States could cause significant, adverse, population-level effects in native species'⁷.

A review by the European Food Safety Authority (2017) concluded that whilst sample sizes were small, there was sufficient evidence to support that declines in fire salamanders at Bunderos in the Netherlands was attributable to *Bsal*. The review concluded that further research was required to provide definitive evidence that *Bsal* was associated with other declines in wild populations.

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 salamanders from declining populations tested positive for *Bsal* by PCR, in contrast to 0 of 51 swabs from a stable population.

⁷ <https://www.federalregister.gov/documents/2016/01/13/2016-00452/injurious-wildlife-species-listing-salamanders-due-to-risk-of-salamander-chytrid-fungus>

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 United Kingdom (Cunningham *et al.*, 2015) demonstrated transboundary spread via movement of live animals. Skin samples from 1,765 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 North 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 four continents (Martel *et al.*, 2014). Positive results were obtained from Southeastern 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. A survey of 30 species of amphibians (665 samples) from 15 provinces in China found no evidence of *Bsal* (Zhu *et al.*, 2014). 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 highly 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.

Annex 29 (contd)

AND

C. Diagnosis

Criterion No. 8. A repeatable and robust means of detection/diagnosis exists.

Assessment:

Methods developed for the culture of *Bd* were successfully used to culture *Bsal*. Culture at various temperatures indicated that incubation at 20°C on tryptone-gelatin hydrolytose-lactose (TGH) broth produced the best results (Martel *et al.*, 2013).

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 analytical specificity.

Blooi *et al.* (2013) further developed a duplex real-time PCR to detect the same target (5.8S ribosomal RNA gene). Amphibian samples originated from experimentally infected and wild populations (declining and healthy). Precision was evaluated by intra- and inter-assay variability testing and shown to be high and reproducible. Analytical specificity was evaluated by assaying DNA extracts from 10 different isolates of Chytridiomycota. The PCR only produced positive results from *Bsal* samples indicating a high level of analytical specificity. The limit of detection was determined to be 0.1 genomic equivalent (GS) of zoospores. The small samples constrain the ability to generate accurate estimates of the duplex PCR test's diagnostic characteristics. Nevertheless, a review by the European Food Safety Authority (2017) concluded that 'the data strongly suggest a good level of performance'.

The duplex real-time PCR has been sufficiently validated sufficiently, recognising the limited number of samples available, to reliably detect *Bsal* so we can conclude that the test can accurately, reliably and robustly detect *Bsal*. Its demonstrated characteristics (notably level of specificity and limit of detection) make the test suitable for screening surveys and confirmation in affected individuals.

As this new method is applied it is expected that additional information contributing to validation will be generated.

Conclusion: the criterion is satisfied.

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TECHNICAL DISEASE CARD

TILAPIA LAKE VIRUS (TiLV)–A NOVEL ORTHOMYXO-LIKE VIRUS

PATHOGEN INFORMATION

1. CAUSATIVE AGENT

1.1. Pathogen type

Virus.

1.2. Disease name and synonyms

Tilapia lake virus (TiLV) disease.

1.3. Pathogen common names and synonyms

Tilapia lake virus (TiLV).

1.4. Taxonomic affiliation

The taxonomic affiliation has not been definitively concluded; however, TiLV has been described as a novel virus in the Family *Orthomyxoviridae* (Eyngor *et al.*, 2014).

1.5. Authority (first scientific description, reference)

The virus was first described by Eyngor *et al.* (2014).

1.6. Pathogen environment (fresh, brackish, marine waters)

Fresh and brackish water.

2. MODES OF TRANSMISSION

2.1. Routes of transmission (horizontal, vertical, indirect)

Co-habitation studies have demonstrated that direct horizontal transmission is an important route of transmission. There is no evidence of vertical transmission. The biophysical characteristics of the virus are not well characterised so it is difficult to determine the significance of indirect transmission by fomites.

2.2. Reservoir

Infected populations of fish, both farmed and wild, are the only established reservoirs of infection. The original source of TiLV is not known.

2.3. Risk factors (temperature, salinity, etc.)

Disease has been associated with transfer between ponds and thus may be associated with stress (Ferguson *et al.*, 2014). No other risk factors (temperature, salinity, etc.) have been identified as potential risk factors.

Annex 30 (contd)**3. HOST RANGE****3.1. Susceptible species**

Mortalities attributed to TiLV have been observed in wild tilapia *Sarotherodon (Tilapia) galilaeus*, farmed tilapia *Oreochromis niloticus* and commercial hybrid tilapia (*O. niloticus* X *O. aureus*) (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Eyngor *et al.*, 2014). To date only tilapines have been shown to be susceptible. It is possible that other species will be found to be susceptible.

3.2. Affected life stage

In the outbreak reported by Ferguson *et al.* (2014) fingerlings were mainly affected. Other reports have not commented on different levels of mortality by life stage (Eyngor *et al.*, 2014).

3.3. Additional comments

There is some evidence that certain genetic strains of tilapia are resistant. Ferguson *et al.* (2014) noted that one strain of tilapia (genetically male tilapia) incurred a significantly lower level of mortality (10-20%) compared with other strains (~80% mortality).

4. GEOGRAPHICAL DISTRIBUTION

TiLV has been reported in Colombia, Ecuador and Israel (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Tsofack *et al.*, 2016) and most recently in Egypt (Fathi *et al.*, 2017). However, a lack of thorough investigation of all mortality incidents means that the geographic distribution of TiLV may be wider than currently. For example, reports of mortality in tilapia in Ghana and Zambia in 2016 have not been attributed to TiLV but the available information does not indicate that the presence of the virus has been investigated.

5. CLINICAL SIGNS AND CASE DESCRIPTION**5.1. Host tissues and infected organs**

The main organs where pathology is observed are the eyes, brain and liver (Eyngor *et al.*, 2014).

5.2. Gross observations and macroscopic lesions

Gross lesions included ocular alterations, including opacity of the lens and in advanced cases ruptured lens. Other lesions included skin erosions, haemorrhages in the leptomeninges and congestion of the spleen (Eyngor *et al.*, 2014).

5.3. Microscopic lesions and tissue abnormality

Histologic lesions have been observed in the brain, eye and liver (Eyngor *et al.*, 2014). Lesions in the brain included oedema, focal haemorrhages in the leptomeninges, and capillary congestion in both the white and grey matter and neural degeneration. Foci of gliosis and occasional perivascular cuffs of lymphocytes have been detected. Ocular lesions included ruptured lenticular capsule and cataractous changes. Foci of hepatocellular swelling were observed. The spleen was hyperplastic, with proliferating lymphocytes. Melanomacrophage centres (MMCs) were increased in size and number in both the liver and the spleen. Transmission electron microscopy confirmed the presence of an orthomyxo-like virus within diseased hepatocytes and thus confirmed earlier reports of syncytial hepatitis (del-Pozo *et al.*, 2016).

5.4. OIE status

Under consideration for listing but currently does not meet all of the criteria for listing as described in Chapter 1.2. of the *Aquatic Animal Health Code* (OIE, 2016).

6. SOCIAL AND ECONOMIC SIGNIFICANCE

Tilapines, comprising more than 100 species, are the second most import group of farmed fish worldwide after carp. Global production is estimated at 4.5 million metric tons with a current value in excess of U.S.\$7.5 billion (FAO, 2014). In some regions they are ecologically important (algae and mosquito control and habitat maintenance for shrimp farming) and an important wild capture species. Introduction of the virus has been shown to cause significant mortality (up to 80%) and thus result in serious economic losses to both farmers and fishers (Eyngor *et al.*, 2014).

7. ZOOTIC IMPORTANCE

None

8. DIAGNOSTIC METHODS

8.1. Definition of suspicion

High levels of mortality in tilapine species, associated with ocular alterations (opacity of the lens or more severe pathology), should be considered suspicious of TiLV. Skin erosions, haemorrhages in the leptomeninges and moderate congestion of the spleen and kidney may be observed on post-mortem.

8.2. Presumptive test methods

TiLV can be cultured in primary tilapia brain cells or in an E-11 cell line, inducing a cytopathic effect at 5-10 days (Eyngor *et al.*, 2014). Tsofack *et al.* (2016) describe optimal conditions for culturing TiLV.

8.3. Confirmatory test methods

A PCR primer set has been designed and a reverse transcriptase (RT) PCR has been developed (Eyngor *et al.*, 2014); however, the test was not fully validated. A more highly sensitive, nested RT-PCR has been published and is suitable for the detection of TiLV in clinical cases (Tsofack *et al.*, 2016).

9. CONTROL METHODS

Restrictions on the movement of live tilapines from farms and fisheries where the virus is known to occur will limit the spread of the disease. Generic biosecurity measures to minimise fomite spread via equipment, vehicles or staff (i.e. cleaning and disinfection) should also be implemented.

Currently, no published methods have been shown to be effective in limiting the impact of an outbreak on an infected farm. It has been suggested that breeding for resistance or the development of a vaccine may offer the long term prospects for managing the disease (Ferguson *et al.*, 2014). A breeding programme would need to select and test a range of different strains of tilapia with a view to finding those least susceptible.

10. TRANSMISSION RISK

As TiLV has been horizontally transmitted through cohabitation, disease transmission is likely with movement of live aquatic animals. There is limited information about TiLV biophysical properties and the risks associated with aquatic animal products. However, it may be assumed that it will share properties of other aquatic orthomyxoviruses, such as infectious salmon anaemia virus. Current evidence suggests that the eye, brain and liver are likely to contain highest concentrations of TiLV and thus solid and liquid waste is likely to be contaminated. However, it is possible that the pathogenic agent may also be found in musculature of infected fish.

Annex 30 (contd)**11. REFERENCES**

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AQUATIC ANIMAL HEALTH STANDARDS COMMISSION WORK PLAN 2017–2018

Task	September 2016	February 2017	GS May 2017	September 2017
AQUATIC CODE				
Glossary	Amended some definitions and circulated for comments	Reviewed comments	Present for adoption	
Chapter 1.2.–Criteria for listing	Reviewed comments and recirculated for comment	Reviewed comments	Present for adoption	
Chapter 1.3.–List of diseases	Reviewed comments and recirculated for comment. Reviewed assessment of tilapi lake virus for listing	Reviewed comments	Present for adoption	
Criteria for listing species as susceptible (Chapter 1.5.)	Developed a new Art 1.5.9. to address diseases with a broad host range	Reviewed comments and recirculated for comment		Review comments
Disinfection of aquaculture establishments and equipment (Chapter 4.3.)	Reviewed comments and circulated for comment	Reviewed Member comments	Present for adoption	
Recommendations for surface disinfection of salmonid eggs (Chapter 4.4.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
General obligations related to certification (Chapter 5.1.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Crayfish plague (<i>Aphanomyces astaci</i>) (Chapter 9.1.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Yellow head disease genotype 1 (Chapter 9.2.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Infectious myonecrosis (Chapter 9.4.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	

Annex 31 (contd)

Task	September 2016	February 2017	GS May 2017	September 2017
AQUATIC CODE (contd)				
Necrotising hepatopancreatitis (Chapter 9.5.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Taura syndrome (Chapter 9.6.)	Reviewed comments and circulated for comment	Review Members comments	Present for adoption	
White spot disease (Chapter 9.7.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
White tail disease (Chapter 9.8.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Acute hepatopancreatic necrosis disease (new Chapter 9.X.)	Reviewed comments and circulated for comment.	Review Member comments	Present for adoption	
Revised Article X.X.8.	Reviewed comments and recirculated for comment	Review Member comments	Present for adoption	
Develop revised lists of susceptible species – all fish chapters	Convene <i>ad hoc</i> Group to undertake assessments of susceptible species lists for OIE listed fish diseases	Reviewed <i>ad hoc</i> group report (January 2017) and requested <i>ad hoc</i> group to continue their work		Review <i>ad hoc</i> group report (April 2017 meeting) and amend relevant chapters of the Code
Develop principles for determining surveillance periods in disease-specific chapters and provide advice on amendments for Chapter 1.4.	<i>Ad hoc</i> group met in January 2017. Will meet again prior to September 2017	Reviewed <i>ad hoc</i> group report and requested Group meet again prior to September 2017 to progress work		Review <i>ad hoc</i> group report
New chapter on Biosecurity (Chapter 4.X.)	Developed ToR for a new <i>ad hoc</i> Group to develop text for this new chapter	<i>Ad hoc</i> Group to be convened prior to September 2017		Review <i>ad hoc</i> Group report

Annex 31 (contd)

Task	September 2016	February 2017	GS May 2017	September 2017
AQUATIC CODE (contd)				
Revision of Chapters 4.2. – 4.4.				Prioritise this work after new chapter on biosecurity underway
New chapter on emergency disease preparedness				Prioritise this work after new chapter on biosecurity underway
Chapter 5.3. – Update in line with <i>Terrestrial Code</i> changes				Update in line with amendments if adopted in the <i>Terrestrial Code</i>
Review Chapter 5.4.		Reviewed to align with text in disease-specific chapters and circulated for comment		Review comments and recirculate for comment
Change relevant terms to pathogenic agent throughout the Code		Implement progressively as chapters are reviewed		
AQUATIC MANUAL				
Acute hepatopancreatic necrosis disease (Chapter 2.2.X.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Crayfish plague (<i>Aphanomyces astaci</i>) (Chapter 2.2.1.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Infectious myonecrosis (Chapter 2.2.4.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Necrotising hepatopancreatitis (Chapter 2.2.5.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	
Taura syndrome (Chapter 2.2.6.)	Reviewed comments and circulated for comment	Review Member comments	Present for adoption	

Annex 31 (contd)

Task	Sept 2016	Feb 2017	GS May 2017	Sept 2017
AQUATIC MANUAL (contd)				
White tail disease (Chapter 2.2.8.)	Reviewed comments and circulated for comment.	Review Member comments	Present for adoption	
White spot disease (Chapter 2.2.7.)	Reviewed comments on proposed changes to title and scope.	Amended case definition. Circulate for comment		Review comments
Develop revised lists of susceptible species – all fish chapters	Convene <i>ad hoc</i> Group to undertake assessments of susceptible species lists for OIE listed fish diseases.	Reviewed <i>ad hoc</i> Group report (January 2017) and requested <i>ad hoc</i> Group to continue their work		Review <i>ad hoc</i> Group report and amend relevant Sections of the <i>Manual</i>
<i>Ad hoc</i> Group on <i>Aquatic Manual</i>	Review <i>ad hoc</i> Group report. Propose a second meeting.	Review <i>ad hoc</i> Group report. Propose amendments to draft template.		Review final template and any chapters that have been revised using it
REFERENCE LABORATORIES (in collaboration with the Biological Standards Commission)				
SOPs for approval and maintenance of Reference Laboratory status	Developed and reviewed draft SOPs.	Finalise SOPs	Labs Commission to present for adoption	
Collaborating Centres		Develop table of potential gaps		Review
OTHER WORK				
Guidance document for assessments of new disease listings		Develop a guidance document and circulate before September 2017		Review

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