Annex 2

Organisation Mondiale de la Santé Animale | World Organisation for Animal Health | Organización Mundial de Sanidad Animal

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September 2016

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

Paris, 12–16 September 2016

EU comment

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

A number of general comments on this report of the September 2016 meeting of the Aquatic Animals Commission are inserted in the text below, while specific comments are inserted in the text of the respective annexes to the report.

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

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at OIE Headquarters in Paris from 12 to 16 September 2016. The list of participants is attached as Annex 1.

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts for the *Aquatic Code* and *Aquatic Manual* circulated after the Commission's February 2016 meeting: Argentina, Australia, Canada, Chile, China (People's Rep. of), Chinese Taipei, Colombia, Korea (Rep. of), Mexico, New Zealand, Norway, Saudi Arabia, Singapore, Switzerland, Thailand, the United States of America and the Member States of the European Union (EU).

The Aquatic Animals Commission reviewed comments that Member Countries had submitted prior to 5 August 2016 and amended texts in the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (the *Aquatic Manual*) where appropriate. The amendments are shown in the usual manner by 'double underline' and '~~strikethrough~~' and may be found in the Annexes to this report. In Annexes previously circulated for comment, the amendments made at this meeting are highlighted with a coloured background in order to distinguish them from those made at the February 2016 meeting.

The Aquatic Animals Commission considered all Member Country comments. However, the Commission was not able to draft a detailed explanation of the reasons for accepting or not each of the proposals received and focused its explanations on the most significant issues.

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 the reports of *ad hoc* Groups, which include important information and encourages Member Countries to review these reports together with the report of the Commission, where relevant.

Furthermore, Member Countries are reminded that comments submitted without a rationale or obvious logic are difficult to evaluate and respond to.

The table below summarises the texts as presented in the Annexes. Member Countries should note that texts in Annexes 3 to 27 are presented for Member Countries' comments and Annexes 28 to 32 are presented for Member Countries' information.

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

Comments on Annexes 3 to 27 of this report must reach OIE Headquarters by the **25th January 2017** to be considered at the February 2017 meeting of the Aquatic Animals Commission.

All comments should be sent to the OIE International Trade Department at: **standards.dept@oie.int Please note the change of email address**.

Item number	Items for Member Country comment:	Annex number	Page numbers
	AQUATIC CODE		
1	General comments – inclusion of other species where disinfection for eggs is practised and important for ensuring safe trade.	NOTE: No Annex is provided but comments are requested.	
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5	Diseases listed by the OIE (Chapter 1.3.)	Annex 5	31
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7	Recommendations for surface disinfection of salmonid eggs (Chapter 4.4.)	Annex 7	43
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9.1	Crayfish plague *(Aphanomyces astaci)* (Chapter 9.1.)	Annex 9	47
9.2	Infection with yellow head virus genotype 1 (Chapter 9.2.)	Annex 10	53
9.3	Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)	Annex 11	59
9.4	Infectious myonecrosis (Chapter 9.4.)	Annex 12	65
9.5	Necrotising hepatopancreatitis (Chapter 9.5.)	Annex 13	71
9.6	Taura syndrome (Chapter 9.6.)	Annex 14	77
9.7.	White tail disease (Chapter 9.8.)	Annex 15	83
10	Acute hepatopancreatic necrosis disease (new Chapter 9.X.)	Annex 16	89
11	Revised Article X.X.8. (clean text and track changes text)	Annex 17A (clean) and 17B (tracked changes)	95 and 97
12	White spot disease (Chapter 9.7.)	Annex 18	99

13	Criteria for listing species as susceptible (Chapter 1.5.)	Annex 19	107
AQUATIC MANUAL			
17	Acute hepatopancreatic necrosis disease (new draft Chapter 2.2.X.)	Annex 20	111
18	Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.)	Annex 21	125
19	Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)	Annex 22	147
20	Infectious myonecrosis (Chapter 2.2.4.)	Annex 23	169
21	Necrotising hepatopancreatitis (Chapter 2.2.5.)	Annex 24	181

Item number	**Items for Member Country comment:**	**Annex number**	**Page numbers**
AQUATIC MANUAL (contd)			
22	Taura syndrome (Chapter 2.2.6.)	Annex 25	195
23	White tail disease (Chapter 2.2.8.)	Annex 26	215
24	White spot disease (Chapter 2.2.7.)	Annex 27	231
ANNEXES FOR MEMBER COUNTRY INFORMATION			
5	Revised assessment for listing infection with *Batrachochytrium salamandrivorans*	Annex 28	233
5	Assessment for listing Tilapia lake virus	Annex 29	237
12	Report of the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases	Annex 30	241
10	Report of the electronic *ad hoc* Group on safety of products derived from aquatic animals	Annex 31	269
J	Aquatic Animal Health Standards Commission Work Plan for 2016/2017	Annex 32	287

A. MEETING WITH THE DIRECTOR GENERAL

Dr Monique Eloit, Director General, welcomed Aquatic Animals Commission members and thanked them for their support and commitment to achieving OIE objectives related to aquatic animal health.

Dr Eloit introduced Dr Matthew Stone, who has recently joined the OIE as the new Deputy Director General International Standards and Science and Ms Ann Backhouse, new Head of the Standards Department. Dr Eloit informed the Commission that the Standards Department will be dedicated to strengthening collaboration and coordination across the four Specialist Commissions and reinforcing the role of the Secretariat to better support the work of the Commissions.

Among other matters, Dr Eloit reiterated the commitment of the OIE to the implementation of the key objectives of the Sixth Strategic Plan. She explained that improved processes for selection of OIE experts will be developed, including for membership of the Specialist Commissions. Dr Eloit noted that the forthcoming session of the Council will consider a paper on the proposed draft procedure for the selection of experts.

Dr Eloit also informed the Aquatic Animals Commission that a decision had been made to provide all of the OIE *ad hoc* Group reports as stand-alone documents on the OIE website. She also noted that the date of adoption and last revision will be added to the end of all chapters in the *Aquatic Code* and *Manual*.

Dr Ingo Ernst, President of the Aquatic Animals Commission, thanked Dr Eloit for her welcome comments. Dr Ernst explained that this was an important meeting for the Commission to build the momentum of its work plan. He noted that support of the Commission by OIE Headquarters, including the work of *ad hoc* Groups, was important in order to complete the planned work items within expected time frames.

B. ADOPTION OF THE AGENDA

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

C. MEETING WITH THE PRESIDENT OF THE TERRESTRIAL 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; proposed revised Chapters 1.2. (criteria for listing); proposed revised Chapters 1.3. (listed diseases); proposed restructuring of Section 4 (disease prevention and control); and proposed development of a guidance document for *ad hoc* Groups on the application of the criteria for listing an OIE disease.

The Aquatic Animals Commission agreed that the alignment of the Aquatic Animals Commission and Code Commission meetings to enable the Presidents to meet should continue in the future to facilitate harmonisation of relevant chapters when under review by the respective Commissions.

D. MEETING WITH THE PRESIDENT OF THE BIOLOGICAL STANDARDS COMMISSION

The President of the Aquatic Animals Commission and the President of the Biological Standards Commission (Laboratory Commission) held a conference call on the 17 August 2016, prior to the meeting of the Laboratory Commission (30th August to 2nd September), to discuss issues of mutual interest, notably: Reference Laboratories - procedures for designating and delisting; Reference Laboratories - evaluating "equivalency" to ISO 17025 accreditation.

The Aquatic Animals Commission agreed that this conference call was very useful and should continue in the future to facilitate the harmonisation of relevant work of the respective Commissions.

E. EXAMINATION OF MEMBER COUNTRY COMMENTS AND WORK OF RELEVANT *AD HOC* GROUPS

Item 1. General comments

The Aquatic Animals Commission thanked Member Countries for their comments and acknowledged their contributions as an important part of the process to develop standards. The Commission particularly welcomed the receipt of comments from Member Countries that had not previously provided comments.

EU comment

Even if not currently up for comments, the EU would like to point out an inaccuracy in the Aquatic Code Chapter 10.8. on Red sea bream iridoviral disease. Indeed, the list of species in Article 10.8.2. includes the following: "Albacore (*Thunnus thynnus*)". We would like to point out that the scientific name for Albacore is *Thunnus alalunga*. If the recommendations in Chapter 10.8. apply to *Tunnus thynnus*, then the word "Albacore" should be replaced by "bluefin tuna" in Article 10.8.2. (Please note that in the Aquatic Manual both species are mentioned correctly.)

Comments of a general nature were received from Australia, China (People's Rep. of), the EU, New Zealand and Thailand.

Headquarters staff informed the Aquatic Animals Commission that some Member Countries continue to submit comments without providing a supporting rationale. Given that such comments are very difficult for the Commission to evaluate and respond to, the decision has been taken, by the Director General, that any comments submitted by Member Countries without a rationale will not be provided to the Commission, and therefore will not be considered when revising relevant texts.

In response to a Member Country comment requesting that the disease naming convention 'infection with pathogen X' be used throughout the *Aquatic Code* and *Manual*, the Aquatic Animals Commission noted that the intent is to adopt this naming format progressively as chapters are reviewed. The process has commenced for all crustacean disease-specific chapters in both the *Aquatic*

Code and *Manual* simultaneously with the revision of the lists of susceptible species. This work is planned for all disease-specific chapters.

In response to a Member Country comment requesting that the chapter on compartmentalisation be reviewed, the Aquatic Animals Commission noted that this is included in their work plan as part of the revision of Section 4.

The Aquatic Animals Commission considered a Member Country comment on the need to evaluate the disease transmission risks presented by filter feeders that may test PCR positive for pathogenic agents. The Commission recognised there may be a need for research to evaluate transmission from putative vectors. With respect to potential disease risks, Member Countries should consider guidance provided in point 3 of Article X.X.3. in disease-specific chapters (i.e. to conduct a risk analysis).

The Aquatic Animals Commission noted that some Member Countries had requested that Chapter 4.4. *Recommendations for surface disinfection of salmonid eggs* be expanded to address disinfection protocols for other aquatic animal species. In its previous report, the Commission had requested that Member Countries provide suggestions on the species for which disinfection of eggs is a priority to prevent disease transmission based on industry practice and trade. However, no specific proposals or supporting information has been provided by Member Countries in order to address this issue.

The Aquatic Animals Commission encouraged Member Countries to make specific proposals regarding other species for which disinfection of eggs is practised and important for ensuring safe trade. Given the significant value and volume of trade in shrimp, they suggested that a procedure for disinfection of crustacean eggs and larvae may be warranted and invited Member Countries to provide relevant disinfection protocols or other supporting information.

EU comment

The EU supports further work on Chapter 4.4. as indicated in the paragraphs above. In particular, we would welcome OIE standards on disinfection methods for fish eggs of non-salmonid species, such as zander (*Sander lucioperca*) and perch (*Perca fluviatilis*). This latter further to the repeated emergence of rhabdovirus outbreaks of perch establishments in the EU. This is all the more justified given the toxicity of iodophores for this species, as indicated in a recent paper published in the Journal of Applied Aquaculture (2016 - 28:1, 47-51, http://www.tandfonline.com/doi/full/10.1080/10454438.2016.1164649).

In addition, the EU would like to suggest the following marine fish species for consideration:

- Lumpfish (Lumpsucker) – *Cyclopterus lumpus*

- Wrasse species - *Labrus bergylta, Crenilabrus melops, Labrus mixus, Ctenolabrus rupestris, Centrolabrus exoletus*

- Atlantic halibut - *Hippoglossus hippoglossus*

We understand industry currently uses the following methods and products, which however have not been validated:

- Standard practice may include the use of hydrogen peroxide / periacetic acid at 4000 ppm for 40 seconds before rinsing in clean seawater – this has been used for both cod and Atlantic halibut;

- More recently a switch to bronopol based products have been tried at 50 ppm for halibut / cod / lumpfish and wrasse. Note that contact times for such treatments have not been established but it is reported that multiple and sometimes daily treatments are undertaken during incubation;

- A formalin bath of 200 ppm for 30 minutes has been used for wrasse, however due to health and safety concerns the EU would not support use of formalin.

- The method given currently in Chapter 4.4., using iodophore solution, has been applied to the eggs of *Cyclopterus lumpus*, with no negative impact on egg viability reported.

Particularly – wild trade has opened up with the Lumpfish and Wrasse becoming more widely used as cleaner fish for sea lice management in Atlantic salmon farms, egg disinfection would make an important contribution to ensuring safe trade in this area.

Item 2. Glossary

Comments were received from Argentina, Colombia, the EU, New Zealand, Norway and Thailand.

Aquatic animals

Some Member Countries suggested that the definition of aquatic animal should distinguish between farmed and wild aquatic animals, as is the case in the *Terrestrial Code*. The Aquatic Animals Commission did not agree with this proposal because it considered that the definition must serve the purposes of its use in the *Aquatic Code*, including to facilitate safe trade of aquatic animals and their products. There are distinct differences between trade in aquatic animals and terrestrial animals, with 50% of traded aquatic animals originating from the wild, and the risk for disease transmission is more dependent on species susceptibility rather than origin from wild or farmed populations.

The Aquatic Animals Commission did not agree with a proposal from a Member Country to delete the last phrase referring to the origin of animals as they considered this phrase provides clarity for the defined term, i.e. includes both aquaculture and wild animals.

Zoning

The Aquatic Animals Commission noted that the current definition for zone was not appropriate and differed markedly from the *Terrestrial Code* definition. The Aquatic Animals Commission revised the definition taking into account the existing definition of compartment in the *Aquatic Code*, and amendments being proposed by the Code Commission for the term 'zone' in the *Terrestrial Code*.

Other relevant information

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, where other terms such as pathogen and aetiological agent needed to be replaced by pathogenic agent. The Commission plan to define more clearly where these replacements are required and will report further on this work at their February 2017 meeting.

The Aquatic Animals Commission was informed that the Code Commission had proposed a significant review of the definitions in the glossary of the *Terrestrial Code* which will be implemented in a gradual process. The Aquatic Animals Commission noted the importance of ensuring alignment between relevant definitions used in both Codes and will follow this work closely.

The Aquatic Animals Commission acknowledged the Headquarters' decision to postpone discussion on the proposed definitions of OIE standard and OIE guideline until the OIE Council considers this issue at its September 2016 meeting. The Commission will be updated on outcomes of the Council at its February 2017 meeting.

The revised Glossary definitions are attached as Annex 3 for Member Country comment.

EU comment

The EU in general supports the proposed changes to the glossary. Comments are inserted in the text of Annex 3.

In addition, the EU would like to refer the Aquatic Animals Commission to the EU comments on proposals to modify the glossary of the Terrestrial Code, as presented in the Code Commission's September 2016 meeting report. The EU comments in relation to some of the proposed Terrestrial Code definitions would also be relevant for the Aquatic Code, as corresponding definitions in the Aquatic Code are the identical or very similar.

Item 3. Notification of diseases, and provision of epidemiological information (Chapter 1.1.)

A comment was received from Australia.

The Aquatic Animals Commission did not agree with a Member Country comment to include 'fomites' in point 6 of Article 1.1.2. as they considered this was covered by the existing wording 'other miscellaneous objects'.

Item 4. Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)

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

The Aquatic Animals Commission considered Member Country comments and amendments being proposed by the Code Commission in the corresponding chapter in the *Terrestrial Code*.

The Aquatic Animals Commission wished to note that no additional amendments were made to the version circulated in their February 2016 report.

A Member Country commented that the revised criteria may not continue to support the listing of all the aquatic animal diseases currently on the OIE list, in particular HPR0 genotypes of infectious salmon anaemia (ISAV). The Aquatic Animals Commission wished to remind Member Countries that the purpose of the revision was to simplify the criteria and align the format with the *Terrestrial Code* while also ensuring that that objective of listing diseases in the *Aquatic Code* continues to be served. The intended meaning of the revised criteria is essentially unchanged from the existing criteria and no changes to the list of diseases are anticipated with adoption of the revised criteria. In addition, the Aquatic Animals Commission noted that 'Infection with HPR-deleted or HPR0 ISAV' is listed in Chapter 1.3. of the *Aquatic Code* as a single disease and HPR0 ISAV is not listed separately.

In response to a Member Country comment to align criterion 1 of Article 1.2.2. with text used in the *Terrestrial Code*, i.e. to insert 'has been proven' rather than 'is likely', the Aquatic Animals Commission reminded Member Countries that they had responded to a similar comment in their February 2016 report. 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 revised Chapter 1.2. is attached as Annex 4A (as clean text) and Annex 4B (showing track changes in Chapter 1.2. of the 2016 *Aquatic Code*) for Member Country comment.

EU comment

The EU in general supports the proposed changes to this chapter. A comment is inserted in the text of Annex 4A.

Item 5. Diseases listed by the OIE (Chapter 1.3.)

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

In response to a Member Country comment regarding the need to consider listing diseases of reptiles in the *Aquatic Code*, the Aquatic Animals Commission noted that reptiles were added to the definition for animals in the *Terrestrial Code* and therefore reptile issues will be addressed by the Code Commission.

Amended crustacean names

The Aquatic Animals Commission did not agree with a Member Country to amend the name of "Acute hepatopancreatic necrosis disease' to include the causal agents in the format 'infection with pathogen X'. The Commission reminded Member Countries that this approach to listing was taken because the scientific information on the disease aetiology continues to evolve.

Batrachochytrium salamandrivorans (*Bsal*)

Several Member Countries supported the listing of this disease. However, some Member Countries questioned whether criterion 8 of Article 1.2.2. was met i.e. availability of repeatable and robust diagnostic tests. The Aquatic Animals Commission considered that this criterion has been met as there are repeatable and robust diagnostic tests available for this pathogen. Since the original assessment was undertaken (February 2016), the Aquatic Animals Commission has identified further information on diagnostic methods to strengthen this assessment. The Commission revised the assessment to incorporate this information.

A Member Country questioned the conclusion that several countries may be declared free of the disease. The Aquatic Animals Commission considered that given available evidence provided in the assessment it would be likely that countries would be able to demonstrate freedom, satisfying criterion 7 of Article 1.2.2.

Some Member Countries commented that it may be desirable to delay any proposals to list new diseases whilst the criteria for listing were under revision. The Aquatic Animals Commission does not consider it necessary to suspend consideration of diseases for listing until the new criteria are adopted because the intended meaning of the revised criteria is essentially unchanged from the existing criteria (see Item 4).

The revised assessment for *Bsal* is provided for Member Country information in support of the proposed listing at Annex 28.

Infection with Ranavirus

In response to a Member Country comment, the Aquatic Animals Commission agreed to amend the listed name for 'infection with ranavirus' to 'infection with *Ranavirus* spp.' to more accurately reflect the scope of this disease which is any virus species of the Genus *Ranavirus*. The Commission noted that once this revision is adopted relevant changes in Chapter 8.2. will be made.

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

At their February 2016 meeting, the Aquatic Animals Commission noted the identification of a novel orthomyxo-like virus which had been named tilapia lake virus (TiLV). The Commission had agreed to further consider this virus, including possible listing in the *Aquatic Code*.

At this meeting, the Commission considered available scientific information and concluded that, due to the lack of specific diagnostic methods for TiLV, the disease could not be proposed for listing at this time. The Commission encourages further research on this virus, in particular the development and validation of diagnostic methods.

The Aquatic Animals Commission recognised the potential significance of TiLV to many countries given the worldwide importance of tilapia farming. The Commission noted that this disease is likely to meet the definition of an "emerging disease" and, as such, should be reported in accordance with

Article 1.1.4. of the *Aquatic Code*. It encourages Member Countries to investigate mortality and morbidity events in tilapines and report any detections of TiLV to the OIE, as an understanding on the geographic distribution of this disease is essential for efforts to control its possible spread. The Aquatic Animals Commission noted that previous disease events associated with this virus had not been reported to the OIE in accordance with the obligations described in Chapter 1.1.

The Aquatic Animals Commission agreed to develop a Technical Disease Card for TiLV to provide information for Member Countries on available detection methods and transmission risks for this virus. The Technical Disease Card will be made available on the OIE website when completed.

The assessment for TiLV against the listing criteria is provided for Member Country information at Annex 29.

Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopanaei*

The Network of Aquaculture Centres in Asia-Pacific (NACA) brought to the attention of the Aquatic Animals Commission that Hepatopancreatic microsporidiosis (HPM) had been included on its Quarterly Aquatic Animal Disease Reporting programme. The Aquatic Animals Commission noted this disease, which is caused by *Enterocytozoon hepatopenaei*, is an important cause of production losses in shrimp farming in Asia and that the geographic distribution of the parasite is uncertain.

The Aquatic Animals Commission encouraged Member Countries to refer to the disease card on Hepatopancreatic microsporidiosis that has been developed by NACA and is available at:

http://enaca.org/publications/health/disease-cards/ehp-disease-card-2015.pdf

Marteilia cochillia

In its February 2016 report, the Aquatic Animals Commission concluded that there was insufficient evidence to meet criterion 6 and 7 (of Article 1.2.2. in the *Aquatic Code*) to support listing of *Marteilia cochillia*. In response to a Member Country comment to reconsider this assessment, the Commission noted that the worldwide distribution of the susceptible host species, *Cerastoma edule*, is limited to Europe and Northwest Africa and trade occurs primarily within Europe. In addition, insufficient work has been done to develop repeatable and robust diagnostic tests for detection of this pathogenic agent.

The Commission maintains its position that this disease does not meet the criteria for listing.

The revised articles of Chapter 1.3. are attached as Annex 5 for Member Country comment.

EU comment

EU comment

The EU in general supports the proposed changes to this chapter, except for the proposed listing of *Batrachochytrium salamandrivorans*. A further comment is inserted in Annex 5.

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

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

The Aquatic Animals Commission considered Member Country comments received as well as interventions made at the OIE 2016 General Session, and made relevant amendments to improve clarity and readability.

The revised Chapter 4.3. is attached as Annex 6 for Member Country comment.

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. Comments are inserted in the text of Annex 6.

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

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

The Aquatic Animals Commission considered Member Country comments and made relevant amendments to improve clarity and readability.

The revised Chapter 4.4. is attached as Annex 7 for Member Country comment.

EU comment

The EU in general supports the proposed changes to this chapter. One comment is inserted in the text of Annex 7.

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

The Aquatic Animals Commission considered Member Country comments on Article 5.1.4. made during the 84th OIE General Session regarding the need for alignment with Chapter 5.1. of the *Terrestrial Code*. The Commission amended point 2 of Article 5.1.4. to improve consistency with point 2 in Article 5.1.4. of the *Terrestrial Code* chapter.

The revised Article 5.1.4. is attached as Annex 8 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 9. Amendments to crustacean disease-specific chapters

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

Given the similarity between the disease-specific crustacean chapters in the *Aquatic Code* the Aquatic Animals Commission considered Member Country comments made in each chapter and applied them in all crustacean chapters, where relevant, to ensure alignment between all chapters.

The Aquatic Animals Commission reviewed and amended, where relevant, the scope for all crustacean disease-specific chapters in the *Aquatic Code* and *Aquatic Manual* to ensure they were aligned with proposed amendments to disease naming, i.e. 'infection with pathogen X'. In response to several Member Country comments regarding the proposed approach, the Aquatic Animals Commission noted that 'infection with pathogen X' is now the name of the disease, i.e. a proper noun, and that the Commission had agreed to apply this approach consistently throughout the *Aquatic Code*. The Commission emphasised that when reading the text of the *Aquatic Code*, 'infection with pathogen X' is equivalent to the previous disease name.

The Aquatic Animals Commission noted that the use of 'infection with pathogen X' had not always been applied correctly and amended text, where relevant.

The Aquatic Animals Commission agreed to put the species listed in Articles X.X.2. in alphabetical order by scientific name.

In response to a Member Country comment, the Aquatic Animals Commission decided to delete the phrase 'For the purposes of this chapter, the terms shrimp and prawn are used interchangeably.' in Articles 9.X.2. as they considered this to be unnecessary.

The Aquatic Animals Commission noted that the FAO recently launched a terminological database, 'FAOTERM' that includes a glossary of terminology (Latin and common names) for an extensive

number of aquatic animal species in English, French and Spanish (accessed at: http://www.fao.org/faoterm/en/).

The Aquatic Animals Commission agreed to use this database as the source for common names used in the *Aquatic Code* and *Manual*.

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

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

In light of the proposed amendment to Chapter 1.5. (see Item 13) the Aquatic Animals Commission agreed not to amend the current list of susceptible species in Article 9.1.2, that had been proposed in their February 2016 report, until they have reviewed comments from Member Countries on the proposed amendments to Chapter 1.5. The outcome of revisions to Chapter 1.5. may have a significant bearing on the list of susceptible species for diseases with a broad host range, such as crayfish plague.

The revised Chapter 9.1. is attached as Annex 9 for Member Country comment.

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. A comment is inserted in the text of Annex 9.

Item 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. is attached as Annex 10 for Member Country comment.

EU comment

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

Item 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 a Member Country comment that *Macrobrachium rosenbergii* should be removed from the list of susceptible species in Article 9.3.2. noting that the assessment undertaken by the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases (provided as Annex 30 in the Commission's February 2016 report) had found that it met the criteria to be listed as a susceptible species. The Aquatic Animals Commission noted that as per Article 1.5.2. susceptibility may include clinical or non-clinical infection.

The revised Chapter 9.3. is attached as Annex 11 for Member Country comment.

EU comment

The EU in general supports the proposed changes to this chapter. One comment is inserted in the text of Annex 11.

Item 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. is attached as Annex 12 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 9.5. Necrotising hepatopancreatitis (Chapter 9.5.)

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

The Aquatic Animals Commission agreed with a Member Country comment to delete the word "Candidatus" throughout the chapter to maintain consistency with other chapters.

The revised Chapter 9.5. is attached as Annex 13 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 9.6. Taura syndrome (Chapter 9.6.)

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

The revised Article 9.6. is attached as Annex 14 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 9.7. 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. is attached as Annex 15 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 10. New chapter on Acute hepatopancreatic necrosis disease (Chapter 9.X.) including the Report of the *ad hoc* Group on safety of products derived from aquatic animals (August 2016)

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

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 also reviewed the report of the electronic *ad hoc* Group on safety of products derived from aquatic animals who had conducted assessments on a range of commodities, commonly traded internationally, against the criteria provided in Chapter 5.4. The Aquatic Animals Commission agreed with the *ad hoc* Group's proposed list of animal products and amended Articles 9.X.3. and 9.X.11. accordingly, and removed the square brackets as this text is no longer under study.

In response to several Member Country comments regarding the aetiology of AHPND as described in Article 9.X.1., the Aquatic Animals Commission noted that although there are reports on the isolation of other *Vibrio* species from clinical cases of AHPND, only *Vibrio parahaemolyticus* (Vp_{AHPND}) has been characterised and demonstrated as a causative agent of AHPND. Therefore, the Aquatic Animals Commission agreed that *V. harveyi* and other bacterial isolates associated with AHPND should not be included as aetiological agents in Article 9.X.1.

The report of the electronic *ad hoc* Group on safety of products derived from aquatic animals is attached as Annex 31 for Member Country information.

The revised Chapter 9.X. is attached as Annex 16 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 11. Revised Article X.X.8.

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

The Aquatic Animals Commission considered Member Country comments and made relevant amendments noting that these amendments were of an editorial nature to improve readability.

The Aquatic Animals Commission reminded Member Countries that once this model article is adopted, Articles X.X.8. of all disease-specific chapters will be amended accordingly. They noted that the proposed amendments have been applied to the revised crustacean chapters which will be circulated for Member Country comment (see Items 9, 10 and 12).

The revised model Article X.X.8. is attached as Annex 17A (as clean text) and Annex 17B (with track changes) for Member Country comment.

EU comment

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

F. OTHER *AQUATIC ANIMAL HEALTH CODE* ISSUES

Item 12. White spot disease (Chapter 9.7.) and the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases

The Aquatic Animals Commission considered the report of the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases (see Annex 30) and wished to acknowledge the excellent and thorough work undertaken by the members of this group.

The Aquatic Animals Commission agreed not to amend the current list of susceptible species in Article 9.7.2. until they have reviewed comments from Member Countries on the proposed amendments to Chapter 1.5. (see Item 13). The outcome of revisions to Chapter 1.5. may have a significant bearing on the list of susceptible species for diseases with a broad host range, such as white spot disease.

However, the Aquatic Animals Commission did agree to apply amendments of a horizontal nature, as noted in Item 9, to ensure their alignment in all crustacean disease-specific chapters.

The report of the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases is provided in Annex 30 for Member Country information.

The revised Chapter 9.7. is attached as Annex 18 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 13. Criteria for listing species as susceptible (Chapter 1.5.)

Several Member Country comments supported the Aquatic Animals Commission proposal, noted in their February 2016 report, to review the criteria in Chapter 1.5. 'Criteria for listing species as susceptible', to address their application to diseases with a wide host range. Application of the current criteria to diseases with a broad host range may result in substantial reductions in the list of susceptible species, which may have implications for international trade (e.g. infection with *Aphanomyces astaci* and infection with white spot syndrome virus).

The Aquatic Animals Commission reviewed Chapter 1.5. with the view to including a mechanism to list taxonomic groups of species as susceptible, when many species within a taxon have been determined to be susceptible and none has been found to be refractory to infection. The Aquatic Animals Commission drafted text for a new Article 1.5.9.

The revised Chapter 1.5. is attached as Annex 19 for Member Country comment.

EU comment

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

Item 14. Review prawn taxonomy literature for *Penaeus*

In its February 2016 meeting report, the Aquatic Animals Commission noted that some Member Countries requested changes to the generic names of shrimp species in the genus *Penaeus*.

The Aquatic Animal Commission noted that a change to penaeid shrimp taxonomy was proposed by Pérez Farfante & Kensley (1997), which included the elevation of former sub-genera in the genus *Penaeus* to generic rank. This change resulted in 24 of the 27 *Penaeus* species being assigned to 5 new genera. However, there has been controversy among scientists regarding the revised nomenclature (e.g. Balwin *et al*., 1998; Lavery *et al*., 2004; Flegel, 2007, 2008; McLaughlin *et al*., 2008) and the taxonomy proposed by Pérez Farfante and Kensley (1997) has been adopted inconsistently by scientists, scientific journals and organisations. This has resulted in a level of instability regarding *Penaeus (sensu lato)* taxonomy.

The Aquatic Animals Commission reviewed recent literature on penaeid shrimp taxonomy and noted that some recent molecular studies do not support the taxonomy proposed by Pérez Farfante and Kensley (1997). In particular, Ma *et al.* (2011) in a comprehensive phylogenetic study recommended that the "old" *Penaeus* classification be restored and the six genera proposed by Pérez Farfante and Kensley (1997) be dismissed. More recent studies (e.g. Zhang *et al*., 2015) support the phylogenetic relationships of Ma *et al.* (2011) but recommend additional studies with more taxa to further understand the phylogeny of the family Penaeidae.

Previously, the Aquatic Animals Commission had chosen not to change the classification of *Penaeus* species in the *Aquatic Code* and *Aquatic Manual* pending resolution of the scientific disagreement regarding *Penaeus* taxonomy. Based on their review of the literature on penaeid taxonomy, the Commission agreed that the "old" *Penaeus* taxonomy (i.e. a single genus *Penaeus*, with 6 sub genera) should continue to be used consistently in the *Aquatic Code* and *Aquatic Manual*.

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4) Holthuis L.B. (1980). Shrimps and Prawns of the World. An Annotated Catalogue of Species of Interest to Fisheries. FAO, Rome. 271 pp. (available online at: http://www.fao.org/docrep/009/ac477e/ac477e00.htm)

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7) McLaughlin P.A., Lemaitre R., Ferrari F.D., Felder D. & Bauer R. (2008). A reply to T.W. Flegel. *Aquaculture*, **275**, 370–373.

8) Pérez Farfante I. & Kensley B. (1997). Penaeoid and Sergestoid Shrimps and Prawns of the World. Paris: Muséum National d'Histoire Naturelle.

9) Zhang D., Gong F., Liu T, Guo H., Zhang N., Zhu K. é Jiang S. (2015). Shotgun assembly of the mitochondrial genome from *Fenneropenaeus penicillatus* with phylogenetic consideration. *Marine Genomics*, **24**, 379–386.

Item 15. ***Ad hoc*** **Group on aquatic animal biosecurity**

The Aquatic Animals Commission considered the priority activities for the revision of Section 4 of the *Aquatic Code* 'Disease prevention and control', that it had proposed in its February 2016 meeting report, i.e. (1) finalise and adopt Chapter 4.3. Disinfection of aquaculture establishments and equipment; (2) develop a new chapter on biosecurity; (3) revise Chapters 4.1. and 4.2. on zoning and compartmentalisation; and (4) develop a new chapter on emergency disease preparedness.

The Aquatic Animals Commission noted that the revised Chapter 4.3. had been adopted in May 2016. The Commission developed the Terms of Reference for an *ad hoc* Group to develop a new chapter on aquatic animal biosecurity for aquaculture establishments for Section 4. They reiterated that this is an important chapter for Section 4 of the *Aquatic Code* on disease prevention and control as it will complement and integrate with other chapters in this section, such as disinfection and compartmentalisation.

The Aquatic Animals Commission requested that an *ad hoc* Group on aquatic animal biosecurity for aquaculture establishments be convened to commence this work.

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

The Commission has recognised that the time periods specified in the disease-specific chapters of the *Aquatic Code* that need to be met (for surveillance and basic biosecurity conditions), before a self-declaration of freedom can be made, are not based on clear criteria and are inconsistent among diseases.

The Aquatic Animals Commission developed the Terms of Reference for this work and requested that an *ad hoc* Group on demonstration of disease freedom be convened to develop criteria for determining these periods and to review the periods for each disease-specific chapter of the *Aquatic Code*.

G. MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS

The Aquatic Animals Commission wished to remind Member Countries that the *ad hoc* Group on the *Aquatic Manual* is currently revising the template for all *Aquatic Manual* chapters (see Item 25). Once the new template has been agreed by the Aquatic Animals Commission it will be applied progressively to all disease-specific chapters of the *Aquatic Manual*, resulting in a comprehensive revision of both their structure and content. The Aquatic Animals Commission agreed that revisions to the crustacean disease chapters should proceed without delay to ensure consistency is retained with equivalent *Aquatic Code* chapters i.e. changing the disease name format to "Infection with pathogen X" and changes to the lists of susceptible species (see Item 9).

The Aquatic Animals Commission reviewed all Member Country comments on the crustacean disease chapters and applied them in all crustacean chapters, where relevant, to ensure alignment between all chapters.

The Aquatic Animals Commission received a Member Country comment suggesting that "quarantine" be included in the title of Section 6 of all *Aquatic Manual* chapters, i.e. "*Test*(*s*) *recommended for targeted surveillance and quarantine to declare freedom from* [*disease name*]". The Commission agreed that this issue

will be addressed by the *ad hoc* Group on the *Aquatic Manual* (see Item 25), which will review the format of Sections 5 (*Rating of tests against purpose of use*) and 6.

The Aquatic Animals Commission considered a Member Country comment that polymerase chain reaction (PCR) should be included as a method for Section 7.1 *Definition of a suspect case* in each disease-specific chapter of the *Aquatic Manual*. The Commission agreed that this would, in most cases, be an appropriate method for defining a suspect case. The Commission had requested that the *ad hoc* Group on the *Aquatic Manual* consider the format of case definitions as a part of its work. This work is ongoing and changes to the case definitions will be addressed progressively when the new case definition format is applied to each disease-specific chapter. However, the Commission noted that for the crustacean disease chapters currently under revision, some case definitions were deficient and required immediate revision. The Commission made these revisions where necessary.

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

Comments had been received from Australia, Canada, China (People's Rep. of), Chinese Taipei, Korea (Rep. of), New Zealand and Saudi Arabia.

In response to several Member Country comments regarding the aetiology of acute hepatopancreatic necrosis disease **(**AHPND), as described in the scope of the draft *Aquatic Manual* Chapter 2.2.X., the Aquatic Animals Commission noted that, although other *Vibrio* species have been isolated from clinical cases of AHPND, only *Vibrio parahaemolyticus* has been demonstrated to be a causative agent of AHPND. For other *Vibrio* species, the Commission is not aware of any publications documenting challenge experiments that have demonstrated that bacteria, other than *V. parahaemolyticus*, can cause AHPND.

The Aquatic Animals Commission agreed that, based on available evidence, *V. harveyi* and other bacterial isolates associated with AHPND should not be included as aetiological agents in the draft *Aquatic Manual* chapter, nor in Article 9.X.1. of the *Aquatic Code* (see Item 10).

The revised Chapter 2.2.X. is attached as Annex 20 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 18. Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.)

The Aquatic Animals Commission agreed to retain the current list of susceptible species in Section 2.2.1. until they have reviewed comments from Member Countries on the proposed amendments to *Aquatic Code* Chapter 1.5. *Criteria for listing species as susceptible to infection with a specific pathogen* (see Item 13). The outcome of revisions to Chapter 1.5. of the *Aquatic Code* may have a significant bearing on the list of susceptible species for diseases with a broad host range, such as crayfish plague.

The Aquatic Animals Commission reviewed Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert. The Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 2.2.1. is attached as Annex 21 for Member Country comment.

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. However, comments are inserted in the text of Annex 21.

Item 19. Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)

Comments had been received from Australia, China (People's Rep. of), Chinese Taipei, Korea (Rep. of) and New Zealand.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory experts. The Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 2.2.3. is attached as Annex 22 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 20. Infectious myonecrosis (Chapter 2.2.4.)

Comments had been received from Australia, China (People's Rep. of), Korea (Rep. of), New Zealand, and the United States of America.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert. The Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 2.2.4. is attached as Annex 23 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 21. Necrotising hepatopancreatitis (Chapter 2.2.5.)

Comments had been received from Australia, China (People's Rep. of), Korea (Rep. of) and New Zealand.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert. The Commission agreed to apply amendments of a horizontal nature as noted above.

The Aquatic Animals Commission agreed that the word "Candidatus" should be deleted from the name of the pathogenic agent, which would be *Hepatobacter penaei.*

The revised Chapter 2.2.5. is attached as Annex 24 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 22. Taura syndrome (Chapter 2.2.6.)

Comments had been received from Australia, China (People's Rep. of), Korea (Rep. of) and New Zealand.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert. The Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 2.2.6. is attached as Annex 25 for Member Country comment.

EU comment

The EU in general supports the proposed changes to this chapter, however wishes to reiterate its comments submitted previously, which were not addressed (available here: https://ec.europa.eu/food/sites/food/files/safety/docs/ia_standards_oie_eu_position_aahsc_report_201602_en.pdf, p. 203).

Item 23. White tail disease (Chapter 2.2.8.)

Comments had been received from Australia, China (People's Rep. of), Korea (Rep. of) and New Zealand.

The Aquatic Animals Commission reviewed the Member Country comments and amended the text accordingly in consultation with the Reference Laboratory expert. The Commission agreed to apply amendments of a horizontal nature as noted above.

The revised Chapter 2.2.8. is attached as Annex 26 for Member Country comment.

EU comment

The EU in general supports the proposed changes to this chapter. A comment is inserted in the text of Annex 26.

Item 24. White spot disease (Chapter 2.2.7.) and the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases

The Aquatic Animals Commission considered the report of the *ad hoc* Group on susceptibility of crustacean species to infection with OIE listed diseases (see Annex 30) and wished to acknowledge the excellent and thorough work undertaken by the members of this Group.

The Aquatic Animals Commission agreed to retain the current list of susceptible species in Section 2.2.1. until they have reviewed comments from Member Countries on the proposed amendments to *Aquatic Code* Chapter 1.5. *Criteria for listing species as susceptible to infection with a specific pathogen* (see Item 13). The outcome of revisions to Chapter 1.5. of the *Aquatic Code* may have a significant bearing on the list of susceptible species for diseases with a broad host range, such as white spot disease.

The Aquatic Animals Commission agreed to amend the title and revise the scope of the chapter in line with amendments being proposed for other crustacean chapters but would put further revisions of this chapter on hold pending the outcome of the proposed revision of *Aquatic Code* Chapter 1.5.

The *ad hoc* Group report on susceptibility of crustacean species to infection with OIE listed diseases is provided in Annex 30 for Member Country information.

The revised title and scope of Chapter 2.2.7. is attached as Annex 27 for Member Country comment.

EU comment

The EU supports the proposed changes to this chapter.

Item 25. Review of the report of the *ad hoc* Group on the *Manual of Diagnostic Tests for Aquatic Animals*

The Aquatic Animals Commission considered the report of the *ad hoc* Group on the OIE *Aquatic Manual*, which had met in April 2017. The *ad hoc* Group's principal task was to review specific issues in the *Aquatic Manual* and to propose an amended structure (new disease chapter template) to improve consistency among chapters and improve the quality and completeness of information. These issues included the structure of Sections 4 *Diagnostic methods*, 5 *Rating of tests against purpose of use*, and 7 *Corroborative diagnostic criteria* of the disease-specific chapters of the OIE *Aquatic Manual*.

The Aquatic Animals Commission reviewed the report and its recommendations and provided feedback for the Group. The Commission also reviewed the revised disease chapter template that had been developed by the *ad hoc* Group, along with the three model chapters for a fish, a mollusc and a crustacean disease that were practical examples of the use of the template.

The Aquatic Animals Commission provided its comments to the *ad hoc* Group on the template and the three model chapters. The Commission agreed that the *ad hoc* Group should meet again before the Commission's next meeting, to further refine the template and model chapters.

H. OIE REFERENCE CENTRES

Item 26. Applications for OIE Reference Centre status or changes of experts

The Aquatic Animals Commission noted the important role that the OIE Reference Centre network provides in supporting the work of the OIE and serving the needs of OIE Member Countries.

The Aquatic Animals Commission was pleased to receive an application for an OIE Reference Laboratory for Koi herpesvirus (KHV). The Commission reviewed the dossier and identified areas where further information was required to facilitate the Commission's assessment. The applicant would be invited to submit this additional information so that it could be considered at the Commission's next meeting in February 2017.

The following nominations for changes to the experts at three OIE Reference Laboratories were submitted by the OIE Delegates of Member Countries concerned. The Aquatic Animals Commission recommended their acceptance:

Viral encephalopathy and retinopathy

Dr Anna Toffan to replace Dr Giovanni Cattoli at the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, ITALY

Red sea bream iridoviral disease

Dr Yasuhiko Kawato to replace Dr Kazuhiro Nakajima at the National Research Institute of Aquaculture, Fisheries Research Agency, Minami-Ise, JAPAN

Oncorhynchus masou *virus disease*

Dr Hisae Kasai to replace Dr Mamoru Yoshimizu at the Graduate School of Fisheries Science, Hokkaido University, Hokkaido, JAPAN

Item 27. Feedback on progress re Reference Laboratory quality management systems: towards ISO 17025 accreditation

The Aquatic Animals Commission reviewed information received from six OIE Reference Laboratories for aquatic animal diseases that are currently not accredited to ISO 17025 or equivalent, regarding their intention to implement such a quality management system. Two of the six laboratories indicated that they were at the initial phase in the process to achieve ISO 17025. Four did not provide a reply or indicated that their primary activity is research rather than provision of diagnostic services and that they would not pursue accreditation.

For those Reference Laboratories that have not achieved accreditation to ISO 17025 or an equivalent quality management system by the end of 2017, the Biological Standards Commission proposed suspending their OIE Reference Laboratory status with the possibility to be reinstated within two years should they achieve accreditation in that time. Laboratories that have still not achieved accreditation two years after suspension would have to re-apply for OIE Reference Laboratory status. The Aquatic Animals Commission supported this approach.

Item 28. Further development of standard operating procedures for the approval and maintenance of Reference Laboratory status

A procedure for designation and ongoing management of Reference Laboratories is under development between the Biological Standards and the Aquatic Animals Commission, and will eventually be subjected to Member Country consultation.

The Aquatic Animals Commission agreed with the Biological Standards Commission's proposal to add two new points to the criteria for de-listing: no response to requests from the OIE Headquarters for scientific expertise (e.g. revision of the OIE *Manual* chapters); and a pattern revealing a lack of diagnostic activity or production and supply of reference material in support of the diagnosis of the disease.

The Aquatic Animals Commission also agreed with the Biological Standards Commission's recommendation on the timeline and different steps to be taken in the process of de-listing an OIE Reference Laboratory.

Item 29. Reference Laboratory network GAP analysis

The Aquatic Animals Commission reviewed the current list of OIE Reference Laboratories for aquatic animal diseases and identified gaps in the network for listed diseases. These gaps exist because there is no OIE Reference Laboratory, or there may be no OIE Reference Laboratory after December 2017 because some have indicated that they may not achieve ISO 17025 or equivalent accreditation by that deadline.

Highest priority was given to listed diseases for which there is currently no OIE Reference Laboratory, or where a gap for a listed disease may occur after December 2017. Other disease categories that were considered include non-listed diseases for which there is a chapter in the *Aquatic Manual*, non-listed diseases that require accurate diagnosis to facilitate safe trade, and non-listed diseases that have significant production impacts.

The Aquatic Animals Commission identified the need to designate OIE Reference Laboratories for the following diseases:

1. Acute hepatopancreatic necrosis disease
2. Infectious haematopoietic necrosis
3. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)
4. Infection with *Xenohaliotis californiensis*
5. Infection with *Batrachochytrium dendrobatidis*

For these diseases, the Commission considered potentially suitable institutes that might be able to achieve OIE Reference Laboratory status. The Commission proposed that the Delegates of the Member Countries concerned be asked to consider supporting an application from these institutes. However, the Aquatic Animals Commission invites applications from any Member Country with expertise in these diseases.

The Aquatic Animals Commission reviewed a document provided by the Biological Standards Commission on the current OIE Reference Laboratory network system. The aim is to develop a strategic plan for the future role of the network. The Aquatic Animals Commission agreed to work together with the Biological Standards Commission, and with staff from the OIE Headquarters with responsibilities in the area, to progress development of this document.

Item 30. Twinning Projects

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

As of September 2016, one project has been completed (Canada with Chile for Infectious salmon anaemia) and five are underway (USA with China [People's Rep. of] for Infectious haematopoietic necrosis; Norway with Brazil for infectious salmon anaemia; Japan with Indonesia for Koi Herpesvirus; USA with Indonesia for shrimp diseases; Denmark with Republic of Korea for Viral haemorrhagic septicaemia).

The Aquatic Animals Commission noted that a new proposal between the Aquaculture Pathology Laboratory, University of Arizona, USA (Parent Laboratory) and the Fish Health and Safety Laboratory, Jeddah Fish Research Centre, Kingdom of Saudi Arabia (Candidate Laboratory) for shrimp diseases with emphasis on Taura Syndrome and White spot disease had been received. The Aquatic Animals Commission reviewed the proposal and provided technical comments.

I. OTHER ISSUES

Item 31. 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 Aquatic Animals Commission agreed that it is important to continue to follow this important topic, and update the *Aquatic Code* and *Manual* as relevant.

J. AQUATIC ANIMALS COMMISSION WORK PLAN FOR 2016/2017

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 32 for Member Country information.

K. AQUATIC ANIMALS COMMISSION ACTIVITIES

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

Members of the Commission have participated in the following activities:

Dr Ingo Ernst held a teleconference on 18 July 2016 for OIE Delegates and Aquatic Animal Focal Points in the Asia Pacific region. The purpose of the teleconference was to brief Member Countries on the report of the February 2016 meeting of the Aquatic Animals Commission, particularly annexes that had been provided for Member Country comment.

Dr Edmund Peeler attended the annual meeting of EU National Reference laboratory for Mollusc Diseases organised by the EU Reference Laboratory for mollusc diseases in Nantes in April 2016. He gave an overview of the work of the OIE Aquatic Animals Commission, highlighting the role of the aquatic animal focal points, and discussed the reporting and management of emerging molluscan diseases. In June 2016 he attended the annual meeting of EU National Reference laboratory for Fish Diseases organised by the EU fish disease Reference Laboratory in Copenhagen. He gave an overview of the work of the OIE Aquatic Animals Commission and presented a review of the use of serology in aquatic animal health.

Dr Alicia Gallardo participated in a Regional Commission of the America's meeting (July 2016) to discuss the February 2016 report of the Aquatic Animals Commission. She presented an overview of the report and answered questions.

Prof. Mohamed Shariff Bin Mohamed Din represented the OIE at: the FAO Second International Technical Seminar/Workshop on Acute hepatopancreatic necrosis disease (AHPND) in Bangkok from 23 to 25 June 2016. He presented an overview of OIE Aquatic Animal Commission highlighting the relevant activities regarding AHPND and the chronological process of listing it in the *Aquatic Code*. In July 2016 he attended the 9th FAO/OIE Regional Steering Committee Meeting of GF-TADs for Asia and the Pacific: "OIE initiatives on Transboundary Animal Diseases, control" in Tokyo. He presented challenges in the control of transboundary aquatic animal diseases.

Dr Maxwell Barson was invited by AU-IBAR to represent the OIE at the Stakeholder Consultation on Fish Certification Procedures, Standards and Regulations to promote Intra-Regional Fish Trade for West and Central Africa - *"Improving Food Security and Reducing Poverty through intra-regional Fish Trade in sub-Saharan Africa"*, held in Accra (Ghana) from 7 to 9 March 2016. He presented a talk on "The Role of OIE in Fish Disease Control and Trade".

L. COLLABORATION

Item 32. FAO

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 acute hepatopancreatic necrosis disease in Asia and Latin America and epizootic ulcerative syndrome in Africa. Dr Ernst provided an update on relevant activities of the Aquatic Animals Commission.

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

M. NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 20–24 February 2017 inclusive.

…/Annexes

Annex 1

MEETING OF THE OIE

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 12–16 September 2016

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Annex 2

MEETING OF THE OIE

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 12–16 September 2016

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Adopted agenda

A) MEETING WITH THE DIRECTOR GENERAL

B) ADOPTION OF THE AGENDA

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

D) MEETING WITH THE PRESIDENT OF THE BIOLOGICAL STANDARDS COMMISSION

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

Item 1. General comments

Item 2. Glossary

Item 3. Notification of diseases, and provision of epidemiological information (Chapter 1.1.)

Item 4. Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)

Item 5. Diseases listed by the OIE (Chapter 1.3.)

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

- Item 9.1. Crayfish plague *(Aphanomyces astaci)* (Chapter 9.1.)
- Item 9.2. Infection with yellow head virus genotype 1 (Chapter 9.2.)
- Item 9.3. Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)
- Item 9.4. Infectious myonecrosis (Chapter 9.4.)
- Item 9.5. Necrotising hepatopancreatitis (Chapter 9.5.)
- Item 9.6. Taura syndrome (Chapter 9.6.)
- Item 9.7. White tail disease (Chapter 9.8.)

Item 10. New chapter on Acute hepatopancreatic necrosis disease (Chapter 9.X.) including Report of the *ad hoc* Group on Safety of Products Derived from Aquatic Animals (August 2016)

Item 11. Revised Article X.X.8.

Annex 2 (contd)

F) OTHER *AQUATIC ANIMAL HEALTH CODE* ISSUES

Item 12. White spot disease (Chapter 9.7.) and the *ad hoc* Group on Susceptibility of Crustacean Species to Infection with OIE Listed Diseases

Item 13. Criteria for listing species as susceptible (Chapter 1.5.)

Item 14. Review prawn taxonomy literature for *Penaeus*

Item 15. A*d hoc* Group on Aquatic Animal Biosecurity

Item 16. A*d hoc* Group on Demonstration of disease freedom

G) *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS*

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

Item 18. Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.)

Item 19. Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)

Item 20. Infectious myonecrosis (Chapter 2.2.4.)

Item 21. Necrotising hepatopancreatitis (Chapter 2.2.5.)

Item 22. Taura syndrome (Chapter 2.2.6.)

Item 23. White tail disease (Chapter 2.2.8.)

Item 24. White spot disease (Chapter 2.2.7.) and the *ad hoc* Group on Susceptibility of Crustacean Species to Infection with OIE Listed Diseases

Item 25. Review of the *ad hoc* Group report on the *Aquatic Manual*

H) OIE REFERENCE CENTRES

Item 26. Applications for OIE Reference Centre status or changes of experts

Item 27. Feedback on progress Reference Laboratory quality management systems: towards ISO 17025 accreditation

Item 28. Reference Laboratory network GAP analysis

Item 29. Twinning Projects

I) OTHER ISSUES

Item 30. Update on OIE activities on antimicrobial resistance

J) AQUATIC ANIMALS COMMISSION WORK PLAN FOR 2016/2017

K) AQUATIC ANIMALS COMMISSION ACTIVITIES

L) COLLABORATION

Item 31. FAO

N) NEXT MEETING

Annex 3

GLOSSARY

EU comment

The EU in general supports the proposed changes to the glossary. Comments are inserted in the text below.

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~~.

ZONE

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

EU comment

The EU suggests deleting the word "clearly", as that word is superfluous. Indeed, it is enough to say that the zone is defined and documented by the Competent Authority.

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— Text deleted.

Annex 4A

CHAPTER 1.2.

CRITERIA FOR THE INCLUSION OF DISEASES IN THE OIE LIST

EU comment

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

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.

EU comment

The EU suggests italicising the word "vectors", as that term is defined in the glossary.

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* exist.

AND

4)

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

OR

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

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— Text deleted.

Annex 4B

CHAPTER 1.2.

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

Article 1.2.1.

Introduction

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

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

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

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

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

Article 1.2.2.

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

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

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

Annex 4B (contd)

~~No.~~		~~Criteria for listing~~	~~Explanatory notes~~
AND			
~~3.~~4.	a.~~Or~~	~~The agent is of public health concern.~~ Natural transmission to humans has been proven, and human infection is associated with severe consequences.	
~~And B. Spread~~			
~~4.~~	~~-~~	~~Infectious aetiology of the disease is proven.~~	
~~5.~~	~~Or~~	~~An infectious agent is strongly associated with the disease, but the aetiology is not yet known.~~	~~Infectious diseases of unknown aetiology can have equally high risk implications as those diseases where the infectious aetiology is proven. Whilst disease occurrence data are gathered, research should be conducted to elucidate the aetiology of the disease and the results be made available within a reasonable period of time.~~

~~No.~~		~~Criteria for listing~~	~~Explanatory notes~~
~~And B. Spread~~			
~~6.~~1.	~~And~~	~~Likelihood of i~~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			
~~7.~~2.	~~And~~	At least one ~~Several~~ country~~ies~~ ~~or a country with a *zone*~~ may or countries with zones ~~has~~ demonstrate~~d~~ 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 Chapter~~s~~ 1.4. ~~and 1.5.~~	~~Free countries/zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a disease can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread diseases, or the protection of the last remaining free zones from a widespread disease.~~
~~And C. Diagnosis~~			
AND			
~~8.~~3.		~~A repeatable and robust~~ A precise *case* definition is available and a r~~R~~eliable means of detection~~/~~ and diagnosis exist~~s~~ ~~and a precise *case* definition is available to clearly identify cases and allow them to be distinguished from other *diseases.*~~	~~A diagnostic test should be widely available and preferably has undergone a formal standardisation and validation process using routine field samples (See *Aquatic Manual*.) or a robust case definition is available to clearly identify cases and allow them to be distinguished from other pathologies.~~

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— Text deleted.

Annex 5

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

EU comment

The EU in general supports the proposed changes to this chapter, except for the proposed listing of *Batrachochytrium salamandrivorans* (*Bsal*). Reference is made to the EU comments provided to the OIE previously, which are maintained for the time being (these comments are available on DG SANTE's website, on p. 73: https://ec.europa.eu/food/sites/food/files/safety/docs/ia_standards_oie_eu_position_aahsc_report_201602_en.pdf).

In this regard, the EU would like to inform the OIE that the European Food Safety Authority (EFSA) has been asked by the European Commission for a scientific and technical assistance on *Bsal* which is expected to be delivered in February 2017. A copy of the EFSA mandate as available on the EFSA website is attached for information. The EU will provide a copy of the EFSA scientific report to the OIE once it has been published. The final EU position on the listing of *Bsal* will be established in the light of that EFSA report.

Furthermore, we would like to reiterate our previous comment on the importance of establishing an OIE reference laboratory for *Batrachochytrium* spp., as there currently is no support for member countries as regards diagnostic confirmation, nor are there any reference materials available (biological reference products and reagents) which are a necessary prerequisite for the laboratory diagnosis and control of these pathogens.

Finally, the EU draws the attention of the Aquatic Animals Commission to the changes proposed by the Code Commission to the corresponding chapter of the Terrestrial Code, regarding the wording of the preamble.

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

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

[…]

Article 1.3.3.

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

– Acute hepatopancreatic necrosis disease

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

– Infection with yellow head virus genotype 1

– Infection with ~~Infectious~~ infectious hypodermal and haematopoietic necrosis virus

– Infection with ~~Infectious~~ infectious myonecrosis virus

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

– Infection with Taura syndrome virus

– Infection with ~~White~~ white spot syndrome virus ~~disease~~

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

Article 1.3.4.

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

– Infection with *Batrachochytrium dendrobatidis*

– Infection with *Batrachochytrium salamandrivorans*

– Infection with *Ranavirus* spp. ~~ranavirus~~.

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— Text deleted.

Annex 6

CHAPTER 4.3.

DISINFECTION OF AQUACULTURE ESTABLISHMENTS AND EQUIPMENT

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. Comments are inserted in the text below.

Article 4.3.1.

Purpose

To provide recommendations on planning and implementation of *disinfection* procedures to prevent the introduction, 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 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.

EU comment

The EU suggests adding the words "or compartments" after the words "aquaculture establishments" in the paragraph above, in order to include the notion of natural occurences.

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

a) Cleaning and washing

Cleaning and washing of surfaces and equipment is an important part of the *disinfection* process and should always precede the application of *disinfectants*. It is necessary to remove solid waste, organic matter (including biofouling) and chemical residues as these may reduce the 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 before application of *disinfectants*.

EU comment

The EU suggests that the use of detergents is more greatly emphasised at this stage, by inserting the following sentence in the paragraph above (between the second and third sentence):

"The use of detergent is important for effective cleaning and washing, in order to break down biofilms."

Indeed, cleaning with detergent is important to ensure effective breakdown of biofilms for successful exposure of the surfaces and equipment to the disinfectant.

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, so proper maintenance of equipment is essential. For permeable surfaces and materials (e.g. woven material, nets and soil), a higher *disinfectant* concentration and a longer contact time is required because the surface area is greater, chemicals cannot penetrate easily and residual organic matter may be present.

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

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

c) Removal or inactivation of the disinfectant

Removal or inactivation of chemical residues is important to avoid toxicity to *aquatic animals*, corrosion of equipment and environmental impacts. Processes that may be employed for the removal or inactivation of chemical residues may include: rinsing of surfaces, dilution to acceptable levels,

treatment to inactivate chemical agents or, time to allow deactivation or dissipation of the active compound. These processes may be used in isolation or in combination.

2) *Disinfectants* should be used in accordance with relevant legislation. *Disinfectants* may present *risks* to the health of 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 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.

   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)

   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 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 tiling of the base of unlined ponds will also aid in incorporation of liming compounds and drying.

2. Tanks

Tank construction material (e.g. fibreglass, concrete or plastic) will determine the type of *disinfection* method used. Bare concrete tanks are susceptible to corrosion by acids and potential damage by high pressure

sprayers. They are also porous and therefore require longer application of chemicals to ensure *disinfection*. Plastic, painted and fibreglass tanks are more easily disinfected because they have smooth, non-porous surfaces that facilitate thorough cleaning and are resistant to most chemicals.

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

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

3. Pipes

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

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

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

4. Cage nets and other fibrous materials

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

All water should be drained from the *container* and any *aquatic animals*, faecal matter and other organic material removed by flushing with clean water 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.~~

~~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.).~~

Annex 6 (contd)

~~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. 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.

10. Husbandry equipment

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

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

EU comment

The EU suggests amending the title of point 10 above as follows:

"10. Husbandry and harvesting equipment".

Furthermore, the words "or harvesting" should be inserted after the words "disinfection of husbandry" in the paragraph above.

Indeed, the equipment used in fishing from the shore should be included (rakes, sieves, trolleys, etc.).

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 equipment such as dive equipment requires special attention and 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 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.

— Text deleted.

Annex 7

CHAPTER 4.4.

RECOMMENDATIONS FOR SURFACE DISINFECTION OF SALMONID EGGS

EU comment

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

Article 4.4.1.

Introduction

The practice of disinfecting salmonid *eggs* at hatcheries is an essential part of ensuring that ~~endemic~~ ~~*diseases*~~ *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.

EU comment

The EU suggests adding the recommendation to neutralise the povidone-iodine solutions before they are discarded, in order to avoid any toxicity for the aquatic environment.

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) rinse~~d~~ in pathogen free 0.9% to 1.1% ~~pathogen free~~ saline (30–60 seconds) to remove organic matter; then

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

3) rinse~~d~~ 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 ($NaHCO_3$) if the pH is low.

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— Text deleted.

Annex 8

CHAPTER 5.1.

GENERAL OBLIGATIONS RELATED TO CERTIFICATION

EU comment

The EU supports the proposed changes to this chapter.

[…]

Article 5.1.4.

Responsibilities in case of an incident related to importation

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

2) If a *disease* 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*.

3~~4~~) 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.

— Text deleted.

Annex 9

CHAPTER 9.1.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. A comment is inserted in the text below.

Article 9.1.1.

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

EU comment

The changes above are unclear. It seems that the first sentence of the paragraph would read as follows, which does not make sense:

"[...] means *infection* with the *pathogenic agent Aphanomyces astaci* the Phylum Oomycota (water moulds). [...].

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

Article 9.1.3.

Importation or transit of aquatic animals ~~and~~ 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* from the species referred to in Article 9.1.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

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

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

e) crayfish oil;

f) crayfish *meal*;

g) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.1.2., other than those referred to in point 1 of Article 9.1.3., *Competent Authorities* should require the conditions prescribed in Articles 9.1.7. to 9.1.11. relevant to the infection with *A. astaci* ~~crayfish plague~~ status of the *exporting country*, *zone* or *compartment*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.1.2. but which could reasonably be expected to pose a *risk* of transmission ~~spread~~ of ~~infection with~~ *A. astaci* ~~crayfish plague~~, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this 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 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 for at least the last five years without detection of ~~infection with~~ *A. astaci* ~~crayfish plague~~;

OR

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

a) on detection of ~~the *disease*~~ *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 ~~destroyed or removed~~ 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

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 ~~infection with~~ *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 ~~infection with~~ *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 ~~destroyed or removed~~ 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

   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~~.

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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.1.2. 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* should be in accordance with the Model Certificate in Chapter 5.11.

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

Article 9.1.8.

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

1) When importing ~~live~~ *aquatic animals* 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, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate *A. astaci* (in accordance with Chapter 4.7).

~~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.~~

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 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*.~~

~~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* or *aquatic animal products* 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, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.1.3., or products described in point 1 of Article 9.1.11., or other products authorised by the *Competent Authority*; and

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

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

Annex 9 (contd)

Article 9.1.10.

Importation of ~~live~~ aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with *A. astaci* ~~crayfish plague~~

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, ~~live~~ *aquatic animals* of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from 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* facilities for slaughter and processing into products authorised by the *Competent Authority*; and

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

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

Article 9.1.11.

Importation of aquatic animals ~~and~~ 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 *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* which have been prepared and packaged for retail trade and which comply with Article 5.4.2.:

   – no *commodities* listed.

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from 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.

Annex 10

CHAPTER 9.2.

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

EU comment

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

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 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 meet the criteria for listing species as susceptible in 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* from the species referred to in Article 9.2.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

3) When considering the importation or transit of *aquatic animals* ~~and~~ *or aquatic animal products* of a species not 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.

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 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.

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*.

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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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.2.2. 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* should be in accordance with the Model Certificate in Chapter 5.11.

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

Article 9.2.8.

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

1) When importing ~~live~~ aquatic animals of 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) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate YHV1 (in accordance with Chapter 4.7).

~~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.~~

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 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.~~

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* or *aquatic animal products* of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with YHV1, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.2.3., or products described in point 1 of Article 9.2.11., or other products authorised by the *Competent Authority*; and

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

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

Article 9.2.10.

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

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

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

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

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

Article 9.2.11.

Importation of aquatic animals ~~and~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.2.2. from a country, *zone* or *compartment* not declared free from infection with YHV1, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

Annex 11

CHAPTER 9.3.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

EU comment

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

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~~ Family *Parvoviridae,* Genus ~~genus~~ *Brevidensovirus* ~~in the~~ Family ~~family *Parvoviridae*~~.

EU comment

The EU suggests inserting a comma before the words "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 ~~susceptible~~ species which meet the criteria for listing species as susceptible in 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 leg shrimp *(Penaeus vannamei)*, and blue shrimp *(P. stylirostris)*, yellow leg *(P. californiensis)*, northern white shrimp *(P. setiferus)* and giant river prawn *(Macrobrachium rosenbergii)*. 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 ~~infection with~~ 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* from the species referred to in Article 9.3.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 90°C for at least 20 minutes (or any time/temperature equivalent which has been demonstrated to inactivate IHHNV);

   c) crustacean oil;

   d) crustacean *meal*.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.3.2., other than those referred to in point 1 of Article 9.3.3., *Competent Authorities*

should require the conditions prescribed in Articles 9.3.7. to 9.3.11. relevant to the 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* of a species not covered in Article 9.3.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of transmission of ~~infection with~~ 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~~.

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 ~~infection with~~ 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 ~~infection with~~ 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* *have been killed and* disposed of ~~destroyed or removed~~ 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 ~~infection with~~ 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 ~~infection with~~ 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 ~~infection with~~ 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 ~~destroyed or removed~~ 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 ~~infection with~~ IHHNV ~~IHHN~~.

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 infection with IHHNV IHHN~~ provided that conditions ~~that~~ are conducive to clinical expression of infection with IHHNV ~~IHHN~~, as described in the corresponding chapter of the *Aquatic Manual*, ~~exist,~~ and *basic biosecurity conditions* are continuously maintained.

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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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* declared free from infection with IHHNV ~~IHHN~~, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country*. 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* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.3.3.

Article 9.3.8.

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

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from infection with IHHNV ~~IHHN~~, the *Competent Authority* of the *importing country* should assess the *risk* ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate IHHNV (in accordance with Chapter 4.7).

~~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.~~

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 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;~~

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

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

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

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

~~g) if IHHNV is not detected, pests are not present, and the general health/disease status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country*, *zone* or *compartment*, the F-1 stock may be defined as 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.~~

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* or *aquatic animal products* of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from infection with IHHNV ~~IHHN~~, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.3.3., or products described in point 1 of Article 9.3.11., or other products authorised by the *Competent Authority*; and

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

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

Article 9.3.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with IHHNV

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

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

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

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

Article 9.3.11.

Importation of aquatic animals ~~and~~ 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 ~~infection with~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.3.2. from a country, *zone* or *compartment* not declared free from infection with IHHNV ~~IHHN~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

Annex 12

CHAPTER 9.4.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

EU comment

The EU supports the proposed changes to this chapter.

Article 9.4.1.

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

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 ~~susceptible~~ species which meet the criteria for listing species as susceptible in Chapter 1.5.: brown tiger prawn *(Penaeus esculentus)*, banana prawn *(Penaeus merguiensis)*, ~~Pacific white shrimp~~ and ~~white leg~~ 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* from the species referred to in Article 9.4.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

   c) crustacean oil;

   d) crustacean *meal*;

   e) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.4.2., other than those referred to in point 1 of Article 9.4.3., *Competent Authorities* should require the conditions prescribed in Articles 9.4.7. to 9.4.11. relevant to the infection with IMNV status of the *exporting country*, *zone* or *compartment*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.4.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of transmission ~~of infection with~~ 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~~.

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 infection with 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 infection with 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 destroyed or removed 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 infection with 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 ~~infection with~~ 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 ~~infection with~~ 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 ~~destroyed or removed~~ 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 ~~infection with~~ IMNV.

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 infection with 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.

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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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.4.2. 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* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.4.3.

Article 9.4.8.

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

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

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate IMNV (in accordance with Chapter 4.7.

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

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

   a) In the *exporting country*:

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

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 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 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* or *aquatic animal products* of 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, require that:

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

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

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

Article 9.4.10.

Importation of ~~live~~ aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with IMNV

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

Annex 12 (contd)

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

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

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

Article 9.4.11.

Importation of aquatic animals ~~and~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from infection with IMNV, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

Annex 13

CHAPTER 9.5.

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

EU comment

The EU supports the proposed changes to this chapter.

Article 9.5.1.

For the purposes of the *Aquatic Code*, infection with *Hepatobacter penaei* ~~necrotising hepatopancreatitis (NHP)~~ means infection with 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 ~~susceptible~~ species which meet the criteria for listing species as susceptible in Chapter 1.5.: ~~Pacific white~~ ~~white leg~~ 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 ~~infection with *H. penaei* NHP~~, regardless of the infection with *H. penaei* ~~NHP~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of the following *aquatic animal products* from the species referred to in Article 9.5.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 100˚C for at least three minutes (or any time/temperature equivalent which has been demonstrated to inactivate ~~Candidatus~~ *H. penaei*);

   c) pasteurised crustacean products that have been subjected to heat treatment at 63˚C for at least 30 minutes (or any time/temperature equivalent which has been demonstrated to inactivate ~~Candidatus~~ *H. penaei*);

   d) crustacean oil;

   e) crustacean *meal*;

   f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.5.2., other than those referred to in point 1 of Article 9.5.3., *Competent Authorities* should require the conditions prescribed in Articles 9.5.7. to 9.5.11. relevant to the infection with *H. penaei* ~~NHP~~ status of the *exporting country*, *zone* or *compartment*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.5.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of transmission of ~~infection with *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 ~~destroyed or removed~~ 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~~.

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.

Annex 13 (contd)

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 ~~infection with~~ *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 ~~infection with~~ *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* have been killed and disposed of ~~destroyed or removed~~ 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~~.

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.

Annex 13 (contd)

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 infection with *H. penaei* NHP~~ provided that conditions ~~that~~ are conducive to clinical expression of infection with *H. penaei* ~~NHP~~, as described in the corresponding chapter of the *Aquatic Manual*, ~~exist,~~ and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with *H. penaei* ~~NHP~~, *targeted surveillance* 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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* declared free from infection with *H. penaei* ~~NHP~~, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country*. 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* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.5.3.

Article 9.5.8.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with *H. penaei* ~~necrotising hepatopancreatitis~~

1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from infection with *H. penaei* ~~NHP~~, the *Competent Authority* of the *importing country* should assess the *risk* ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate *H. penaei* (in accordance with Chapter 4.7.

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

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

   a) In the *exporting country*:

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

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

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* or *aquatic animal products* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from infection with *H. penaei* ~~NHP~~, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.5.3., or products described in point 1 of Article 9.5.11., or other products authorised by the *Competent Authority*; and

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

Annex 13 (contd)

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

Article 9.5.10.

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

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, ~~live~~ *aquatic animals* of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from 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* facilities for slaughter and processing into products authorised by the *Competent Authority*; and

2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of ~~Candidatus~~ *H. penaei*.

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

Article 9.5.11.

Importation of aquatic animals ~~and~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.5.2. from a country, *zone* or *compartment* not declared free from infection with *H. penaei* ~~NHP~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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— Text deleted.

Annex 14

CHAPTER 9.6.

INFECTION WITH TAURA SYNDROME VIRUS

EU comment

The EU supports the proposed changes to this chapter.

Article 9.6.1.

For the purposes of the *Aquatic Code*, infection with Taura syndrome virus ~~(TS)~~ means infection with the *pathogenic agent* Taura syndrome virus (TSV),~~ Taura syndrome virus is classified as a species of the~~ 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 meet the criteria for listing species as susceptible in Chapter 1.5.::greasyback shrimp *(Metapenaeus ensis),* northern brown shrimp (*P aztecus*), giant tiger prawn *(P. monodon),* northern white shrimp *(P. setiferus),* blue shrimp *(P. stylirostris)* 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* from the species referred to in Article 9.6.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 70°C for at least 30 minutes (or any time/ temperature equivalent which has been demonstrated to inactivate TSV);

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

   d) crustacean oil;

   e) crustacean *meal*;

   f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.6.2., other than those referred to in point 1 of Article 9.6.3., *Competent Authorities* should require the conditions prescribed in Articles 9.6.7. to 9.6.11. relevant to the infection with TSV ~~TS~~ status of the *exporting country*, *zone* or *compartment*.

Annex 14 (contd)

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.6.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of transmission of ~~infection with~~ TSV ~~TS~~, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this 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 ~~infection with~~ TSV ~~TS~~;

OR

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

   a) on detection of 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 ~~destroyed or removed~~ by means that minimise the ~~*risk*~~ likelihood of further transmission ~~spread of~~ ~~infection with~~ 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 ~~infection with~~ 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 ~~TS~~ if all the relevant *Competent Authorities* confirm that all relevant conditions have been met.

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

1) none of the *susceptible species* referred to in Article 9.6.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.6.2. are present in the *zone* or *compartment* and the following conditions have been made:

   a) there has not been any ~~observed~~ occurrence of 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 ~~TS~~ and subsequently lost its ~~*disease*~~ free status due to the detection of ~~infection with~~ TSV ~~TS~~ 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 ~~destroyed or removed~~ 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 ~~infection with~~ TSV ~~TS~~.

Article 9.6.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TSV ~~TS~~ following the provisions of points 1 or 2 of Articles 9.6.4. or 9.6.5. (as relevant) may maintain its status as free from infection with TSV ~~TS~~ provided that *basic biosecurity conditions* are continuously maintained.

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 infection with TSV TS~~ provided that conditions ~~that~~ are conducive to clinical expression of infection with TSV ~~TS~~, as described in the corresponding chapter of the *Aquatic Manual*, ~~exist,~~ and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with TSV ~~TS~~, *targeted surveillance* 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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* declared free from infection with TSV ~~TS~~, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country*. 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* should be in accordance with the Model Certificate in Chapter 5.11.

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

Article 9.6.8.

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

1) When importing~~, for *aquaculture*, live~~ *aquatic animals* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from infection with TSV ~~TS~~, the *Competent Authority* of the *importing country* should assess the *risk* ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate TSV (in accordance with Chapter 4.7.

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

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

   a) In the *exporting country*:

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

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 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 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* or *aquatic animal products* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from infection with TSV ~~TS~~, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.6.3., or products described in point 1 of Article 9.6.11., or other products authorised by the *Competent Authority*; and

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

Annex 14 (contd)

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

Article 9.6.10.

Importation of ~~live~~ aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with TSV

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, ~~live~~ *aquatic animals* of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from 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* facilities for slaughter and processing into products authorised by the *Competent Authority*; and

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

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

Article 9.6.11.

Importation of aquatic animals ~~and~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.6.2. from a country, *zone* or *compartment* not declared free from infection with TSV ~~TS~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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— Text deleted.

Annex 15

CHAPTER 9.8.

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

EU comment

The EU supports the proposed changes to this chapter.

Article 9.8.1.

For the purposes of the *Aquatic Code*, infection with *Macrobrachium rosenbergii* nodavirus means infection with the *pathogenic agent Macrobrachium rosenbergii* nodavirus (MrNV), ~~of the~~ Family *Nodaviridae*. The *disease* is commonly known as white tail disease. ~~white tail disease (WTD) means *infection* with macrobrachium nodavirus (MrNV). This virus has yet to be formally classified.~~

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

Article 9.8.2.

Scope

The recommendations in this chapter apply to the following ~~susceptible~~ species which meet the criteria for listing species as susceptible in Chapter 1.5.: ~~the~~ giant ~~fresh water~~ 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 ~~infection with~~ MrNV ~~WTD~~, regardless of the infection with MrNV ~~the WTD~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of the following *aquatic animal products* from the species referred to in Article 9.8.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

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

   d) crustacean oil;

   e) crustacean *meal*;

   f) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.8.2., other than those referred to in point 1 of Article 9.8.3., *Competent Authorities*

should require the conditions prescribed in Articles 9.8.7. to 9.8.11. relevant to the infection with MrNV ~~WTD~~ status of the *exporting country*, *zone* or *compartment*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.8.2. but which could reasonably be expected to pose a *risk* of transmission ~~of spread~~ of ~~infection with~~ MrNV ~~WTD~~, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this 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:

   a) 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

   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 for at least the last two years without detection of ~~infection with~~ MrNV ~~WTD~~;

OR

4) it previously made a *self-declaration of freedom* from infection with MrNV ~~WTD~~ and subsequently lost its ~~*disease*~~ free status due to the detection of MrNV ~~WTD~~ 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 ~~destroyed or removed~~ 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

   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 ~~infection with~~ 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 ~~infection with~~ 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 ~~infection with~~ 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 ~~destroyed or removed~~ by means that minimise the ~~*risk*~~ likelihood of further transmission ~~spread~~ of ~~infection with~~ 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 ~~infection with~~ MrNV ~~WTD~~.

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.

Annex 15 (contd)

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 infection with MrNV WTD~~ provided that conditions ~~that~~ are conducive to clinical expression of infection with MrNV ~~WTD~~, as described in the corresponding chapter of the *Aquatic Manual*, ~~exist,~~ and *basic biosecurity conditions* are continuously maintained.

However, for declared free *zones* or *compartments* in infected countries and in all cases where conditions are not conducive to clinical expression of infection with MrNV ~~WTD~~, *targeted surveillance* 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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* declared free from infection with MrNV ~~WTD~~, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *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* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.8.3.

Article 9.8.8.

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

1) When importing~~, for *aquaculture*, live~~ *aquatic animals* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV ~~WTD~~, the *Competent Authority* of the *importing country* should assess the *risk* ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate MrNV (in accordance with Chapter 4.7.

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

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

   a) In the *exporting country*:

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

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 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 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 aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with MrNV ~~white tail disease~~

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV ~~WTD~~, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.8.3., or products described in point 1 of Article 9.8.11., or other products authorised by the *Competent Authority*; and

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

Annex 15 (contd)

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

Article 9.8.10.

Importation of ~~live~~ aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with MrNV ~~white tail disease~~

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, ~~live~~ *aquatic animals* of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV ~~WTD~~, the *Competent Authority* of the *importing country* should require that:

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

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

This Article does not apply to *commodities* referred to in point 1 of Article 9.8.3.

Article 9.8.11.

Importation of aquatic animals ~~and~~ 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.8.2. from a country, *zone* or *compartment* not declared free from infection with MrNV ~~WTD~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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— Text deleted.

Annex 16

CHAPTER 9.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

EU comment

The EU supports the proposed changes to this chapter.

Article 9.X.1.

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

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

Article 9.X.2.

Scope

The recommendations in this chapter apply to the following ~~*susceptible*~~ *species* which meet the criteria for listing species as susceptible in Chapter 1.5.: ~~white leg shrimp (*Penaeus vannamei*) and~~ giant tiger prawn (*Penaeus monodon*) 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* from the species referred to in Article 9.X.2. which are intended for any purpose and which comply with Article 5.4.1.:

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

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

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

   c~~d~~) crustacean oil;

   d~~e~~) crustacean *meal*;

   e~~f~~) chemically extracted chitin.~~]~~

2) When authorising the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species referred to in Article 9.X.2., other than those referred to in point 1 of Article 9.X.3., *Competent Authorities* should require the conditions prescribed in Articles 9.X.9 to 9.X.11. relevant to the AHPND status of the *exporting country*, *zone* or *compartment*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not covered in Article 9.X.2. but which could reasonably be expected to pose a *risk* ~~of spread~~ of 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~~.

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.

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 16 (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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* declared free from AHPND, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country*. 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* should be in accordance with the Model Certificate in Chapter 5.11.

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

Article 9.X.8

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

1) When importing~~, for *aquaculture*, live~~ *aquatic animals* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* ~~and, if justified, apply the following risk mitigation measures:~~ in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate *Vp*$_{AHPND}$ (in accordance with Chapter 4.7.

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

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

   a) In the *exporting country*:

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

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

   b) In the *importing country*:

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

      ii) test the F-0 population for *Vp*$_{AHPND}$ 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 the *Aquatic Manual*) and test for *Vp*$_{AHPND}$ in accordance with Chapter 1.4.;

v) if *Vp*$_{AHPND}$ is not detected in the F-1 population, it may be defined as free from AHPND and may be released from *quarantine*;

vi) if *Vp*$_{AHPND}$ 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 *Vp*$_{AHPND}$, pests and general health/disease status;~~

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

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

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

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

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

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

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

Article 9.X.9.

Importation of aquatic animals ~~and~~ 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* or *aquatic animal products* of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.X.3., or products described in point 1 of Article 9.X.11., or other products authorised by the *Competent Authority*; and

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

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

Article 9.X.10.

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

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

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

Annex 16 (contd)

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

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

Article 9.X.11.

Importation 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.X.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

— Text deleted.

Annex 17A (contd)

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'

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

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

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

   b) the treatment of transport water, equipment, effluent and waste materials to inactive 'pathogen X' (in accordance with Chapter 4.7.).

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

   a) In the *exporting country*:

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

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

Annex 17B

'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)

EU comment

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

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

1) When importing ~~live~~ for *aquaculture*, aquatic animals of 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 2) and 3) below.

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate 'pathogen X' (in accordance with Chapter 4.~~3~~7.) ~~and biosecure disposal of effluent and waste~~.

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

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

   a) In the *exporting country*:

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

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

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.

Annex 18

CHAPTER 9.7.

INFECTION WITH WHITE SPOT SYNDROME VIRUS ~~DISEASE~~

EU comment

The EU supports the proposed changes to this chapter.

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~~ 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* from the 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);

   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 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 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* 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*.

3) When considering the importation or transit of *aquatic animals* ~~and~~ or *aquatic animal products* of a species not 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* 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~~.

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.

Annex 18 (contd)

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* 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 ~~infection with~~ WSSV ~~WSD~~.

Annex 18 (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* ~~and~~ or *aquatic animal products* of species referred to in Article 9.7.2. 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* should be in accordance with the Model Certificate in Chapter 5.11.

This Article does not apply to *commodities* listed in point 1 of Article 9.7.3.

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~~

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

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

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

   b) the treatment of transport water, equipment, ~~used in transport and of all~~ effluent and waste materials ~~in a manner that ensures inactivation of~~ to inactivate WSSV (in accordance with Chapter 4.7.

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

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

a) In the *exporting country*:

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

ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 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 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 18 (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* or *aquatic animal products* 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, if justified, require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.7.3., or products described in point 1 of Article 9.7.11., or other products authorised by the *Competent Authority*; and

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

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

Article 9.7.10.

Importation of ~~live~~ aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with WSSV ~~disease~~

When importing, for use in animal *feed* or for agricultural, industrial or pharmaceutical use, ~~live~~ *aquatic animals* of species referred to in Article 9.7.2. 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* facilities for slaughter and processing into products authorised by the *Competent Authority*; and

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

This Article does not apply to *commodities* referred to in point 1 of Article 9.7.3.

Article 9.7.11.

Importation 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 have been prepared and packaged for retail trade and which comply with Article 5.4.2.

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

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

2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of species referred to in Article 9.7.2. 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.

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— Text deleted.

Annex 19

CHAPTER 1.5.

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

EU comment

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

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. of each *disease*-specific chapter in the *Aquatic Code*.

EU comment

As disease-specific chapters of the Aquatic Code do not contain an Article 1.5.2., the EU suggests amending the article above as follows:

"The purpose of this chapter is to provide criteria for determining which species are listed as susceptible in ~~Article 1.5.2.~~ the second article 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 a species as susceptible should be based on a finding that the evidence is definite. However, possible susceptibility of a species is also important information and this should also be included in Section 2.2.1. 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 Article1.5.6.).

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.

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.

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 classification higher than species (e.g. genus, family).

1) A decision to conclude susceptibility for a taxonomic level above species should only be made where:

   A. more than one species within the taxonomic group has been found to be susceptible in accordance with the criteria above;

   AND

   B. no species within the taxonomic group has been found to be refractory to infection.

   The taxa chosen should be the lowest level supported by this evidence.

2) Evidence that a species is refractory to infection may include:

   A. absence of *infection* in species exposed to the *pathogenic agent* in natural settings where the pathogen is known to be present and it causes disease in susceptible species;

   B. absence of *infection* in species exposed to the *pathogenic agent* through controlled challenges using experimental procedures.

— Text deleted.

Annex 20

CHAPTER 2.2.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$) ~~and *V. harveyi*~~ that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB. 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 *Photorhabdus* insect-related (Pir) binary toxin, PirA and PirB (Gomez-Gil *et al.,* 2014; Gomez-Jimenez *et al*., 2014; Han *et al.,* 2015a; Kondo *et al.,* 2014; Lee *et al.,* 2015; Yang *et al.,* 2014). The plasmid within ~~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 *Vp*$_{AHPND}$ 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 *pnd*A gene, which is associated with a post-segregational killing (psk) system. For a *Vp*$_{AHPND}$ bacterium that harbours a plasmid with the psk system (PSK$^+$), only progeny that inherit the PSK$^+$ plasmid will be viable (Lee *et al.,* 2015). Progeny that do not inherit the PSK$^+$ plasmid will die because the stable *pnd*A mRNA will be translated to PndA toxin that will kill the bacterium. The presence of a psk system on a plasmid thus ensures that the plasmid is inherited during bacterial replication. The pVA1 plasmid will therefore be passed on to subsequent generations of *Vp*$_{AHPND}$ producing PirA^{vp} and PirB^{vp}.

2.1.2. Survival outside the host

~~AHPND-causing strains of *V. parahaemolyticus* (*Vp*$_{AHPND}$)~~ would be 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).

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, 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 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.

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. Although AHPND can ~~be found~~ occur all year round in South-East Asia, the hot and dry season from April to July seems to be the peak. Overfeeding, poor seed quality, poor water quality, poor feed quality, algal blooms or crashes are also factors that may lead to occurences 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 20 (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

Samples, 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

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:

i) The acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (FAO, 2013; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013a; 2013b; 2014a; 2014b).

ii) The terminal phase is characterised by marked intra-tubular haemocytic inflammation and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells (FAO, 2013; Leaño & Mohan, 2013; NACA, 2012; 2014; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013a; 2013b; 2014a; 2014b).

4.2.4. 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

Vp$_{AHPND}$ ~~Pir toxin-producing strains of *V. parahaemolyticus* (and other bacterial species)~~ can be isolated on standard media used for isolation of bacteria from diseased shrimp (Lee *et al.,* 2015; Soto-Rodriguez *et al.,* 2015). Bacterial identification may be carried out using 16S rRNA PCR (Weisburg *et al.,* 1991) or *tox*R-targeted PCR (Kim *et al*., 1999) and sequencing ~~(Weisburg *et al.,* 1991)~~, and ~~their probable ability to cause AHPND using~~ AHPND-specific PCR methods described in section 4.3.1.2.3.

4.3.1.2.1. Cell culture or artificial media

See sections 4.3.1.2.3.1.1 and 4.3.1.2.3.1.2.

4.3.1.2.2. Antibody-based antigen detection methods

None is available to date (October 2015).

Annex 20 (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

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 ~~PCR primers for~~ One-step PCR detection of AHPND-causing bacteria

Four one-step PCR methods (AP3, TUMSAT-Vp3, VpPirA-284 and VpPirB-392) are described here for detection of Pir toxin genes 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
AP3	AP3-F: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP3-R: 5'-GTG-GTA-ATA-GAT-TGT-ACA-GAA-3'	*pirA*^{vp}	333bp	Sirikharin *et al*., 2014, 2015
TUMSAT-Vp3	TUMSAT-Vp3 F: 5'-GTG-TTG-CAT-AAT-TTT-GTG-CA-3' TUMSAT-Vp3 R: 5'-TTG-TAC-AGA-AAC-CAC-GAC-TA-3'	*pirA*^{vp}	360bp	Tinwongger *et al*., 2014
VpPirA-284	VpPirA-284F: 5'-TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG-3' VpPirA-284R: 5'-CAC-GAC-TAG-CGC-CAT-TGT-TA-3'	*pirA*^{vp}	284bp	Han *et al.,* 2015a
VpPirB-392	VpPirB-392F: 5'-TGA-TGA-AGT-GAT-GGG-TGC-TC-3' VpPirB-392R: 5'-TGT-AAG-CGC-CGT-TTA-ACT-CA-3'	*pirB*^{vp}	392bp	Han *et al.,* 2015a

4.3.1.2.3.1.~~7~~5 Protocol for the AP3 PCR method

This protocol follows the method described by Sirikharin et al. (2015). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM $MgCl_2$, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP3-F1, 0.5 µl 10 µM AP3-R1, 0.2 µl Taq DNA polymerase and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds with a final extension step at 72°C for 5 minutes and then the reaction mixture can be held at 4°C.

4.3.1.2.3.1.~~8~~6 Protocol for the VpPirA-284 and VpPirB-392 PCR methods

This protocol follows the method described by Han et al. (2015) and uses PuReTaq ready-to-go PCR beads (GE Healthcare). A 25 µl PCR reaction mixture is prepared with PuReTaq ready-to-go PCR beads. Each reaction contains 0.2 µM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, and 1 µl of extracted DNA. For PCR a 3-minute denaturation step at 94°C is followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

4.3.1.2.3.1.~~9~~7 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.~~5~~8 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 $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM AP4-F1, 0.5 µl 10 µM AP4-R1, 0.3 µl of Taq DNA pol (5 units μl^{-1}) and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension step at 72°C for 2 minutes and hold at 4°C.

The nested PCR reaction mixture consists of 2.5 µl 10x PCR mix, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.375 µl 10 µM AP4-F2, 0.375 µl 10 µM AP4-R2, 0.3 µl Taq DNA pol (5 units μl^{-1}) and 2 µl of the first PCR reaction in a total volume of 25 µl. The nested PCR protocol is 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds and hold at 4°C.

Annex 20 (contd)

The nested PCR primers, designed using the China (People's Rep. of) isolate of AHPND bacteria (Yang *et al.,* 2014), are shown in Table 4.2. The expected amplicon sizes are 1269 bp for the outer primers (AP4-F1 and AP4-R1) and 230 bp for the inner primers (AP4-F2 and AP4-R2). At high concentrations of target DNA, additional amplicons may occur as the product of residual primer AP4-F1 pairing with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

Table 4.2. *Primers for the AP4, nested PCR method for detection of AHPND-causing bacteria*

Method name	Primers	Expected amplicon size	Reference
AP4 Step 1	AP4-F1: 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' AP4-R1: 5'-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'	1269	Dangtip *et al.,* 2015
AP4 Step 2	AP4-F2: 5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3' AP4-R2: 5'- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'	230	

4.3.1.2.3.1.~~11~~9 Analysis of conventional PCR products by agarose gel electrophoresis

After PCR, amplicons are visualised by agarose gel electrophoresis. Twenty µl of the PCR reaction mixture, with 6x loading dye added, is loaded onto a 1.5% agarose gel and electrophoresis is carried out at 90 volts for 40 minutes. Amplicons are visualised with SYBR Safe gel stain (Invitrogen, Cat. No. 33102) according to the manufacturer's instructions. Amplicons of the expected size appropriate for the PCR methods used (Tables 4.1, and 4.2 ~~and 4.3~~) indicate a positive result. Positive results must be confirmed by sequence analysis.

4.3.1.2.3.1.~~12~~10 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 4.3.

Table 4.3. *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 *et al.,* 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 $MgCl_2$, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP3-F1, 0.5 µl 10 µM AP3-R1, 0.2 µl Taq DNA polymerase and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds with a final extension step at 72°C for 5 minutes and then the reaction mixture can be held at 4°C.~~

~~*4.3.1.2.3.1.8 Protocol for the VpPirA 284 and VpPirB 392 PCR methods*~~

~~This protocol follows the method described by Han *et al.* (2015) and uses PuReTaq ready-to-go PCR beads (GE Healthcare). A 25 µl PCR reaction mixture is prepared with PuReTaq ready-to-go PCR beads. Each reaction contains 0.2 µM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, and 1 µl of extracted DNA. For PCR a 3-minute denaturation step at 94°C is followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.~~

~~*4.3.1.2.3.1.9 Protocol for the TUMSAT Vp3 PCR method*~~

~~This protocol follows the method described by Tinwongger *et al.* (2014). A 30 µl PCR mixture is prepared containing 1 µl DNA template, 10× PCR buffer, 0.25 mM dNTP mixture, 0.6 µM of each primer and 0.01 U Taq polymerase. PCR conditions consist of an initial preheating stage of 2 minutes at 95°C, followed by 30 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 56°C and 30 seconds extension at 72°C.~~

~~*4.3.1.2.3.1.10 Protocol for the AP4 nested PCR method*~~

~~This protocol follows the method described by Sritnyalucksana *et al.* (2015) and Dangtip *et al.* (2015). The first PCR reaction mixture consists of 2.5 µl 10× PCR mix, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM AP4-F1, 0.5 µl 10 µM AP4-R1, 0.3 µl of Taq DNA pol (5 units μl^{-1}) and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension step at 72°C for 2 minutes and hold at 4°C.~~

~~The nested PCR reaction mixture consists of 2.5 µl 10x PCR mix, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.375 µl 10 µM AP4-F2, 0.375 µl 10 µM AP4-R2, 0.3 µl Taq DNA pol (5 units μl^{-1}) and 2 µl of the first PCR reaction in a total volume of 25 µl. The nested PCR protocol is 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds and hold at 4°C.~~

~~*4.3.1.2.3.1.11 Analysis of conventional PCR products by agarose gel electrophoresis*~~

~~After PCR, amplicons are visualised by agarose gel electrophoresis. Twenty µl of the PCR reaction mixture, with 6x loading dye added, is loaded onto a 1.5% agarose gel and electrophoresis is carried out at 90 volts for 40 minutes. Amplicons are visualised with SYBR Safe gel stain (Invitrogen, Cat. No. 33102) according to the manufacturer's instructions. Amplicons of the expected size appropriate for the PCR methods used (Tables 4.1, 4.2 and 4.3) indicate a positive result. Positive results must be confirmed by sequence analysis.~~

~~*4.3.1.2.3.1.12 Protocol for the AHPND specific 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.~~13~~11 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 shrimp are ~~processed~~ required for histology and bacterial re-isolation ~~and AHPND PCR and sequence analysis~~.

5. Rating of tests against purpose of use

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

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PL	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Bioassay	d	d	d	d	d	a
Histopathology	d	c	a	c	a	b
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	d	d	d	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 suspected if at least one of the following criteria is met:

i) Mortality associated with clinical signs of AHPND
ii) Histopathology indicative of AHPND
iii) Detection of Pir toxin genes 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 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 (clinical signs, mortality, histopathology, PCR and sequence)

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Annex 20 (contd)

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Annex 21

CHAPTER 2.2.1.

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

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. However, we note that the whole Chapter needs to be reviewed taking into account the latest scientific data. For example, there are now five known genotypes of the pathogenic agent. In addition, there are instances where European species act as asymptomatic carriers, for example narrow clawed crayfish (*Astacus leptodactylus*), see references below.

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Further comments are inserted in the text below.

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci* ~~Schikora~~, ~~a member of the~~ Phylum ~~Class~~ Oomycota. 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-Uribeondo *et al.,* 1995; Huang *et al.,* 1994): Group A (the so called *Astacus* strains) comprises a number of strains that were isolated from *Astacus astacus* and *Astacus leptodactylus*; these strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus strains* I) includes isolates from both *A. astacus* in Sweden and *Pacifastacus leniusculus* from Lake Tahoe, USA. Imported 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-Uribeondo *et al*., 1995). *Aphanomyces astaci* strains that have been present in

Europe for many years (group A strains) appear to be less pathogenic than strains introduced more recently with crayfish imports from North America since the 1960s.

2.1.2. Survival outside the host

~~Although *A. astaci* is not an obligate parasite and will grow well under laboratory conditions on artificial media (Alderman & Polglase, 1986; Cerenius *et al*., 1988), in the natural environment it does not survive well for long periods in the absence of a suitable host.~~

Aphanomyces astaci zoospores remain motile for up to 3 days and cysts survive for 2 weeks in distilled water (Svensson & Unestam, 1975; Unestam, 1966). As *A. astaci* can go through three cycles of zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension kept at 2°C for 2 months (Unestam, 1966).

2.1.3. Stability of the agent (effective inactivation methods)

Aphanomyces astaci, both in culture and in infected crayfish, is killed by a short exposure to temperatures of 60°C or to temperatures of –20°C (or below) for 48 hours (or more) (Alderman, 2000; Oidtmann *et al*., 2002). Sodium hypochlorite and iodophors are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection, since organic matter was found to decrease the effectiveness of iodophors (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation.

2.1.4. Life cycle

The life cycle of *A. astaci* is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores. These initially encyst, but then release a biflagellate zoospore (secondary zoospore). Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. Free-swimming zoospores appear to be chemotactically attracted to crayfish cuticle (Cerenius & Söderhäll, 1984a) and often settle on the cuticle near a wound (Nyhlen & Unestam, 1980). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infective viability (Cerenius & Söderhäll, 1984b). Growth and sporulation capacity is strain-and temperature-dependent (Dieguez-Uribeondo *et al*., 1995).

2.2. Host factors

2.2.1. Susceptible host species

~~Species that fulfil the criteria for listing 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 crawfish (*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 & Soylu, 1989; Unestam, 1969b; 1976; Unestam & Weiss, 1970). Laboratory challenges have demonstrated that Australian species of crayfish are also highly susceptible (Unestam, 1976). North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Orconectes* spp. are infected by *A. astaci*, but under normal conditions the infection does not cause clinical disease or death. All North American crayfish species investigated to date have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (Oidtmann *et al*., 2006; Unestam, 1969b; Unestam & Weiss, 1970) and it is therefore currently assumed that this is the case for any other North American species.

The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) ~~but this was reported only under laboratory conditions~~ (Benisch, 1940; Schrimpf *et al.*, 2014).

EU comment

Freshwater crab (*Potamon potamios*) should be added in the paragraph above after the Chinese mitten crab (*Eriocheir sinensis*).

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 uropds 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, 1969~~a~~; 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

There is good field and experimental evidence that movements of finfish from areas in which there is a clinical outbreak of disease due to infection with *A. astaci* ~~crayfish plague is active~~ can transmit infection from one watershed to another (Alderman *et al*., 1987; Oidtmann *et al*., 2002~~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.

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 leads to infection and eventually to death. The minimal infectious dose has still not been established, but it may be as low as a single spore per animal (B. Oidtmann, unpublished data). Prevalence of infection within a population in the early stage of an outbreak may be low (only one or a few animals in a river population may be affected). However, the pathogen is amplified in affected animals and subsequently released into the water; usually leading to 100% mortality in a contiguous population. The velocity 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).

EU comment

Please delete the word "and" after "2001" in the last sentence of the paragraph above (linguistic).

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-Uribeondo *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).

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. $CaCl_2$ stimulates zoospore emergence from primary cysts, whereas $MgCl_2$ has an inhibitory effect. In general, zoospore emergence is triggered by transferring the vegetative mycelium into a medium where nutrients are absent or low in concentration (Cerenius & Söderhäll, 1984b).

2.4. Control and prevention

Once *A. astaci* has been introduced into a population of highly susceptible crayfish species in the wild, the spread within the affected population cannot be controlled. Therefore, prevention of introduction is essential. 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 source water 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. 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 escape ~~spread~~ of crayfish ~~from such sites~~. Therefore, a risk assessment ~~should careful consideration needs to~~ be conducted to determine ~~given as to~~ whether ~~such~~ facilities should be established.

Certain pathways of introduction, such as the release of North American crayfish by private individuals are difficult to control.

2.4.1. Vaccination

Currently, there is no evidence that vaccines offer long-term protection in crustaceans and even if this were not to be the case, vaccination of natural populations of crayfish is ~~impossible~~ 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~~ develop~~ing~~ 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 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 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).

5 ~~5~~. Any equipment that is brought onto site should be disinfected.

~~6. General biosecurity measures should be in place (e.g. controlled access to premises; disinfection of boots when site is entered; investigation of mortalities if they occur; introduction of live animals (crayfish, fish) only from sources known to be free of *crayfish plague* infection with *A. astaci*).~~

3. Sampling

3.1. Selection of individual specimens

In the case of a suspected outbreak of 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 temperatures deleterious to the animals. These can be attached at the inside bottom of the transport container. The crayfish must however be protected from direct contact with freezer packs. This can be achieved using, for instance, cardboard or a several layers of newspaper.

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

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

Should only dead animals be found at the site of a suspected outbreak, these might still be suitable for diagnosis. Depending on the condition they are in, they can either be: a) transported chilled (if they appear to have died only very recently), or, b) placed in non-methylated ethanol (minimum concentration 70%; see Section 3.2).

Animals showing advanced decay are unlikely to give a reliable result, however, if no other animals are available, these might still be tested.

3.2. Preservation of samples for submission

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

3.3. Pooling of samples

Not recommended.

3.4. Best organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended.

3.5. Samples 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 laboratory (Oidtmann *et al*., 1999). Molecular methods are now available that are less dependent on speed of delivery and can deal with a greater range of samples compared with methods relying on agent isolation (Oidtmann *et al*., 2006; Vrålstad *et al*., 2009).

4.1. Field diagnostic methods

4.1.1. Clinical signs

Highly susceptible species

Gross clinical signs are ~~extremely~~ variable and depend on challenge severity and water temperatures. The first sign of 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~~ rapid~~ly~~ disease spread, particularly at summer water temperatures, ~~infection will spread quickly and~~ stretches of over 50 km may lose all their highly susceptible native crayfish in less than 21 days 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).

4.2. Clinical methods

4.2.1. Gross pathology

Highly susceptible species

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

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example the soft abdominal cuticle. However, ~~it must be stressed that~~ these melanisations can be caused by mechanical injuries or infections with other water moulds and are very unspecific. Conversely, visible melanisation is not always associated with carrier status. Infected animals can appear completely devoid of visible melanisations.

4.2.2. Clinical chemistry

No suitable methods available.

4.2.3. Microscopic pathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. Additionally, such material does not prove that any hyphae observed are those of ~~the primary pathogen~~ *A. 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 H_2O) clean disposable paper towel. Simple aseptic excision of infected tissues, which are then placed as small pieces (3–5 mm^2) on the surface of isolation medium plates , will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 hours) animals. Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low-power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

Provided that care is taken in excising infected tissues for isolation, contaminants need not present significant problems. Small pieces of cuticle and muscle may be transferred to a Petri dish of sterile water and there further cut into small pieces with sterile instruments for transfer to isolation medium (IM). Suitable instruments for such work are scalpels, fine forceps and scissors.

To reduce potential contamination problems, disinfection of the cuticle with ethanol and melting a sterile glass ring 1–2 mm deep into the isolation medium can improve isolation success (Nyhlen & Unestam, 1980; Oidtmann *et al.,* 1999). The addition of potassium tellurite into the area inside the glass ring has been described (Nyhlen & Unestam, 1980).

Annex 21 (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. Parafilm[1]) 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.

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*.

Encysted primary spores are spherical, (8)9–11(15) µm in diameter, and are relatively few in number, (8)15–30(40) per sporangium in comparison with other *Aphanomyces* spp. Spores remain encysted for 8–12 hours. Optimum temperatures for sporangial formation and discharge for the majority of European isolates of *A. astaci* are between 16 and 24°C (Alderman & Polglase, 1986). For some isolates, particularly from Spanish waters, slightly higher optimal temperatures may prevail (Dehus *et al*., 1999). The discharge of secondary zoospores from the primary cysts peaks at 20°C and does not occur at 24°C. In new isolates of *A. astaci*, it is normal for the majority of primary spore cysts to discharge as secondary zoospores, although this varies with staling in long-term laboratory culture. Sporangial formation and discharge occurs down to 4°C. *A. astaci* does not survive at –5°C and below for more than 24 hours in culture, although –20°C for >48 hours may be required in infected crayfish tissues, nor does it remain viable in crayfish tissues that have been subject to normal cooking procedures (Alderman, 2000; Oidtmann *et al*., 2002).

In many cases, some of the primary spores are not discharged from the sporangium and many sporangia do not discharge at all. Instead, the primary spores appear to encyst *in situ* within the sporangium, often develop a spherical rather than elongate form and certainly undergo the same changes in refractive index that mark the encystment of spores outside the sporangium. This within-sporangial encystment has been observed on crayfish. Spores encysted in this situation appear to be capable of germinating to produce further hyphal growth.

Release of secondary zoospores is papillate, the papilla developing shortly before discharge. The spore cytoplasm emerges slowly in an amoeboid fashion through a narrow pore at the tip of a papilla, rounds up and begins a gentle rocking motion as a flagellar extrusion begins and the spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral (Scott, 1961); zoospores are typical saprolegniaceous secondary zoospores measuring 8 × 12 µm. Active motility takes some 5–20 minutes to develop (dependent on temperature) and, at first, zoospores are slow and uncoordinated. At temperatures between 16 and 20°C, zoospores may continue to swim for at least 48 hours (Alderman & Polglase, 1986).

Test sensitivity and specificity of the cultivation method can be very variable depending on the experience of the examiner, but in general will be lower than the PCR.

North American crayfish species

Isolation of *A. astaci* by culture following the methods described for the highly susceptible species usually fails. Currently, the recommended method for detecting infection in such species is by PCR.

4.3.1.2.2. Antibody-based antigen detection methods

None available.

4.3.1.2.3. Molecular techniques

Animals

In the case of a suspected outbreak of the disease in highly susceptible crayfish species, moribund or recently dead (<24 hours) crayfish are preferably selected for DNA extraction. Live crayfish can be killed using chloroform. If the only animals available are animals that have died a few days prior to DNA extraction, they can be tested, but a negative PCR result must be interpreted with caution as DNA degradation may have occurred. Endogenous controls can be used to assess whether degradation may have occurred. These should preferably use host tissues richer in host cells compared to the cuticle; cuticle itself contains very few host cell nuclei. If circumstances prevent delivery of crayfish to the specialist laboratory within 24 hours, fixation in 70% ethanol (≥~~3~~10:1 ethanol to crayfish tissue) is possible, but may result in a reduction of the DNA yield.

Annex 21 (contd)

DNA extraction

Where animals of the highly susceptible species are analysed, the soft abdominal cuticle is the preferred sample tissue for DNA extraction. Any superficial contamination should first be removed by thoroughly wiping the soft abdominal cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. The soft abdominal cuticle is then excised and 30–50 mg ground in liquid nitrogen to a fine powder using a pestle and mortar (alternative grinding techniques may be used, but should be compared with the liquid nitrogen method before routine use). For carrier identification, 30–50 mg tissue from each soft abdominal cuticle, and telson and uropods are sampled and processed separately. DNA is extracted from the ground cuticle using a proteinase K-based DNA extraction method (e.g. DNeasy tissue kit; Qiagen, Hilden, Germany; protocol for insect tissue) following the manufacturer's instructions (Oidtmann *et al*., 2006) or using a CTAB (cetyltrimethylammonium bromide-based)-based assay (Vrålstad *et al.,* 2009). Negative controls should be run alongside the samples. Shrimp tissues may be used as negative controls.

4.3.1.2.3.1. PCR

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here that have proven highly sensitive and specific. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome. As should be standard for any PCR-based diagnostic tests, negative controls should be run alongside the samples to control for potential contamination. Environmental controls (using for example shrimp tissue as described above) and extraction blank controls from the DNA extraction should be included along with 'no template' PCR controls (template DNA replaced with molecular grade water). The no template PCR controls should include an environmental PCR control left open during pipetting of sample DNA.

Method 1:

This conventional PCR assay uses species-specific primer sites located in the ITS1 and ITS2 regions. Forward primer (BO 42) 5'-GCT-TGT-GCT-GAG-GAT-GTT-CT-3' and reverse primer (BO 640) 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3'. The PCR is carried out in a 50 µl reaction volume containing 1 × PCR buffer 75 mM Tris/HCl, pH 8.8, 20 mM $[NH_4]_2SO_4$, 0.01% (v/v) Tween 20), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 0.5 µM of each primer, and 1.25 units of DNA polymerase (e.g. Thermoprime Plus DNA Polymerase; AB Gene, Epsom, UK) or equivalent Taq polymerase and 2 µl DNA template. The mixture is denatured at 96°C for 5 minutes, followed by 40 amplification cycles of: 1 minute at 96°C, 1 minute at 59°C and 1 minute at 72°C followed by a final extension step of 7 minutes at 72°C. Amplified DNA is analysed by agarose gel electrophoresis. The target product is a 569 bp fragment. Confirmation of the identity of the PCR product by sequencing is recommended. The assay consistently detects down to 500 fg of genomic target DNA or the equivalent amount of ten zoospores submitted to the PCR reaction (Tuffs & Oidtmann, 2011).

Method 2:

This assay is a TaqMan minor groove binder (MGB) real-time PCR assay that targets a 59 bp unique sequence motif of *A. astaci* in the ITS1 region. Forward primer AphAstITS-39F (5'-AAG-GCT-TGT-GCT-GGG-ATG-TT-3'), reverse primer AphAstITS-97R (5'-CTT-CTT-GCG-AAA-CCT-TCT-GCT-A-3') and TaqMan MGB probe AphAstITS-60T (5'-6-FAM-TTC-GGG-ACG-ACC-CMG-BNF-Q-3') labelled with the fluorescent reporter dye FAM at the 5'-end and a non-fluorescent quencher MGBNFQ at the 3'-end. Real-time PCR amplifications are performed in a total volume of 25 µl containing 12.5 µl PCR Master Mix (e.g. Universal PCR Master Mix or Environmental PCR Master Mix, Applied Biosystems), 0.5 µM of the forward (AphAstITS-39F) and reverse (AphAstITS-97R) primers, 0.2 µM 200 nM of the MGB probe (AphAstITS-60T), 1.5 µl molecular grade water and 5 µl template DNA (undiluted and tenfold diluted). Amplification and detection is performed in Optical Reaction Plates sealed with optical adhesive film or similar on a real-time thermal cycler. The PCR programme consists of an initial decontamination step of 2 minutes at 50°C to allow optimal UNG enzymatic activity, followed by 10 minutes at 95°C to activate the DNA polymerase, deactivate the UNG and denature the template DNA, and successively 50 cycles of 15 seconds at 95°C and 60 seconds at 58°C. A dilution series with reference DNA of known DNA content 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 ~~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.

Annex 21 (contd)

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.

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	~~d~~ n/a	~~d~~ n/a
Antibody-based assays	n/a	n/a
***In-situ* 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]

Annex 21 (contd)

Table 5.2 (contd). *Methods for targeted surveillance in highly susceptible crayfish species to declare freedom from ~~crayfish plague~~ infection with A. astaci*

Method	Screening method	Confirmatory method
Sequencing of PCR products	n/a	a
Transmission EM	~~d~~ n/a	~~d~~ n/a
Antibody-based assays	n/a	n/a
***In-situ* 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	~~d~~ n/a	~~d~~ n/a
Antibody-based assays	n/a	n/a
***In-situ* 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 fish 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 farm setting, an infection with ~~*crayfish plague*~~ *A. astaci* should be noticed relatively quickly, due to a relatively quick onset of mortalities in the farmed crayfish population.

To undertake targeted surveillance, regular inspections are recommended, where samples are collected if there is any suspicion of mortality or disease. If moribund or dead animals are found, it is recommended that samples are analysed by PCR and if PCR returns a positive result, that PCR products generated using primers 42 and 640, or ITS-1 and -4 are sequenced and the sequences analysed.

6.2. North American crayfish species

In North American crayfish species, animals 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 suspected if at least one of the following criteria is met:

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 real-time PCR.

EU comment

Please replace the word "or" by the word "by" in point v) above (syntax).

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 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 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 real-time PCR.

EU comment

Please replace the word "or" by the word "by" in point iii) above (syntax).

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 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 real-time PCR

EU comment

Please replace the word "or" by the word "by" in point ii) above (syntax).

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 (*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

Annex 22

CHAPTER 2.2.3.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Infection with infectious hypodermal and haematopoietic necrosis virus ~~(IHHN) disease~~ means ~~is caused by~~ infection with the *pathogenic agent* infectious hypodermal and haematopoietic necrosis virus (IHHNV), ~~of the~~ 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.,* 2003~~b~~): Type 1 is from the Americas and East Asia (principally the Philippines). Type 2 is from South-East Asia. These genotypes are infectious to *Penaeus vannamei* and *P. monodon*. Two putative related sequences are found embedded in the genome of penaeids: Type 3A from East Africa, India and Australia, and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al*., 2007). ~~There is evidence that these sequences are not infectious to *P. vannamei* and *P. monodon* (Tang & Lightner, 2002; Tang *et al*., 2003b; 2007).~~ IHHNV type 3A and type 3B related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al*., 2007). The putative IHHNV sequences in the *P. monodon* genome are not infectious to the 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.

2.2. Host factors

2.2.1. Susceptible host species

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

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

~~IHHNV infections are most severe in the Pacific blue shrimp, *P. stylirostris*, where the virus can cause acute epizootics and mass mortality (> 90%). In *P. stylirostris,* the juvenile and subadult life stages are the most severely affected (Bell & Lightner, 1984; 1987; Brock & Lightner 1990; Brock *et al.,* 1983; Lightner, 1996a; Lightner & Redman, 1998a; Lightner *et al.,* 1983a).~~

~~IHHNV causes the chronic disease runt deformity syndrome (RDS) in *P. vannamei* in which reduced, irregular growth and cuticular deformities, rather than mortalities, are the principal effects (Bray *et al.,* 1994; Browdy *et al.,* 1993; Castille *et al.,* 1993; Kalagayan *et al.,* 1991; Lightner, 1996a; 1996b; Motte *et al.,* 2003). IHHNV infection in *P. monodon* is usually subclinical, but RDS, reduced growth rates and reduced culture performance have been reported in IHHNV-infected stocks (Chayaburakul *et al.,* 2004; Primavera & Quinitio, 2000).~~

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing a species as susceptible to infection with IHHNV according to Chapter 1.5. of the *Aquatic Code* include: northern brown shrimp (*Penaeus aztecus*). ~~Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.~~

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: northern pink shrimp (*Penaeus. duorarum*), western white shrimp (*P. occidentalis*), *kuruma prawn* (*P. japonicus*), green tiger prawn (*P. semisulcatus*), *Hemigrapsus penicillatus,* Argentine stiletto shrimp (*Artemesia longinaru~~i~~s*), Cuata swimcrab (*Callinectes arcuatus*), Mazatlan sole (*A~~r~~chirus mazatlanus*), *yellowfin mojarra* (*Gerres cinereus*), tilapias (*Oreochromis* sp.), Pacific piquitinga (*Lile stolifera*) and blackfin snook (*Centropomus medius*).

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

IHHNV has been detected ~~demonstrated~~ in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei.* Eggs, produced by IHHNV-infected females with high virus loads, were found to generally fail to develop and hatch. Those nauplii produced from infected broodstock that do hatch have a high prevalence of infection with IHHNV ~~infection~~ (Motte *et al.,* 2003).

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

See Sections 2.2.1 and 2.2.2.

2.2.5~~4~~. Target organs and infected tissue

IHHNV infects and has been shown to replicate (using *in-situ* hybridisation [ISH] with specific DNA probes) in tissues of ectodermal and mesodermal origin from the embryo. Thus, the principal target organs include: the gills, cuticular epithelium (or hypodermis), all connective tissues, the haematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, and striated muscle show no histological signs of infection with ~~by~~ IHHNV and are usually negative ~~for IHHNV~~ by ISH (Lightner, 1993; 1996a; 2011; Lightner *et al.,* 1992b).

Annex 22 (contd)

2.2.5~~6~~. 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).

2.2.6~~7~~. Vectors

No vectors are known ~~in natural infections~~.

~~2.2.8. Known or suspected wild aquatic animal carriers~~

~~IHHNV is common in wild penaeid shrimp in South-East Asia (*P. monodon*) and in the Americas (*P. vannamei, P. stylirostris* and other Pacific side wild penaeid species) (Fegan & Clifford, 2001; Lightner, 1996a; Lightner *et al.,* 2009; Morales-Covarrubias *et al.,* 1999).~~

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of IHHNV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water (Lightner, 1996a; Lightner *et al.,* 1983a; 1983b; 1985), and vertical transmission via infected eggs (Motte *et al.,* 2003) have been demonstrated.

2.3.2. Prevalence

In regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *P. stylirostris* in the lower and upper Gulf of California, respectively (Pantoja *et al.,* 1999); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (Morales-Covarrubias *et al.,* 1999); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (Nunan *et al.,* 2001); and from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte *et al.,* 2003). Other penaeids collected during some of these surveys and found to be IHHNV positive included the brown shrimp, *P. californiensis* and the Western white shrimp *P. occidentalis*. In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence, approaching 100%, is typical (Chayaburakul *et al.,* 2004; Lightner, 1988; 1996a; 1996b; Lightner *et al.,* 1992a; 1983a; Martinez-Cordova, 1992).

2.3.3. Geographical distribution

IHHNV appears to have a world-wide distribution in both wild and cultured penaeid shrimp (Brock & Lightner, 1990; Lightner, 1996a; 1996b; Owens *et al.,* 1992). In the Western Hemisphere, 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).

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 10^2 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.

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 ~~by~~ IHHNV (see Section 4.2.1.2). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV. As *P. vannamei*, *P. stylirostris*, and *P. monodon* can be infected by IHHNV and not present obvious signs of infection (e.g. they may show markedly reduced growth rates or 'runting'), molecular tests are recommended when evidence of freedom from infection with IHHNV ~~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 in juveniles of this species. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size 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).

Annex 22 (contd)

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 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 22 (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 IHHNV.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See section 4.2.6.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture or artificial media

IHHNV has not been grown in vitro. No crustacean cell lines exist (Lightner, 1996a; Lightner & Redman, 1998a: 1998b).

4.3.1.2.2. Antibody-based antigen detection methods

None has been successfully developed.

4.3.1.2.3. Molecular techniques

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. PCR tests for IHHNV have been developed and a number of methods and commercial ~~products using these methods~~ PCR detection kits are readily available.

DNA probes for dot-blot and ISH applications: gene probe and PCR methods provide greater diagnostic specificity and sensitivity than ~~do more~~ traditional techniques ~~for IHHN diagnosis~~ that employ classic histological approaches. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp. A haemolymph sample may be taken with a tuberculin syringe, or an appendage (a pleopod for example) may be biopsied (Bell *et al*., 1990), and used as the sample for a ~~direct~~ dot-blot hybridisation test.

~~*Dot-blot hybridisation procedure for IHHNV:* the probe is labelled with a non-radioactive label, digoxigenin-11-dUTP (DIG-11-dUTP). The system using DIG to label nucleic acid probes was developed by Boehringer Mannheim Biochemicals (this company is now owned by Roche Diagnostic Corporation), which is described in the Roche *DIG Nonradioactive Labeling and Detection Product Selection Guide* and *DIG Application Manual for Filter Hybridization™ System User's Guide for Membrane Hybridization* and from Boehringer Mannheim's *Nonradioactive In Situ Hybridization Application Manual* (Roche Applied Science, 2006a; 2006b). The protocols given below use a DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (**Lightner, 1996a**; Mari *et al.,* 1993). An alternative method for producing DIG-labelled probes uses specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit™.~~

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

Annex 22 (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 ~~10~~ 5 minutes and quench on ice for ~~5~~ 1–2 minutes. ~~Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein.~~ Keep on ice until samples are dotted on to the membrane.

iv) Dot 1–3 µl of each sample on to an appropriate place on the filters. Allow to air-dry and then fix samples on to the membrane by baking at 80°C for 30 minutes or by UV cross-linking using a DNA transilluminator for 3 minutes.

~~v) Adjust a water bath to 68°C and prepare the prehybridisation solution. For a 10 × 15 cm membrane, prepare 8 ml per membrane. Set a stirring hot plate to 'low' and stir while warming the solution for 30 minutes until the blocking agent has dissolved and the solution is cloudy. Also, prepare some heat-seal bags that are slightly larger in size than the membrane: five to six bags will be needed per membrane.~~

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 ~~10~~ 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~~	~~1 ×~~	~~30 minutes at room temperature~~
~~Buffer I~~	~~1 ×~~	~~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 Diagnostics[2] ~~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 22 (contd)

In-situ hybridisation (ISH) procedure: the ISH method given below uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al*. (1993) and Lightner (1996a). ~~Formulas for the required reagents are given after the protocols.~~

i) Embed tissue in paraffin and cut sections at 4–6 µm thickness. Place sections on to positively charged microscope slides (do not put gelatine in water to float sections; just use water).

ii) Put slides in a slide rack, such as a Tissue-Tek rack. Heat the slides in an oven for 45 minutes at 60°C. In the staining centre, rehydrate the tissue as follows:

Xylene (or suitable substitute)	3 ×	5 minutes each
Absolute alcohol	2 ×	1 minute each
95% alcohol	2 ×	10 dips each
80% alcohol	2 ×	10 dips each
50% alcohol	1 ×	10 dips
Distilled water	six rinses (do not let slides dry out)	

iii) Wash the slides for 5 minutes in ~~phosphate-buffered-saline (~~PBS (or Tris/NaCl/EDTA [TNE] buffer). Prepare fresh proteinase K at 100 µg ml^{-1} in PBS (or TNE). Place slides flat in a humid chamber, pipette on 500 µl of the proteinase K solution and incubate for 10–15 minutes at 37°C. Drain fluid onto blotting paper.

iv) Return slides to slide rack. Fix sections in 0.4% cold formaldehyde for 5 minutes at room temperature.

v) Incubate slides in 2 × SSC for 5 minutes at room temperature.

vi) With slides flat, add 0.5–1 ml prehybridisation buffer and incubate in a humid chamber for 15–30 minutes at 37°C.

vii) Boil the DIG-labelled probe for ~~10~~ 3–5 minutes and quench on ice; spin briefly in the cold and keep on ice. Dilute the probe to 25 ng ml^{-1} in prehybridisation solution and cover the tissue with 250 µl of the solution. Incubate the slides for 2–4 hours at 42°C or overnight at 37°C in a humid chamber. ~~Drain fluid onto blotting paper. During this incubation, pre-warm the wash buffers at 37°C.~~

viii) Place slides in slide rack. Wash the slides as follows:

2 × SSC	2 ×	5–30 minutes at 37°C
1 × SSC	2 ×	5 minutes at 37°C
0.5 × SSC	2 ×	5 minutes at 37°C

ix) Wash the slides for ~~5~~ 1–3 minutes in Buffer I at room temperature. Put the slides flat in a humid chamber and block with 0.5 ml per slide of Buffer II. Incubate for 15 minutes at 37°C. ~~Drain the fluid on to blotting paper.~~

x) Dilute the anti-DIG alkaline phosphotase conjugate ~~(Roche Applied Science cat. 10686322)~~ at a ratio of 1/1000 in Buffer II ~~(1 µl anti-DIG-AP per 1 ml buffer)~~. Cover tissue with 500 µl of diluted conjugate and incubate in a humid chamber for 30 minutes at 37°C.

xi) Place the slides in a slide rack. Wash in Buffer I twice for 5–10 minutes each time at room temperature. Wash once with Buffer III for ~~5–10~~ 1–2 minutes.

xii) Prepare the development solution by first adding 4.5 µl NBT per 1 ml buffer III. Mix well. Then add 3.5 µl X-phosphate per ml of solution and mix well. Pipette on 500 µl per slide and incubate in a humid chamber in the dark for 2–3 hours at room temperature.

xiii) Stop the reaction by returning the slides to a slide rack and washing in Buffer IV for 15 minutes at room temperature.

xiv) Counterstain the slides by dipping for 5 minutes in 0.5% aqueous Bismarck brown Y.

xv) Dehydrate the slides in the staining centre as follows:

95% alcohol	3 ×	10 dips each
Absolute alcohol	3 ×	10 dips each
Xylene (or suitable substitute)	4 ×	10 dips each

Do not allow the slides to dry out – leave them in the last xylene (or xylene substitute) container until ready for cover-slips.

xvi) Mount with cover-slips and mounting medium (Permount).

xvii) Examine the slides under bright-field for a dark-blue or black precipitate that marks sites where IHHNV DNA is present. Pathodiagnostic intranuclear Cowdry type A inclusions are well marked with the probe. Also often marked are host cell nuclei without obvious inclusions, cytoplasmic inclusions, and accumulation of free virus in the tissue spaces and haemolymph.

~~NOTE: Always run a known positive and negative control.~~

Reagent formulas for ISH method:

~~i) 10 × phosphate buffered saline~~

~~NaCl~~	~~160 g~~
~~KH_2PO_4~~	~~4 g~~
~~Na_2HPO_4~~	~~23 g~~
~~KCl~~	~~4 g~~
~~DD H_2O~~	~~1950 ml (qs to 2 litres)~~

~~pH to 8.2 with NaOH; autoclave to sterilise; store at room temperature. To make 1 × PBS, dilute 100 ml 10 × PBS in 900 ml DD H_2O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.~~

~~i~~) 10 × Tris/NaCl/EDTA (TNE) buffer

Tris base	60.57 g
NaCl	5.84 g
EDTA	3.72 g
H_2O	900 ml (qs to 1 litre)

pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 × TNE in 900 ml ~~DD~~ H_2O~~; Filter 1 × solution through a 0.45 µm filter; store at 4°C~~.

~~iii~~ii) Proteinase K, 100 µg ml^{-1} (prepare just prior to use)

PBS	10 ml 1 × PBS
Proteinase K	1 mg

~~iv) 0.4% formaldehyde~~

~~37% formaldehyde~~	~~5.4 ml~~
~~DD H_2O~~	~~500 ml~~

~~Store at 4°C; can be reused up to four times before discarding.~~

~~v~~iii) Prehybridisation buffer (50 ml final volume)

4 × SSC	10 ml 20 × SSC
50% formamide	25 ml 100% formamide
1 × Denhardt's	2.5 ml 20 × Denhardt's
5% dextran sulphate	10 ml 25% dextran sulphate
Warm to 60°C	

Boil 2.5 ml of 10 mg ml^{-1} salmon sperm DNA and add to buffer for final concentration of 0.5 mg ml^{-1} salmon sperm DNA~~; store at 4°C~~.

~~vi) 20 × SSC buffer~~

~~3M NaCl~~	~~175.32 g NaCl~~
~~0.3 M $Na_3C_6H_5O_7.2H_2O$~~	~~88.23 g Na citrate.$2H_2O$~~
~~DD H_2O~~	~~1000 ml (qs)~~

~~pH to 7.0; autoclave; store at 4°C.~~

~~To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H_2O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H_2O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H_2O. Filter solutions through a 0.45 µm filter; store at 4°C.~~

Annex 22 (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 polyvinylpyrollidine~~
~~DD H_2O~~	~~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_2O~~	~~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_2O~~	~~25 ml~~

~~To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18 gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.~~

~~x~~iv) 10 × Buffer I

1 M Tris/HCl	121.1 g Tris base
1.5 M NaCl	87.7 g NaCl
~~DD~~ H_2O	1000 ml (qs)

pH to 7.5 with HCl. Autoclave~~; store at 4°C~~.

To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml ~~DD~~ H_2O. ~~Filter through a 0.45 µm filter; store at 4°C.~~

~~xi~~v) Buffer II (blocking buffer)

Blocking reagent	0.25 g Blocking reagent (Roche Diagnostics ~~1-096-176~~)
Buffer I	50 ml 1 × Buffer I

~~Store at 4°C for up to 2 weeks.~~

~~xii~~vi) Buffer III

100 mM Tris/HCl	1.21 g Tris base
100 mM NaCl	0.58 g NaCl
~~DD~~ H_2O	100 ml (qs)
pH to 9.5 with HCl	
Then add:	
50 mM $MgCl_2$	1.02 g $MgCl_2.6H_2O$

~~Filter through a 0.45 µm filter; store at 4°C.~~

~~xiii) 10% polyvinyl alcohol (PVA)~~

~~Polyvinyl alcohol~~	~~10 g~~
~~DD H_2O~~	~~100 ml~~

~~To prepare, slowly add PVA to water while stirring on low heat. (It takes 2–3 hours for PVA to go into solution.) Dispense 10 ml per tube and store frozen at –20°C.~~

~~xiv~~vii) Development solution

Mix 90 ml Buffer III with 10 ml of 10% PVA. Store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

4.5 µl NBT	75 mg NBT ml^{-1} in 70% dimethylformamide ~~(Roche Diagnostics 1-383-213)~~
3.5 µl X-phosphate	5-bromo-4-chloro-3-indoyl phosphate, toluidine salt ~~(50 mg ml^{-1} in dimethylformamide) (Roche Diagnostics 1-383-221)~~

~~xv~~viii) Buffer IV

10 mM Tris/HCl	1.21 g Tris base
1 mM EDTA	0.37 g EDTA.2H_2O (disodium salt)
~~DD~~ H_2O	1000 ml

pH to 8.0 with HCl. ~~Filter through a 0.45 µm filter; store at 4°C.~~

~~xvi~~ix) 0.5% Bismarck Brown Y

Bismarck Brown Y	2.5 g
~~DD~~ H_2O	500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

Polymerase chain reaction for IHHNV: several one-step PCR methods (Krabsetsve *et al.,* 2004; Nunan *at al.,* 2000; Shike *et al.,* 2000; Tang *et al.,* 2000; 2007~~; Tang & Lightner 2001~~), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available from commercial sources.

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods ~~for IHHNV~~. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (~~Krabsetsve *et al.,* 2004; Tang & Lightner, 2002~~ Tang *et al*., 2000; 2007). However these tests also detect IHHNV-related sequences called types 3A and 3B, which are inserted into the genome of certain ~~geographic~~ stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (~~Duda & Palumbi, 1999;~~ Saksmerprome *et al.,* 2011; Tang & Lightner, 2006; Tang *et al.,* 2007). PCR primers have been developed that can detect the ~~IHHN viral~~ IHHNV sequence but do not react with IHHNV-related sequences present in the *P. monodon* stocks from Africa, Australia (Tang *et al.,* 2007), or Thailand (Saksmerprome *et al.,* 2011). Primer set 309F/R amplifies only a genomic segment of IHHNV types 1 and 2 (the infectious forms of IHHNV), but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Primer set MG831F/R reacts only with types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang *et al.,* 2007). Hence, confirmation of unexpected positive or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. ~~PCR using primers from another region of the genome, real-time PCR~~ histology, bioassay, ISH) is highly recommended.

Table 4.1. *Recommended primer sets for one-step PCR detection of IHHNV*

Primer	Product	Sequence	G+C%/Temp.	GenBank & References
389F	389 bp	5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3'	50%/72°C	AF218266
389R		5'-GGC-CAA-GAC-CAA-AAT-ACG-AA-3'	45%/71°C	(Tang *et al.,* 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 *et al.,* 2000)
392F	392 bp	5'-GGG-CGA-ACC-AGA-ATC-ACT-TA-3'	50%/68°C	AF218266
392R		5'-ATC-CGG-AGG-AAT-CTG-ATG-TG-3'	50%/71°C	(Tang *et al.,* 2000)
309F	309 bp	5'-TCC-AAC-ACT-TAG-TCA-AAA-CCA-A-3'	36%/68°C	AF218266
309R		5'-TGT-CTG-CTA-CGA-TGA-TTA-TCC-A-3'	40%/69°C	(Tang *et al.,* 2007)
MG831F	831 bp	5'-TTG-GGG-ATG-CAG-CAA-TAT-CT-3'	45%/58°C	DQ228358
MG831R		5'-GTC-CAT-CCA-CTG-ATC-GGA-CT-3'	55%/62°C	(Tang *et al.,* 2007)

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region ~~(ORF 1)~~ of the IHHNV genome. Primers 77012F/77353R are from a region in between the nonstructural and ~~the structural (coat protein)~~ capsid protein-coding region of the genome. In the event that results are ambiguous using the 389F/R 'universal' primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012F/77353R or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.

Annex 22 (contd)

General PCR method for IHHNV: the PCR method described below for IHHNV generally follows the methods outlined in Tang *et al.* (2007) and Nunan *et al.* (2000). Cumulative experience with the technique has led to modifications with respect to template ~~(~~DNA extraction ~~of clinical specimens),~~ methods, choice of primers (Table 4.1), and volume of reaction.

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 IHHNV: (a) DNA from a known negative tissue sample; (b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and (c) a 'no template' control.

iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV-infected material. Prepare primers at ~~100 µl^{-1}~~ 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 PuReTaqTM 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~~
		~~1 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~~viii) ~~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 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 IHHNV.

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

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

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The primers and TaqMan probe are designed by the Primer Express software (~~Applied Biosystems~~ Life Technologies). The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5'-TAC-TCC-GGA-CAC-CCA-ACC-A-3' and 5'-GGC-TCT-GGC-AGC-AAA-GGT-AA-3', respectively. The TaqMan probe (5'-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3'), ~~which corresponds to the region from nucleotide 1632 to 1644,~~ is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end ~~(Applied Biosystems, part no. 450025).~~

ii) Preparation of DNA template: the extraction ~~and purification~~ of DNA template is the same as that described ~~in the section of traditional PCR~~ above.

iii) The 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 IHHNV-infected tissue.

Sequencing: PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified products from the infectious form of the virus and demonstrate the presence of the insertion of non-infectious IHHNV genome in host DNA (Tang & Lighter, ~~2002;~~ 2006).

Through PCR, IHHNV was detected in *P. monodon* from South-East Asia. ~~Most~~ Some of these IHHNV PCR ~~assays~~ primers also ~~detected~~ reacted to IHHNV-related sequences in *P. monodon* populations in Africa, Australia and Thailand (Tang & Lightner, 2006; Saksmerprome *et al*., 2011). To discriminate the IHHNV-related sequences from the actual virus, PCR assays using primers that detect the 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. *Infection with IHHNV surveillance, detection and diagnostic methods*

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	D	d
Bioassay	d	d	d	d	C	c
Direct LM	d	d	d	d	D	d
Histopathology	d	d	c	c	A	b
Transmission EM	d	d	d	d	C	c
Antibody-based assays	d	d	d	c	D	d
In-situ ~~**DNA probes**~~ **hybridisation**	d	d	b	b	A	a
Dot-blot hybridisation	d	d	c	c	A	a
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 haematopoietic necrosis

As indicated in Table 5.1, PCR is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic IHHNV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with IHHNV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

i) Clinical signs indicative of infection with IHHNV ~~and a positive result by *in-situ* hybridisation~~

or

ii) Histopathology indicative of infection with IHHNV ~~and a positive result by *in-situ* hybridisation~~

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 Infectious hypodermal and haematopoietic necrosis (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 Infectious hypodermal and haematopoietic necrosis

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

Annex 23

CHAPTER 2.2.4.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

EU comment

The EU supports the proposed changes to this chapter.

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 *P. 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 – *Mr*NV (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.

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 (*Penaeus esculentus*), banana prawn (*P. merguiensis*), 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 (*Penaeus monodon*) and blue shrimp (*Penaeus 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 (*Penaeus 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).

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 (Sutanto, 2011). 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).

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). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P. vannamei*, and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* most suited to local culture conditions has proven to be the most successful husbandry practice for preventing and controlling other 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.~~

Samples, 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 ~~infection~~ are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue.

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

3.5. Samples 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.

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.

4.2.3. Microscopic pathology

Infection with IMNV ~~IMN disease~~ in the acute and chronic phases can be presumptively diagnosed using histology (Bell & Lightner, 1988; Lightner, 2011; Lightner *et al.,* 2004; Poulos *et al.,* 2006). However, the lesions in striated muscles and LO are not pathognomonic for infection with IMNV ~~IMN~~. White tail disease of penaeid shrimp caused by the nodavirus PvNV can mimic infection with IMNV ~~IMN~~ (Tang *et al.,* 2007). Hence, diagnostic information from other sources (e.g. history, gross signs, morbidity, mortality, or RT-PCR findings) may be required to confirm a diagnosis of infection with IMNV ~~IMN~~.

By histology using routine haematoxylin–eosin (H&E) stained paraffin sections (Bell & Lightner, 1988), tissue sections from shrimp with acute-phase infection with IMNV ~~IMN~~ present myonecrosis with characteristic coagulative necrosis of striated (skeletal) muscle fibres, often with marked oedema among affected muscle fibres. Some shrimp may present a mix of acute and older lesions. In these shrimp, the affected muscle fibres appear to progress from presenting coagulative necrosis to presenting liquefactive necrosis, which is accompanied by moderate infiltration and accumulation of haemocytes. In the most advanced lesions, haemocytes and inflamed muscle fibres are replaced by a loose matrix of fibrocytes and connective tissue fibres that are interspersed with haemocytes and foci of (presumed) regenerating muscle fibres (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

Significant hypertrophy of the LO caused by accumulations of LOS is a highly consistent lesion in shrimp with acute or chronic-phase infection with IMNV ~~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 (i.e. Qiagen RNA extraction kit, Promega and Roche RNA purification kit recommend using 25–50 mg of tissue[3]). Depending on the kit used, the elution volume for Roche and Qiagen and low elution volume RNA isolation Promega extraction kit is 100 µl. The high elution volume RNA isolation Promega extraction kit is 500 µl. Extracted RNA should be maintained at –20°C before testing, however, for long-term storage the RNA should be kept at –70°C.

Following RNA extraction, the method is summarised below:

RNA templates:

1. Frozen or ethanol-fixed tissue (pleopods, cephalothorax, muscle)
2. Haemolymph (less sensitive than when other tissues are used)

RT-PCR reaction mixture (~~Applied Biosystems rTth Enzyme and 5 × EZ Buffer #N808-0178~~ SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Life Technologies):

Reagent	**Volume ~~25 µl reaction~~**	**Final concentration**
~~DD~~ dH_2O	~~6.5~~ 5.5 µl	–
~~5 × EZ Buffer~~ 2 × reaction mix	~~5.0~~ 12.5 µl	1 ×
~~dNTP mix~~ Forward/reverse primer (10mM each)	~~3.0~~ 1.0 µl	~~300 µM each~~ 0.4 µm
~~Primer F (100 ng µl^{-1})~~ RT/Taq enzyme mix	1.0 µl	~~0.62 µM~~
~~Primer R (100 ng µl^{-1})~~	~~1.0 µl~~	~~0.62 µM~~
~~$Mn(Oac)_2$ (25 mM)~~	~~2.5 µl~~	~~2.5 mM~~
~~rTth Enzyme (2.5 U µl^{-1})~~	~~6.5 1.0 µl~~	~~0.1 U µl^{-1}~~
RNA template[1]	~~1–5~~ 5.0 µl	1–50 ng ~~total RNA~~

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

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 23 (contd)

RT-PCR thermal cycling conditions:

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

Nested PCR reaction (~~Amersham Biosciences pure Taq~~ illustra™ PuReTaq™ Ready-To-Go PCR Beads ~~#27-9558-01~~, GE Healthcare):

Reagent	25 µl reaction	Final concentration
DD H_2O	~~22.5~~ 23 µl	–
Primer NF (~~100 ng µl^{-1}~~ 10 µM)	~~1.0~~ 0.5 µl	~~0.465~~ 0.2 µM
Primer NR (~~100 ng µl^{-1}~~ 10 µM)	~~1.0~~ 0.5 µl	~~0.465~~ 0.2 µM
Template[2]	~~0.5~~ 1.0 µl	–

[2]Template for the nested reaction is the product from the first step reaction

Nested PCR thermal cycling conditions:

Primers	Temperature (°C)	Time	No. cycles	Amplicon length
	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, 2006
4914R	ACT-CGG-CTG-TTC-GAT-CAA-GT		
4725 NF	GGC-ACA-TGC-TCA-GAG-ACA	139 bp	
4863 NR	AGC-GCT-GAG-TCC-AGT-CTT-G		

Quantitative (real-time) RT-PCR for detection of IMNV

A real-time qRT-PCR method was developed to detect and quantify IMNV in shrimp tissue. The method can detect as few as 10 IMNV RNA copies per µl total RNA (Andrade *et al.,* 2007). The method as published is summarised below.

The Primer Express software (Applied Biosystems) was used to aid the design of the PCR primers and TaqMan probe targeted to the ORF1 region of the IMNV genome (GenBank accession no. AY570982) (Andrade *et al.,* 2007; Poulos *et al.,* 2006). Primers IMNV412F (5'-GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA-3') and IMNV545R (5'-AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT-3') amplify a 134 bp DNA. The TaqMan probe, IMNVp1 (5'-6FAM-CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG-TAMRA-3'), which corresponds to the nucleotides 467–500, is labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) at its 5'-end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at its 3'-end.

The IMNV genome fragment is amplified using ~~an ABI GeneAmp 5700 sequence detection system~~ StepOnePlus PCR System and the TaqMan ~~One~~ Fast virus 1-Step ~~RT-PCR master mix (Applied Biosystems)~~ Master Mix (Life Technologies). Prior to the ~~real-time~~ qRT-PCR, extracted RNA is boiled at 95–100°C for ~~5~~ 3 minutes to denature the dsRNA and chilled immediately in ~~wet~~ ice. The reaction mixture contains 1 µl RNA sample, ~~12.5 µl~~ TaqMan Master mix (2×), ~~0.625 µl Multiscribe mix (40×),~~ 300 nM each primer IMNV412F and IMNV545R, 200 nM. IMNVp1TaqMan probe in a ~~25~~ 10–20 µl final volume. The ~~RT~~ qRT-PCR thermal cycling conditions used are ~~48~~ 50°C for ~~30~~ 3 minutes, 95°C for ~~10 minutes~~ 20 seconds followed by 40 cycles of 95°C for ~~15~~ 3 seconds and 60°C for ~~1 minute. The IMNV RNA copy number~~ 30 seconds. At the end of the ~~samples~~ reaction, fluorescence intensity is ~~determined using serial dilutions~~ measured, a threshold will be set to be above the baseline. Samples with a Ct value lower than 40 cycles are considered to be positive. It is necessary to include a 'no template' control in each reaction run. This is to rule out the presence of ~~a synthetic~~ fluorescence contaminants in the reaction mixture. A positive control should also be included, and it can be RNA extracted from IMNV-infected tissue, or in vitro transcribed IMNV RNA ~~standard~~ containing the target sequence (see below). ~~and the Gene Amp 5700 sequence detection software.~~

To synthesise an RNA standard for the real-time qRT-PCR, the PCR primers IMNV218F and IMNV682R (5'-GCT-GGA-CTG-TAT-TGG-TTG-AG-3' and 5'-AAC-CAA-GTT-CTT-CTT-CTC-CAG-TT-3', respectively) are used to amplify a 464 bp DNA product from the IMNV genome. The PCR product purified using a QIAuick PCR Purification kit (QIAGEN) was cloned into pGEM-T Easy Vector. A recombinant plasmid, pIMNV-1, confirmed to contain the 464 bp insert by sequence analysis, is linearised by digestion with *Pst*I and used as the template for an *in-vitro* RNA transcription using T7 RNA polymerase and associated reagents (Promega). RNA is synthesised at 37°C for 2 hours in a 50 µl reaction containing 1 µg plasmid DNA, followed by DNase I digestion at 37°C for 30 minutes for remove DNA. The length and integrity of the synthesic ssRNA is confirmed by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The RNA is purified using a Qiaquick PCR Purification kit, quantified by a spectrophotometer, and stored at –70°C

4.3.1.2.4. Agent purification

While IMNV has been purified from infected shrimp tissue by sucrose density gradient ultracentrifugation (Poulos *et al.,* 2006), this is not recommended for diagnostic purposes.

4.3.2. Serological methods

Not applicable because shrimp are invertebrates which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to IMNV.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of 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

Annex 23 (contd)

Table 5.1 (contd). *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Direct LM	d	d	c	c	c	c
Histopathology	d	d	b	b	a	c
Transmission EM	d	d	d	d	d	d
Antibody-based assays	d	d	d	d	c	d
DNA probes (ISH)	d	d	a	a	a	a
Nested RT-PCR or real-time RT-PCR	a	a	a	a	a	a
~~**Real-time RT-PCR**~~	~~d~~	~~c~~	~~a~~	~~a~~	~~a~~	~~a~~

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

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 suspected if at least one of the following criteria is met:

i) 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 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 infectious myonecrosis
(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 infectious myonecrosis

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

Annex 24

CHAPTER 2.2.5.

INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Infection with *Hepatobacter penaei* means infection with the pathogenic agent ~~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 *et al.,* 1996a; 1996b). The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy *et al.,* 1996a; 1996b). Genetic analysis of ~~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 *et al.,* 1996a; 1996b).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent

~~NHPB Candidatus~~ *H. penaei*-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. ~~NHPB Candidatus~~ *H. penaei* frozen at –20°C 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 24 (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 (*Penaeus vannamei*) ~~Most penaeid species can be infected with NHPB, including the principal cultured species in Latin American, *P. vannamei* (Pacific white shrimp) and *P. stylirostris* (Pacific blue shrimp)~~.

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: northern white shrimp (*Penaeus setiferus*), northern pink shrimp (*Penaeus duorarum*), blue shrimp (*P. stylirostris)*, banana prawn (*P. merguiensis*), aloha prawn (*P. marginatus*), northern brown shrimp (*P. aztecus*) and giant tiger prawn (*P. monodon*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following 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* (Frelier *et al.*, 1995; Lightner, 1996). *Penaeus setiferus* is reportedly less susceptible to disease than *P. vannamei* (Frelier *et al.*, 1995).~~

~~In an NHP survey of the Gulf of Mexico, *P.setiferus* and *P.duorarum* in the vicinity of coastal prawn farms along the Yucatan and Campeche coast revealed no histological evidence of NHP (Del Río-Rodríguez *et al.*, 2006).~~

2.2.3~~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 cannibalism; transmission 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-Gámez *et al.,* 2007; Lightner, 1996; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2011).

2.3.4. Mortality and morbidity

In *P. vannamei*, infection ~~by NHPB~~ with *H. penaei* results in an acute, usually catastrophic disease with mortalities approaching 100%.

2.3.5. Environmental factors

The replication rate of ~~NHPB Candidatus~~ *H. penaei* increases at lengthy periods of high temperatures (>29°C) and salinity changes (20–38%). In Mexico, ~~NHPB Candidatus~~ *H. penaei* has been detected at a low prevalence (<7%) in shrimp farms in the months of April, May, July and August. However, in the months of September and October when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

Annex 24 (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 infection with *H. penaei* 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.

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

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.~~

Samples, 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; Frelier *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~~ infection with *H. penaei* may be enhanced in a contained population by rearing shrimps in relatively crowded or stressful conditions. The ‘crowding stress’ factors may include high stocking densities, 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 epicommensal organisms; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic.

4.1.2. Behavioural changes

In acute ~~NHPB~~ disease, *P. vannamei* may present behavioural changes including lethargy and reduced feeding activity.

4.2. Clinical methods

4.2.1. Gross pathology

~~NHPB~~ Infection with *H. penaei* often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adult and broodstock, the incubation period and severity of the disease are somewhat size or age dependent. ~~Infected adults seldom show signs of the 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 field^{-1} organism^{-1}) and cell detachment.

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

Transition phase: infiltration of haemocytes, increased numbers of deformed tubules (11–15 field^{-1} organism^{-1}), melanised tubules, necrotic tubules and a high level of encapsulation present in different regions of the sample. ~~At this stage haemocyte nodules were observed with masses of bacteria in the centre of the nodule.~~

Chronic phase: areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells ~~with masses of bacteria in the cytoplasm~~.

4.2.5. Smears

Not applicable.

4.2.6. Electron microscopy/cytopathology

Not currently applicable for diagnostic purposes

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See section 4.2.4

4.3.1.1.2. Smears

Not applicable

4.3.1.1.3. Fixed sections

See section 4.2.3.

4.3.1.1.4. Bioassay method

Confirmation of ~~NHPB~~ infection with *H. penaei* may be accomplished by bioassay of ~~NHPB~~ suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the intracellular bacteria (Cock *et al.,* 2009; Johnson, 1990; Lee & O'Bryen, 2003; Lightner, 2005). Oral protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped hepatopancreas of suspect shrimp to SPF juvenile *P. vannamei* in small tanks. The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the hepatopancreas feeding (*per os*) protocol is used to bioassay for ~~NHPB Candidatus~~ *H. penaei*, ~~NHPB Candidatus~~ *H. penaei*-positive indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of ~~NHPB disease~~ infection with *H. penaei* and unusual mortalities.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture 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 & Frelier, 1996; Loy *et al.,* 1996b). Gene probes and PCR methods provide greater diagnostic sensitivity than ~~do~~ 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 & Frelier (1996) and Lightner (1996) provides greater diagnostic sensitivity than do more traditional methods for ~~NHPB Candidatus~~ *H. penaei* detection and diagnosis of infection that employ classical histological methods (Johnson, 1990; Lightner, 1996; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2012). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled cDNA probe to ~~NHPB Candidatus~~ *H. penaei*, provides a definitive diagnosis of ~~NHPB~~ infection with *H. penaei* (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.*, 2006). Pathognomonic ~~NHPB Candidatus~~ *H. penaei* positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. (See Chapter 2.2.3 *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 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™ Nucelic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega)[4].

ii) The following controls should be included when perfomring the PCR assay ~~for NHPB~~ a) known ~~NHPB Candidatus~~ *H. penaei* negative tissue sample; b) a known ~~NHPB Candidatus~~ *H. penaei* -positive sample (hepatopancreas); and c) a 'no template' control.

iii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

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*.

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

ii) *Preparation of DNA template:* the extraction and purification of *DNA* template from hepatopancreas, is the same as that described in the section for traditional PCR.

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

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

v) At the end of the reaction, real time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase in PCR product.

vi) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler, 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).

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 *L. 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	c	c	b	d
Bioassay	d	d	d	d	c	d
Direct LM	d	d	c	d	c	d
Histopathology	d	b	b	c	a	b
In-situ DNA probes	a	a	a	a	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
Real-time PCR	a	a	a	a	a	a
PCR	a	a	a	a	a	a
Sequencing	d	d	d	d	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *H. penaei* ~~Necrotising hepatopancreatitis~~

As indicated in Table 5.1, real-time PCR (Section 4.3.1.2.3.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity. When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic ~~NHPB~~

~~Candidatus~~ *H. penaei*-induced lesions in the hepatopancreas by histology (with or without confirmation by ISH with ~~NHPB Candidatus~~ *H. penaei*-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

Annex 25

CHAPTER 2.2.6.

INFECTION WITH TAURA SYNDROME VIRUS

EU comment

The EU in general supports the proposed changes to this chapter, however wishes to reiterate its comments submitted previously, which were not addressed (available here: https://ec.europa.eu/food/sites/food/files/safety/docs/ia_standards_oie_eu_position_aahsc_report_201602_en.pdf, p. 203).

1. Scope

Infection with Taura syndrome virus means infection with the pathogenic agent Taura syndrome virus (TSV), ~~of the~~ Family *Dicistroviridae,* 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).

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 (*P. aztecus*), giant tiger prawn (*P. monodon*), northern white shrimp (*P. setiferus*), blue shrimp (*P. stylirostris*), and whiteleg shrimp (*Penaeus 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 (*Penaeus chinensis*), giant river prawn (*Macrobrachium rosenbergii*), the copepod *Ergasilus 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 (*Penaeus 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~~2~~. 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~~3~~. 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~~4~~. Target organs and infected tissue

TSV infects and has been shown to replicate (using ISH with specific DNA probes) principally in the cuticular epithelium (or hypodermis) of the general exoskeleton, foregut, hindgut, gills and appendages, and often in the connective tissues, the haematopoietic tissues, the lymphoid organ (LO), and antennal gland. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, striated muscle, and the ventral nerve cord, its branches and its ganglia typically show no histological signs of infection 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

TS is best known as a disease of nursery- or grow-out-phase *P. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with TS are typically small juveniles of from ~0.05 g to <5 g. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults (Brock, 1997; Brock *et al.,* 1995; Lightner, 1996a, 1996b; Lotz, 1997).

2.3.1. Transmission mechanisms

Transmission of TSV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (Brock, 1997; Hasson *et al.,* 1995; Lightner, 1996a, 1996b; White *et al.,* 2002). Vertical transmission from infected adult broodstock to their offspring is strongly suspected but has not been experimentally confirmed.

2.3.2. Prevalence

In regions where the virus is 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).

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~~ 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 the these regions (Hasson *et al.,* 1999a; Lightner, 1996a; 2005; 2011; Lightner *et al.,* 2012).

Asia and the Middle East: TSV was introduced into Chinese Taipei in 1999 with infected imported Pacific white shrimp, *P. vannamei*, from Central and South American sources (Tu *et al.,* 1999; Yu & Song, 2000). Since that original introduction, the virus has spread with movements of broodstock and PL to China (People's Rep. of), Thailand, Malaysia, and Indonesia where it has been the cause of major epizootics with high mortality rates in introduced unselected stocks of *P. vannamei* (Chang *et al.,* 2004; Lightner, 2011; Nielsen *et al.,* 2005; Tang & Lightner, 2005). ~~Recently~~ During 2010 and 2011, infection with TSV has also been 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*, 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 TS 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 TSV reaching Mexico in 1994 where it caused crop failures of *P. vannamei,* selected lines of TSV-resistant *P. stylirostris* became the dominant shrimp farmed in western Mexico from 1995. However, in 1998–1999, a new 'strain' of TSV (Type B; Erickson *et al.,* 2002; Fegan & Clifford, 2001; Lightner, 1999; 2005; Zarin-Herzberg & Ascencio, 2001) emerged and caused massive epizootics in *P. stylirostris*. The emergence of this new 'strain' of TSV was soon followed in late 1999 by the introduction of white spot syndrome virus (WSSV) into shrimp farms in western Mexico, to which *P. stylirostris* had no resistance, effectively ending any interest in the culture of *P. stylirostris* in Mexico.

TSV-resistant domesticated stocks of *P. vannamei* and *P. stylirostris* have been developed. Some domesticated lines of TSV-resistant *P. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (Clifford, 1998; Moss *et al.,* 2001; White *et al.,* 2002). After the appearance of TS in Central America, improved 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 ~~infection~~ was reported by expression of the TSV coat protein antisense RNA in *P. vannamei* zygotes. Transgenic juveniles reared from zygotes protected in this manner showed improved resistance to TSV challenge by *per os* or intramuscular (IM) injection routes (Lu & Sun, 2005). Similar results have been produced by injection of short random double-stranded RNAi sequences into juvenile *P. vannamei* (Robalino *et al.,* 2004).

2.4.7. Disinfection of eggs and larvae

It is possible 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 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 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.~~

Samples, 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~~ 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 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 TS 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 ~~TS epizootics~~, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of infection with TSV ~~infection~~. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites of resolving TS 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 ~~infections~~, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.,* 1999b; Lightner 2011), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When LOS are observed by routine histology and chronic infection with TSV ~~infection~~ is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR [see Section 4.3.1.2.7]) is recommended for confirmation of infection with TSV ~~infection~~.

4.2.3.1. Acute phase of Taura syndrome

Diagnosis of infection with TSV in the acute phase of the disease is dependent on the histological demonstration (in haematoxylin and eosin [H&E] stained preparations) of multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres basal to affected cuticular epithelium are occasionally affected. In some severe cases of acute-phase 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 TS 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).

4.2.3.2. Transition (recovery) phase of Taura syndrome

In the transitional phase of infection with TSV, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by *Vibrio* spp. (Hasson *et al.,* 1999b; Lightner, 1996a; 2011). Sections of the LO during the transition phase of infection with TSV may appear normal with H&E staining. However, when sections of the LO are assayed for TSV by ISH with a specific cDNA probe (or by ISH with MAb 1A1 for TSV type A, genotype 1), large quantities of TSV are shown accumulating in the more peripheral parenchymal cells of the LO tubules (Hasson *et al.,* 1999b; Srisuvan *et al.,* 2005).

4.2.3.3. Chronic phase of Taura syndrome

Shrimp in the chronic phase of 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 publication that incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle et al., 2003), two other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (Luo et al., 2004; Pantoja et al., 2004).

4.3.1.2.2. Antibody-based antigen detection methods

An MAb for detection of TSV may be used to assay samples of haemolymph, tissue homogenates, or Davidson's AFA-fixed tissue sections from shrimp (Erickson *et al.,* 2002; 2005; Poulos *et al.,* 1999). TSV MAb 1A1 may be used to distinguish some variants or 'strains' of TSV from other strains (Erickson *et al.,* 2002; 2005).

4.3.1.2.3. Bioassay method

Confirmation of 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, TS-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).

vi) Filter the diluted supernatant fluid from step v using a sterile syringe (size depends on the final volume of diluted supernatant) and a sterile 0.45 µm syringe filter. ~~Multiple filters may have to be used as they clog easily.~~ Filtrate should be collected in a sterile test tube or beaker. The solution can ~~now~~ be stored frozen ~~(recommend –20°C~~ at –20°C (or –80°C for ~~short-term [weeks] storage and –80°C for~~ a long-term ~~[months to years]~~ storage) or used immediately to inject indicator shrimp.

vii) Indicator shrimp should be from TSV-susceptible stocks of SPF *P. vannamei* (such as the 'Kona stock') (Moss *et al.,* 2001), which are commercially available from a number of sources in the Americas, and not from selected lines of known TSV-resistant stocks.

viii) Inject 0.01 ml per gram of body weight using a 1 ml tuberculin syringe. Indicator shrimp should be injected intramuscularly into the third tail segment. If the test shrimp begin to die within minutes post-injection, the inoculum contains excessive amounts of proteinaceous materials and should be further diluted prior to injecting additional indicator shrimp. Sudden death occurring post-injection is referred to as 'protein shock', and is the result of systemic clotting of the shrimp's haemolymph in response to the inoculum (Lightner, 1996a; White *et al.,* 2002).

ix) Haemolymph samples may be diluted (1/10 or 1/20 in TN buffer), filter sterilised (if necessary), and injected into the indicator shrimp without further preparation.

x) If TSV was present in the inoculum, the indicator shrimp should begin to die within 24–48 hours post-injection. Lower doses of virus may take longer to establish a lethal infection and shrimp should be monitored for at least 10–15 days post-injection.

xi) The presence (or absence) of TSV in the indicator shrimp should be confirmed by histological analysis (or ISH by gene probe, if available) of Davidson's fixed moribund shrimp. If additional confirmation is needed beyond demonstration of pathognomonic TSV lesions, RT-PCR with sequencing of the resulting amplicon can be carried out.

4.3.1.2.4. Sentinel shrimp bioassay method

As a variation to the bioassay technique, a 'sentinel shrimp' system may be used. For example, TSV-sensitive stocks of small juvenile SPF P. vannamei may be held in net-pens in tanks, or in the same water system, with other shrimp of unknown TSV status to bioassay for the presence of infectious agents such as TSV.

4.3.1.2.5. Dot-blot immunoassay method

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore~~, South San Francisco, California [CA], USA~~)[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.

vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semi-purified TSV. A negative reaction is one in which no coloured spot is visible in the well.

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

4.3.1.2.6. 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.~~

Primer	**Product**	**Sequence**	**Temperatue**	**~~G+C%~~**
9992F	231 bp	5'-AAG-TAG-ACA-GCC-GCG-CTT-3'	69°C	~~55%~~
9195R		5'-TCA-ATG-AGA-GCT-TGG-TCC-3'	~~63°C~~	~~50%~~
7171F	341 bp	5'-CGA-CAG-TTG-GAC-ATC-TAG-TG-3'	63°C	~~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).

Annex 25 (contd)

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 ~~10~~ 5 µl of total RNA extracted ~~from haemolymph, frozen shrimp tissues, ethanol fixed tissue~~ as the template ~~(concentration of RNA = 1–100 ng ml^{-1})~~.

iii) The following controls should be included in every RT-PCR assay for TSV: (a) known TSV-negative tissue sample; (b) a known TSV-positive sample (tissue or purified virus); and (c) a 'no-template' control.

iv) The ~~GeneAmp® EZ rTth RNA PCR kit (Applied Bioscience, Forster City, CA) was used~~ SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Life Technologies) can be used for all amplification reactions described here. ~~Alternative kits~~ Other commercially available equivalent reagent can also be used ~~and adjusted for use for this assay~~.

v) The optimised RT-PCR conditions (final concentrations in ~~50~~ 25 µl total volume) for detection of TSV in shrimp tissue samples are: ~~primers (0.62 µM each), dNTPs (300 µM each), rTth DNA polymerase (2.5 U 50 µl^{-1}), manganese acetate (2.5 mM), in 5 × EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2).~~

~~vi) If the thermal cycler does not have a heated lid, then light mineral oil (50 µl) is overlaid on the top of the 50 µl reaction mixtures to prevent condensation or evaporation during thermal cycling.~~

Reagent	Volume	Final concentration
dH_2O	5.5 µl	
2× Reaction Mix	12.5 µl	1×
Primer Forward/Reverse (10 M each)	1.0 µl	0.4 µM
RT/Taq enzyme Mix	1.0 µl	
RNA template*	5.0 µl	1–50 ng

vi) The RNA template and all the reagents are combined and reverse transcription is allowed to proceed at 60°C for 30 minutes, followed by ~~94°C for 2 minutes~~ 95°C for 2 minutes. At the completion of reverse transcription, the samples are amplified for 39 cycles under the following conditions: denaturation at 95°C for 45 seconds, and then annealing/extension at 62°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle. in a 4°C soak file.

~~Note: The reaction conditions described here were optimised using an automatic Thermal Cycler GeneAmp 980 (Applied Biosystems). The conditions should be optimised for each thermal cycler using known positive controls.~~

vii) A 6 µl of the ~~completion of reverse transcription, the samples are~~ amplified ~~for 40 cycles under the following conditions: denaturation at 94°C for 45 seconds, and then annealing/extension at 60°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle and the process is terminated in a 4°C soak file.~~

~~ix) Following the termination of RT-PCR, the amplified cDNA solutions are drawn off from beneath the mineral oil and placed into clean 0.5 ml microfuge tubes.~~

~~x) A 10 µl sample of the amplified~~ products can then be added to the well of a ~~2.0~~ 1.5% agarose gel, stained with ethidium bromide (0.5 g ml^{-1}), and electrophoresed in 0.5 × TBE (Tris, boric acid, ethylene diamine tetra-acetic acid [EDTA]).

~~xi) A 1 kb DNA ladder (Invitrogen, Carlsbad, CA) is used as a marker.~~

~~xiii) Details of the composition of the reagents and buffers used here may be found in Chapter 2.2.2 IHHN.~~

4.3.1.2.7.3. Real-time RT-PCR 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 ream-time RT-PCR is ~100 copies of the target sequence from the TSV genome (Dahr *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 AFAF277675) that encodes for nonstructural proteins. The primers and TaqMan probe were designed by the Primer Express software (~~Applied Biosystems~~ Life Technologies). The upstream (TSV1004F) and downstream (TSV1075R) primer sequences are: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3' and 5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'), respectively. The TaqMan probe, TSV-P1 (5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3'), which corresponds to the region from nucleotide 1024 to 1051, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end ~~(Applied Biosystems, catalog no. 450025)~~.

ii) Preparation of RNA template: the extraction and purification of RNA template from haemolymph, or shrimp tissue, is the same as that described in the section for ~~traditional~~ conventional RT-PCR.

iii) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of contaminants in the reaction mixture ~~or in the heat block of the thermal cycler~~. A positive control should also be included, and this can be an *in-vitro* transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV-infected tissue.

iv) The 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 osystems; ABI PRISM 7000, 7300, 7500, or newer models~~ Life Technologies or equivalent ~~thermocycler~~ real-time PCR systems). The cycling consists of reverse transcription at ~~48~~ 50°C for 30 minutes and initial denaturation at 95°C for ~~10 minutes~~ 20 seconds, followed by 40 cycles of denaturation at 95°C for ~~15~~ 3 seconds and annealing/extension at 60°C for ~~1 minute. The levels of fluorescence are measured at the end of each annealing/extension cycle~~ 30 seconds.

vi) At the end of the reaction, real-time fluorescence measurements are analysed. A threshold will be set to be above the baseline ~~that begins to detect the increase in signal associated with an exponential increase in PCR product~~. Samples will be defined as negative if there is no Ct (threshold cycle) value after 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive.

4.3.1.2.7.4. Sequencing

RT-PCR products may be cloned and sequenced when necessary to confirm infection by TSV or to identify false positives or nonspecific amplification (Mari *et al.,* 2002; Nielsen *et al.,* 2005; Srisuvan *et al.,* 2005; Tang & Lightner, 2005; Wertheim *et al.,* 2009).

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.

Annex 25 (contd)

4.3.2. Serological methods

Not applicable because shrimp are invertebrate animals which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to TSV.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of TSV are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended 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. *TSV surveillance, detection and diagnostic methods in penaeids*

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	c
Bioassay	d	d	d	d	c	b
Direct LM	d	d	c	d	c	d
Histopathology	d	b	b	c	a	a
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	c	c	b	b
In-situ DNA probes	d	c	b	b	a	a
RT-PCR, 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-transcriptase polymerase chain reaction.

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

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.7.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic TSV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with TSV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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).~~
- ~~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 Taura syndrome
(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 Taura syndrome

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

Annex 26

CHAPTER 2.2.8.

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

EU comment

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

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*.~~

EU comment

Please put the word "Nodaviridae" in italics (taxonomy).

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; 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 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.~~

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 (*Penaeus 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*).

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

Larvae, PL and early juveniles are susceptible, whereas adults are resistant ~~and act as carriers~~ (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.2.4~~3~~. 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).

2.2.5~~4~~. 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).

2.2.6~~5~~. 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).

2.2.7~~6~~. Vectors

Not known. ~~Penaeid shrimp (*Penaeus indicus, P. monodon, P. japonicus*) (Sudhakaran *et al.*, 2006b), *Artemia* (Sudhakaran *et al.*, 2006c), and aquatic insects (*Belostoma* sp., *Aesohna* sp., *Cybister* sp., and *Notonecta* sp.) are vectors of WTD (Sudhakaran *et al.*, 2008).~~

~~**2.2.8. Known or suspected wild aquatic animal carriers**~~

~~Not known.~~

2.3. Disease pattern

A high prevalence of infection with 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).

2.3.2. Prevalence

Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems, as well as in experimental infection by immersion challenge, and 100% mortality has been reported 5–7 days after

the appearance of the first gross signs in PL in natural or experimental infection (Arcier *et al.,* 1999; Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; b).

2.3.3. Geographical distribution

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), 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).

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). 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). 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) 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 crusteaceans).

3.3. Pooling of samples

Samples, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. Larger prawns 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/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 (moults) in the tanks appear abnormal and resemble 'mica flakes' (Arcier *et al.,* 1999). The infected PL show progressive weakening of their feeding and swimming ability (Sahul Hameed *et al.,* 2004a).

4.2. Clinical methods

4.2.1. Gross pathology

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 para-spherical virus particles of different sizes within the cytoplasm of connective cells and muscle cells. Large viral particles are five- to six-sided, with a diameter of 26–27 nm, and would be characteristic of 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 26 (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) 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). 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, H_2O_2 at a 0.33% final concentration).

vii) Stop the reaction after 15 minutes by adding 25 µl of H_2SO_4 to each well.

viii) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.2.2. TAS-ELISA protocol (Qian et al., 2006)

i) Coat ELISA plates with rabbit polyclonal antibody raised against 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, H_2O_2 at a 0.33% final concentration).

viii) Stop the reaction after 15 minutes by adding 25 µl H_2SO_4 to each well.

ix) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.3. Molecular techniques

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

The protocol for the RT-PCR for detection of 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 (nRT-PCR) is also available and recommended for screening broodstock and seed (Sudhakaran *et al.,* 2006a).

Total RNA extraction

i) Collect 50 mg of PL or 100 mg of an organ piece (gill tissue, abdominal muscle, tail muscle or pleopods) from adult prawns and homogenate in 300 µl TN buffer (20 mM Tris/HCl, 0.4 M NaCl, pH 7.4).

ii) Centrifuge the homogenate at 12,000 ***g*** for 15 minutes at room temperature and collect the supernatant.

iii) Take 150 µl of supernatant and add 1 ml TRIzol. Mix thoroughly and incubate for 5 minutes at room temperature.

iv) After 5 minutes, add 200 µl chloroform to the sample, mix well and centrifuge at 12,000 ***g*** for 15 minutes at room temperature.

v) Collect the aqueous phase and transfer to a fresh tube, and precipitate RNA by mixing with 500 µl isopropanol.

vi) Incubate the sample for 10 minutes at room temperature and centrifuge at 12,000 ***g*** for 10 minutes at 4°C.

vii) Dissolve the RNA pellet in 50 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA [ethylene diamine tetra-acetic acid], pH 7.5) after a wash with 75% ethyl alcohol.

viii) Quantify the RNA by measuring the absorbance at 260 nm using UV spectrophotometer and check the purity by measuring the ratio of OD_{260nm}/OD_{280nm}.

Annex 26 (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 nRT-PCR protocol described by Sudhakaran *et al.* (2006a). This test can be used for screening healthy PL, juveniles and broodstock for viruses. The third protocol is a multiplex RT-PCR procedure adapted from Yoganandhan *et al.* (2005). It can be used for the simultaneous detection of 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):

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 nRT-PCR is more sensitive and useful for screening seed and broodstock (Sudhakaran *et al.,* 2006a):

For the nRT-PCR, the first step of the RT-PCR, as described in protocol 1, should be performed with external primers and the nPCR should be carried out using an RT-PCR product as a template. For nRT-PCR, add 2 ml RT-PCR product to a PCR tube containing 20 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 20 pmol of each internal primer, 1.25 units of heat-stable DNA polymerase). The nRT-PCR protocol for both viruses comprise an initial 95°C for 10 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final extension at 72°C for 5 minutes. Analyse the nRT-PCR products by electrophoresis on a 1% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

If the viral load is sufficiently high, a 425 bp DNA will be amplified for MrNV and 546 bp DNA 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 nRT-PCR is ~1000-fold greater than the one-step RT-PCR.

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 ml 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 for MrNV and 500 bp DNA 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 $MgCl_2$ and 1 µl dye solution).

iv) The PCR programme consists of initial *Taq* polymerase activation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 5 seconds at 60°C and 10 seconds at 72°C. Melting temperatures will be measured by returning to 70°C for 30 seconds and gradual heating to 95°C in 10 minutes. The negative control reactions should contain water in place of cDNA template in each run to ensure the absence of viruses.

v) The number of viral cDNA copies in the sample will be determined using Light Cycler fit point method.

Annex 26 (contd)

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 $MgCl_2$).

xiii) Develop the reaction by incubating the slides in buffer V containing NBT and BCIP in a dark and humid chamber for a minimum of 2 hours or overnight. Stop the reaction by incubating the slides in buffer III 2× for 15 minutes.

xiv) Counterstain the slides with 1% Brown Bismarck, mount with a cover-slip and examine with a bright field microscope.

xv) Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

4.3.1.2.3.3. Loop-mediated isothermal amplification (*Haridas* et al., *2010; Pillai* et al., *2006; Puthawibool* et al., *2010*)

Haridas *et al*. (2010) and Pillai *et al.* (2006) have applied loop-mediated isothermal amplification (LAMP) for rapid diagnosis of 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.

i) Extraction of total RNA from the samples as per the procedure mentioned above.

ii) Carry out the RT-LAMP reaction in the reaction mixture (2 µM each of inner primers FIP and BIP, 0.2 µM each of outer primers F3 and B3, 1400 µM of dNTP mix, 0.6 M betaine, 6 mM $MgSO_4$, 8 U of Bst DNA polymerase along with 1× of the supplied buffer, 0.125 U of AMV RTase and the specified amount of template RNA in a final volume of 25 µl) at 55, 60, 63 and 65°C for 1 each, followed by heat inactivation at 80°C for 2 minutes to terminate the reaction. Uninfected samples and reaction mix without template serve as the negative controls.

iii) Analyse the LAMP products by electrophoresis on a 2% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

iv) Without use of agarose electrophoresis, amplification of DNA can be detected by addition 1.0 µl of 10^{-1} diluted SYBR Green to the reaction mixture and observe the colour change.

4.3.1.2.3.4. Sequencing

For confirmation of suspected new hosts of 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 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 26 (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
***In-situ* DNA probes**	c	b	b	c	a	a
PCR	a	a	a	a	a	a
Sequence	d	d	d	a	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

The method for targeted surveillance to declare freedom from infection with MrNV ~~WTD~~ is nRT-PCR.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with MrNV is considered to be confirmed if two or more 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.

~~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).

~~Suspect cases should first be checked by RT-PCR and confirmed by nRT-PCR, sequencing, TEM and DNA probes.~~

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* *

NB: There is an OIE Reference Laboratory for 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 White tail disease

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

Annex 27

CHAPTER 2.2.7.

INFECTION WITH WHITE SPOT SYNDROME VIRUS ~~DISEASE~~

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

~~For the purpose of this chapter,~~ Infection with white spot ~~disease (WSD) is considered to be infection with~~ white spot syndrome virus ~~(WSSV)~~ means infection with with the pathogenic agent white spot syndrome virus (WSSV), Family *Nimaviridae*, Genus *Whispovirus*.

[. . .]

Annex 28

Revised assessment for listing of *Batrachochytrium salamandrivorans* in the *Aquatic Code*

Overall Assessment

The Aquatic Animal Health Standards Commission assessed *Batrachochytrium salamandrivorans* (*Bsal*) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code*, and agreed that *Bsal* meets the OIE criteria for listing, notably A. Consequences: negative impact on wild amphibian populations, B. Spread: proven infectious aetiology, and high likelihood of spread via international trade, and zones free of the pathogen, and C. Diagnosis: availability of a robust diagnostic test (see Table 1 below).

Table 1. Summary of assessment of *Bsal*

	Listing criteria								Conclusion
	1	2	3	4	5	6	7	8	
Batrachochytrium salamandrivorans	NA	+	NA	+	NA	+	+	+	List

NA = not applicable.

Background

It is well recognised that amphibian populations are in crisis across the globe due to a variety of factors, amongst them diseases. *Batrachochytrium dendrobatidis* (*Bd*), a fungal infection, emerged as an important pathogen of amphibians in recent years and has resulted in declines of more than 200 amphibian populations and reductions in excess of 40% of amphibian species in Central America, and losses in Europe, Australia and North America (Fisher *et al*., 2012). *Bd* was added to the OIE list of diseases in 2008.

A rapid decline of free-living fire salmanders (*Salamandra salamandra*) in The Netherlands was reported in 2013 (Spitzen-van der Sluijs *et al.,* 2013). Initial investigations failed to identify a clear cause but subsequent investigations into the mortality of captive salamanders identified a new species of chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*) (Martel *et al.*, 2013). Martel *et al.* (2014) 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

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) provides very solid evidence that *Bsal* is both a necessary and sufficient cause of disease in fire salamanders in the Netherlands. *Bsal* was isolated from the skin of fire salamanders in affected populations in Bunderos (the Netherlands). Analysis demonstrated that the *Bsal* is a novel chytrid fungus in a clade with *Bd*. Infected animals show severe pathology (multifocal erosions and ulcerations) and die within 7 days. Field observations and experimental studies indicate that case fatality approaches 100%. Between 2010 and 2013 the fire salamander in affected populations in the Netherlands was reduced by 96%.

Annex 28 (contd)

Experimental challenge studies have demonstrated that 41 of 44 Western Paleartic salamander species are susceptible to *Bsal*, and it is lethal to at least some New World salmandrid species (Martel *et al.*, 2014). Thus the disease has the potential to negatively impact many amphibian populations. Yap *et al*. (2015) have modelled the likely impact of *Bsal* in North America and concluded that it is a serious threat to biodiversity there.

Conclusion: *the criterion is satisfied*

OR

Criterion No. *3. The agent is of public health concern.*

***Conclusion*:** Criteria is not applicable

AND

B. Spread

Criterion No. 4. *Infectious aetiology of the disease is proven*

Assessment:

Bsal was isolated from the skin of affected salamanders (Martel *et al*., 2013). Extensive screening was undertaken but no other pathogens were detected. By microscopy, high numbers of colonial thalli were observed. Transmission electron microscopic examination of skin lesions of clinically affected animals demonstrated presence of the pathogen (intracellular structures consistent with colonial thalli) (Martel *et al.*, 2013). The infectious aetiology and role of *Bsal* is further proven by samples from declining and stable populations of fire salamanders (Martel *et al.*, 2013). Thirteen of 33 swabs from live fire salmanders from declining populations tested positive for *Bsal* by PCR, in contrast to 0 of 51 swabs from a stable population.

Transmission studies provided further evidence of the infectious aetiology of the disease. Five salamanders were exposed to *Bsal* zoospores (Martel *et al*., 2013); all animals died. The pathogen was re-isolated from one animal and confirmed by PCR on all five.

Conclusion: *the criterion is satisfied*

OR

Criterion No. *5. An infectious agent is strongly associated with the disease, but the aetiology is not yet known.*

***Conclusion*:** Criteria is not applicable

AND

B. Spread

Criterion No. 6. *Likelihood of international spread, including via live aquatic animals, their products or fomites*

Assessment:

Martel *et al.* (2014) speculated that *Bsal* originated in Asia and spread to Europe via the international salamander pet trade; and identified three actively traded Asian salamander species as reservoirs for *Bsal* (*Cynops cyanurus*, *Cynops pyrrhogaster*, and *Paramesotriton deloustali*) (Martel *et al.*, 2015). The identification of *Bsal* in a collection of amphibians imported to the UK (Cunningham *et al*., 2015) demonstrated transboundary spread via movement of live animals. Skin samples from 1765 amphibians from pet shops, Heathrow Airport and an exporter in Hong Kong yielded 3 positive samples (2 of which were imported into Europe in 2010) (Martel *et al*., 2014). An analysis of the pet salamander trade by Yap *et al.* (2015) concluded that it presents a high risk of *Bsal* introduction to N. America.

Conclusion: *the criterion is satisfied*

AND

Criterion No. 7. ***Several countries or countries with zones may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4.***

Assessment:

Bsal was first described in 2013 and thus there has been limited opportunity to complete surveillance to evidence freedom or put in place sanitary measures to prevent introduction. *Bd* surveillance has been based on a *Bd*-specific qPCR, and cannot be used to assess the current worldwide distribution of *Bsal*. However, a *Bsal* specific PCR was developed by Martel *et al.* (2013) and has been used to screen over 500 wild amphibians from four continents (Martel *et al.,* 2014). Positive results were obtained from SE Asia and, the Netherlands and Belgium (where the pathogen was associated with disease). Two studies in North America found no evidence of *Bsal* in wild salamanders (Bales *et al.,* 2015; Muletz *et al.,* 2014). Yap *et al.* (2015) also consider that North America is free but at risk of *Bsal* introduction. 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*

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-gelation hydrolactose-lactose (TGhL) 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 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. 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 specificity. The limit of detection was determined to be 0.1 genomic equivalent (GS) of zoospores.

The duplex real-time PCR has been sufficiently validated 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.

Annex 28 (contd)

Conclusion: *the criterion is satisfied.*

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Annex 29

Assessment for listing Tilapia lake virus in the *Aquatic Code*

Overall Assessment

The Aquatic Animal Health Standards Commission assessed Tilapia lake virus (TiLV) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code* (see Table 1 below).

Table 1. Summary of assessment of TiLV

	Listing criteria								Conclusion
	1	2	3	4	5	6	7	8	
Tilapia lake virus	+	+	NA	+	NA	+	+	-	do not list

NA = not applicable.

Background

A novel orthomyxo-like virus, named as Tilapia lake virus (TiLV), has been identified as the cause of mass die-offs of tilapia (Eyngor *et al.*, 2014) in both farms and the wild environment. The host range is not well known but a number of tilapines are known to be susceptible (Eyngor *et al.*, 2014). Tilapia is the second most import group of farmed fish after carps. Global production of tilapia, predominantly *Oreochromis niloticus*, is estimated at 4.5 million metric tonnes (FAO data). Farming occurs primarily in tropical and subtropical countries though some production in recirculation systems has started in other regions. *O niloticus* was first introduced to developing countries to support subsistence farming. However, larger scale commercial production is now important and frozen fillet and other tilapia products are traded globally.

Criteria

No		Criteria for listing	*Tilapia lake virus*
A Consequences			
1		The disease has been shown to cause significant production losses at a national or multinational (zonal or regional) level.	Very high levels of mortality (>80%) have been observed in affected populations (both farmed and wild) *(Bacharach et al., 2016; Ferguson et al*., 2014, Gophen *et al.*, 2015). Decreases of catch of tilapines, specifically *Sarotherodon (Tilapia) galilaeus*, from the Sea of Galilee have been observed since 2007. Since 2009 episodic losses of tilapia (*Oreochromis niloticus)* were recorded in fish farms all over Israel (Eyngor *et al*., 2014). Mortality in farmed *O. niloticus* in Ecuador have also been attributed to TiLV (Ferguson *et al*., 2014). Losses are significant regionally and at a national level. **Criteria met**
2	Or	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.	The virus impacts wild populations but causes a lower level of mortality compared to the impact in farms (Eyngor *et al*., 2014). **Criteria met**
3	Or	The agent is of public health concern.	Not applicable

Annex 29 (contd)

And B spread			
4	Or	Infectious aetiology of the disease is proven	A virus has been cultured from affected fish and the genome has been characterised and classified as a novel orthomyxovirus (*Eyngor et al.*, 2014). In-situ hybridisation indicates presence of the agent in association with lesions (Eyngor *et al.*, 2014). Cohabitation of infected and naïve fish demonstrated waterborne transmission between fish with the latter developing a lethal disease (mortality was similar to levels achieved by lethal injection) (Eyngor *et al.*, 2014). **Criteria met**
5		An infectious agent is strongly associated with the disease, but the aetiology is not yet known.	Not applicable
And B spread			
6	And	Likelihood of international spread, including via live aquatic animals, their products or fomites.	The virus has been isolated from affected tilapia in both Israel and Ecuador and, despite geographic separation, strains were highly homologous, suggesting an epidemiological link and international spread. Historically, tilapia have been traded internationally to establish populations for production in new regions. The current driver for international trade is the dissemination of improved genetic strains (though current pattern and volume of trade has not been determined for this assessment). Tilapia products are traded internationally and while a risk of transmission with some product types should be expected, specific risks have not been considered in this assessment. **Criteria met**
7	And	Several countries or countries with zones may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4	Currently, the virus has only been identified in Israel and Ecuador. Mortalities events associated with this virus have not been reported from other regions, e.g. in Zambia (Bwalya1 *et al.*, 2016). The distribution of the virus may be wider (mortality may not have been investigated in other regions); however, due to the broad distribution of tilapia (Asia, Africa and South America) and virulence of the virus, it is almost certain that many countries are currently free. **Criteria met**

And C Diagnosis			
8		A repeatable and robust means of detection/diagnosis exists.	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). A PCR primer set has been designed. However, it is not known whether these primers will detect all strains of the virus (Eyngor *et al*., 2014). **Criteria not met**

Conclusion

TiLV clearly meets criteria with respect to impact (criteria 1 and 2) and infectious aetiology (criteria 4). Routes for international spread undoubtedly exist (criteria 6). Whilst surveillance has not been undertaken, it is almost certain that a considerable proportion of global tilapia production is currently free of the virus (criteria 7). The virus can be cultured but antibody-based tests or nucleic acid-based tests to confirm the identification have not yet been validated sufficiently. A primer set is available but it is not clear whether all strains of the virus can be detected hence confirmation requires genetic sequencing. We cannot conclude that a repeatable robust means of diagnosis (criteria 8) is currently available.

Definition of suspect case

High levels of mortality in tilapine species, associated with ocular alterations (opacity of the lens or more severe pathology). Skin erosions, haemorrhages in the leptomeninges and moderate congestion of the spleen and kidney may be observed on post-mortem.

Definition of confirmed case

Cell culture of the virus on E-II or primary tilapia brain cells followed by virus identification by genetic sequencing.

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Organisation Mondiale de la Santé Animale | World Organisation for Animal Health | Organización Mundial de Sanidad Animal

Annex 30

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REPORT OF THE MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 1–3 June 2016

The OIE *ad hoc* Group on Susceptibility of Crustacean Species to Infection with OIE Listed Diseases (the *ad hoc* Group) met at OIE Headquarters on 1–3 June 2016.

The members of the *ad hoc* Group, the adopted agenda and the Terms of Reference are presented at Annex 1, Annex 2 and Annex 3 respectively.

Dr Gillian Mylrea, Deputy Head of the OIE International Trade Department, welcomed members and thanked them for their willingness to work on this important topic. Dr Mylrea informed members that recommendations from their second meeting in October 2015 regarding the list of susceptible species for seven of the OIE listed crustacean diseases (acute hepatopancreatic necrosis disease; crayfish plague; infectious hypodermal and haematopoietic necrosis; infectious myonecrosis; necrotising hepatopancreatitis; Taura syndrome; and white tail disease) had been considered by the OIE Aquatic Animal Health Standards Commission (Aquatic Animals Commission) at their February 2016 meeting. The Commission had amended the disease-specific chapters for these diseases in the OIE *Aquatic Animal Health Code (Aquatic Code)* and the OIE *Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual)* in line with *ad hoc* group recommendations and had circulated these for Member Countries' comments in their February 2016 report.

The chair of the *ad hoc* Group, Dr Grant Stentiford, thanked the members for all their continued support and for participation in the third meeting of the *ad hoc* Group. Dr Stentiford clarified that the purpose of this meeting was to review the literature and develop a list of susceptible species for white spot syndrome virus for inclusion in the relevant chapters of the OIE *Aquatic Code* and *Aquatic Manual*.

The *ad hoc* Group applied the three-stage approach, outlined in Article 1.5.3. in Chapter 1.5. of the *Aquatic Code*, to assess susceptibility of a species to infection with white spot syndrome virus (WSSV).

The "Criteria for listing species as susceptible to infection with a specific pathogen" as described in Chapter 1.5. of the *Aquatic Code* are as follows:

1) criteria to determine whether the route of transmission is consistent with natural pathways for the infection (as described in Article 1.5.4.);

2) criteria to determine whether the pathogenic agent has been adequately identified (as described in Article 1.5.5.);

3) criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection (as described in Article 1.5.6.).

Hosts that were classified as susceptible species (as described in Article 1.5.7.) were proposed for inclusion in Article 9.7.2. of Chapter 9.7. of the *Aquatic Code* entitled White spot disease.

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Hosts that were classified as species for which there is incomplete evidence for susceptibility (as described in Article 1.5.8.) were proposed for inclusion in a new Section 2.2.2. *Species with incomplete evidence for susceptibility* of Chapter 2.2.7. of the *Aquatic Manual* entitled White spot disease.

In addition, organisms producing pathogen-specific positive PCR results (without confirmation of an active infection) were identified and listed in a new sub point 2.2.2.2. of the *Aquatic Manual* chapter.

The assessment for infection with WSSV conducted by the *ad hoc* Group is provided in Annex 4.

The *ad hoc* Group wished to note the following:

1) In many of the older publications accurate pathogen identification was not carried out because molecular typing techniques were not available at the time. This is particularly so for many of the original studies in Penaeidae. Therefore, for many of these cases, a weight of evidence approach using combined data from relevant studies was used to assess susceptibility.

2) Species categorised as '2' (i.e. species for which there is incomplete evidence for susceptibility (those species for which criteria A-D were not fully met) includes a wide range of species, from those for which susceptibility to disease is low (e.g. reservoir species) through to those that do not meet category 1 because there is insufficient data available.

3) The *ad hoc* Group worked on the assumption that authors had correctly identified the host species on which they were reporting.

The *ad hoc* Group made the following recommendations:

1) That species categorised as '3' (i.e. species for which only PCR based survey results are available) be listed in a new section in the relevant chapter of the *Aquatic Manual* to more clearly differentiate '2s' and '3s' because studies that only detected the nucleic acid of the pathogen (e.g. by PCR) cannot be used as evidence of infection. However, they are important to include because they provide some indication of the presence of the target pathogen in the host or the environment.

   The *ad hoc* Group suggested this approach be included in the relevant *Aquatic Manual* chapter as a new point 2.2.2.2. as shown below:

   > "2.2.2.2. Pathogen-specific positive PCR results (without confirmation of an active infection) have been reported in the following organisms: species X, Y and Z."

2) The following amendment be made to Chapter 1.5. *Criteria for listing species as susceptible to infection with a specific pathogen* to improve the applicability of this criterion:

   > In point A of Article 1.5.6. add the words "(and for viruses in host cells)" to clarify that the pathogen of interest is replicating in host cells and not potentially in symbionts:
   >
   > i.e. "A. the *pathogenic agent* is multiplying in the host (and for viruses in host cells), or developing stages of the *pathogenic agent* are present in or on the host;"

3) The words 'in host cells' be added to criteria A for Table 1. *Criteria for susceptibility to infection with pathogen X* in all Tables developed by this *ad hoc* Group at their October 2015 meeting for: TSV, YHV, IMNV, IHHNV, MrNV and NHP to read as: "A: Replication in host cells".

4) Section 7 of crustacean chapters of the *Aquatic Manual* be amended to take account of the requirement for accurate systematics of the pathogen. At present it confuses confirmation of a case with identification of the pathogen of concern.

_____

…/Annexes

Annex 30 (contd)

Annex 1

MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 1–3 June 2016

List of participants

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Annex 2

MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 1–3 June 2016

Adopted agenda

1. Conduct an assessment for species susceptibility as described in Chapter 1.5. of the *Aquatic Code* entitled White spot disease (Chapter 9.7.).

2. Draft a report to be considered by the Aquatic Animals Commission at their September 2016 meeting.

Annex 30 (contd)

Annex 3

MEETING OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 1–3 June 2016

Terms of Reference

Background

A new Chapter 1.5. *Criteria for listing species as susceptible to infection with a specific pathogen* was introduced into the 2014 edition of the *Aquatic Code*. The purpose of this chapter is to provide criteria for determining which host species are listed as susceptible in Article X.X.2. of each disease-specific chapter in the *Aquatic Code.* The criteria are to be applied progressively to each disease-specific chapter in the *Aquatic Code*.

This *ad hoc* Group on Susceptibility of Crustacean Species to Infection with OIE Listed Diseases has undertaken assessments on susceptibility of crustacean species for eight of the OIE listed crustacean diseases: acute hepatopancreatic necrosis disease; crayfish plague *(Aphanomyces astaci)*; infection with yellow head virus genotype 1; infectious hypodermal and haematopoietic necrosis; infectious myonecrosis; necrotising hepatopancreatitis; Taura syndrome; and white tail disease.

The assessments have been reviewed by the Aquatic Animals Commission to amend in the list of susceptible species in Article X.X.2. of the disease-specific chapters in the *Aquatic Code.*

In addition, for species where there is some evidence of susceptibility but insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3., information has been proposed to be included in the relevant disease-specific chapter in the *Aquatic Manual*.

Terms of Reference

1) Consider standards of evidence required to satisfy the criteria in Chapter 1.5.

2) Review relevant literature documenting susceptibility of species.

3) Propose susceptible species for infection with white spot syndrome virus (WSSV) based on Article 1.5.7.

4) Propose susceptible species for infection with WSSV based on Article 1.5.8.

Expected outputs

1) Develop a list of susceptible species for inclusion in the relevant articles of the chapter in the *Aquatic Code* and *Manual* for white spot disease.

2) Draft a report for consideration by the Aquatic Animals Commission at their September 2016 meeting.

Annex 30 (contd)

Annex 4

ASSESSMENT OF HOST SUSCEPTIBILITY TO INFECTION WITH WHITE SPOT SYNDROME VIRUS (WSSV)

The objectives of this assessment were to: (1) determine susceptibility of given host taxa to infection with white spot syndrome virus (WSSV) by applying the three-stage approach for as described in Article 1.5.3. of the *Aquatic Code* and (2) to provide the OIE with recommendations regarding revision of the relevant sections of the *Aquatic Code* and the *Aquatic Manual* with respect to host species susceptibility.

The *ad hoc* Group based pathogen identification on Section 7

Criteria for susceptibility to infection with WSSV are detailed in Table 1 (as per Article 1.5.6. of the *Aquatic Code*). This table includes Replication in host cells (A), Viability/Infectivity (B), Pathology/Clinical Signs (C) and Location (D).

Hosts were considered to be infected with WSSV if they fulfilled either criterion A, or at least two of criteria B, C and D (as per point 3 of Article 1.5.7. of the *Aquatic Code*).

Table 1. Criteria for susceptibility to infection with WSSV

A: Replication in host cells	B: Viability/Infectivity	C: Pathology/Clinical signs	D: Location
Presence of characteristic inclusion bodies and ideally positive labelling of inclusion bodies by ISH or IFAT; OR Presence of virions in inclusion bodies by TEM; OR Demonstration of increasing copy number over time with qPCR with confirmatory PCR/ sequencing specific for infectious virus; OR Serial passage from individual to SPF individual of the same species*.	Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**.	Inclusions (eosinophilic to basophilic) within nuclei of cells in target organs and tissues. Host nuclei hypertrophic with marginated chromatin with/without the presence of clinical signs (e.g. white spots on cuticle, moribund, lethargic)***.	Cells of tissues and organs of ectodermic and mesodermic origin. Target sites include cuticular epithelium (gills, pleopods, appendages), connective tissues, the haematopoietic tissues, the antennal gland and lymphoid organ****.

Key:

* To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed.

** To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required.

*** Clinical signs accordiner 2.2.7. of the *Aquatic Manual* may not present equally in all host taxa and are not specific for infection with WSSV.

**** Lymphoid organ not present in most non-penaeid host taxa. For non-crustacean host taxa other organs and tissues may show evidence of infection with WSSV.

Annex 30 (contd)

Annex 4 (contd)

The assessment for host susceptibility to infection with WSSV is provided in Table 2.

Table 2. Outcome of assessment for host susceptibility to infection with WSSV

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Alpheidae	*Alpheus*	*brevicristatus*	nd	nest PCR	No	No	No	No	2	63
Alpheidae	*Alpheus*	*brevicristatus*	I	nest PCR/dot blot/ ISH	Yes	Yes	Yes	Yes	2	63, 76
Alpheidae	*Alpheus*	*lobidens*	nd	nest PCR	No	No	No	No	3	63
Ameiridae	*Nitocra*	*sp.*	E (per os)	PCR	No	No	No	No	3	74
Artemiidae	*Artemia*	*salina*	nd	nest PCR	No	No	No	No	3	49
Artemiidae	*Artemia*	sp.	N/E (bath)	dot blot/ISH	No	No	No	No	3	76
Astacidae	*Astacus*	*astacus*	E (per os)/I	nest PCR	No	No	No	No	3	33
Astacidae	*Astacus*	*leptodactylus*	E (per os)	ISH/TEM/dot blot	Yes	No	Yes	Yes	1	12
Astacidae	*Austropotamobius*	*pallipes*	E (per os)/I	PCR/sequencing	Yes	Yes	Yes	Yes	1	2
Astacidae	*Pacifastacus*	*leniusculus*	E (per os)	PCR/sequencing	Yes	Yes	Yes	Yes	1	2
Balanidae	*Balanus*	sp.	N/E (bath)/I	PCR/sequencing/dot blot/ISH	No Yes	No Yes	No Yes	No	3	55, 76
Calanidae	*Calanus*	*pacificus californicus*	E (per os)	RT-qPCR of VP28 transcripts	Yes	No	No	No	1	46
Calappidae	*Calappa*	*lophos*	N/E (per os/bath)	PCR	No	No	No	No	3	66
Calappidae	*Calappa*	*philarigus*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Callianassidae	*Callianassa*	*harmandi*	I	dot blot/ISH	Yes	Yes	Yes	Yes	2	76
Cambaridae	*Orconectes*	*limosus*	E (per os)/I	TEM/dot blot	Yes	No	Yes	Yes	1	12
Cambaridae	*Orconectes*	*punctimanus*	N	PCR/probe	No	No	No	No	3	42
Cambaridae	*Procambarus*	*clarkii*	N/E (per os)/I	PCR/ISH/dot blot	Yes	No Yes	Yes	Yes	1	3, 6, 18, 31, 66, 69, 76

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Cambaridae	*Procambarus*	*zonangulus*	N	PCR/sequencing	Yes	No	Yes	Yes	1	3
Carcinidae	*Carcinus*	*maenas*	E (per os)/I	PCR	Yes	Yes	Yes	Yes	2	2, 12
Cancridae	*Cancer*	*pagurus*	E (per os)/I	ISH/TEM/dot blot	Yes	Yes	Yes	Yes	1	2, 12
Coleoptera (Ephydridae)			N	PCR	No	No	No	No	3	41
Crangonidae	*Crangon*	*affinis*	E (bath)	PCR/monoclonal antibody	No	No	Yes	No	3	26
Cyclopidae	*Apocyclops*	*royi*	E (bath)	PCR/sequencing	Yes	No	No	No	3	8
Decapoda (order)	*Paratelphusa*	*hydrodomous*	E (per os)/I	PCR	Yes	Yes	Yes	Yes	2	52, 57
Decapoda (order)	*Paratelphusa (Barytelphusa)*	*pulvinata*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	57
Diogenidae	*Diogenes*	*nitidimanus*	I	PCR	No	No	No	No	3	9
Dorippidae	*Paradorippe*	*granulata*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Epialtidae	*Doclea*	*muricata (=hybrida)*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Ergasilidae	*Ergasilus*	*manicatus*	E (bath)	qPCR–no sequence	Yes	No	No	No	2	50
Galenidae	*Halimede*	*ochtodes*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Grapsidae	*Grapsus*	*albolineatus*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Grapsidae	*Metopograpsus*	sp.	E (per os)	EM in *P. vannamei*. No PCR or sequence	Yes	Yes	Yes	Yes	2	54
Grapsidae	*Metopograpsus*	*messor*	N	PCR	No	No	No	No	3	29
Grapsidae	*Hemigrapsus*	*sanguineus*	I	dot blot/ISH	Yes	Yes	Yes	Yes	2	76
Leucosiidae	*Philyra*	*syndactyla*	E (per os)	PCR	Yes	No	Yes	Yes	2	58
Lithodidae	*Lithodes*	*maja*	E (per os)	PCR	Yes	No	Yes	Yes	2	58
Macrophthalmidae	*Macrophthalmus*	*sulcatus*	N	PCR	No	No	No	No	3	29

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Matutidae	*Ashtoret*	*miersii*	E (per os)	PCR	Yes	No	Yes	Yes	2	58
Matutidae	*Matuta*	*planipes*	N	PCR	No	No	No	No	3	49
Menippidae	*Menippe*	*rumphii*	E (per os)	PCR	No	No	No	No	3	58
Nephropidae	*Homarus*	*gammarus*	E (per os)/I	PCR/sequencing	Yes	Yes	Yes	Yes	1	1, 2
Nephropidae	*Nephrops*	*norvegicus*	E (per os)/I	PCR/sequencing	Yes	Yes	Yes	Yes	1	2
Nereididae	*Dendronereis*	sp.	N	PCR/sequencing	Yes	No	Yes	No	1	15, 16, 28
Ocypodidae	*Macrophthalmus*	*japonicus*	N	dot blot/ISH	Yes	No	Yes	Yes	2	76
Ocypodidae	*Uca (=Gelasimus)*	*vocans (=marionis nitidus)*	N	PCR	No	No	No	No	3	29
Ocypodidae	*Uca (=Leptuca)*	*pugilator*	E/I	PCR/ISH	Yes	Yes	Yes	Yes	2	35
Paguridae	*Pagurus*	*angustus*	I	PCR	No	No	No	No	3	9
Paguridae	*Pagurus*	*minutus*	N/I	PCR/TEM	Yes	No	No	No	1	9
Palaemonidae	*Exopalaemon*	*carinicauda*	N/E (per os)	RT-qPCR/dot blot/ ISH	Yes	Yes	No Yes	Yes	1	19, 76
Palaemonidae	*Exopalaemon*	*orientis*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	1	7, 66
Palaemonidae	*Macrobrachium*	*idella*	E (per os)	Typical histopathology and Western blot. No PCR	Yes	Yes	Yes	Yes	2	54, 56
Palaemonidae	*Macrobrachium*	*lamerrae*	E (per os)	Typical histopathology and Western blot. No PCR	Yes	Yes	Yes	Yes	2	56
Palaemonidae	*Macrobrachium*	*nipponense*	E (per os)	PCR	Yes	No	Yes	Yes	2	72
Palaemonidae	*Macrobrachium*	*rosenbergii*	E (per os)/I	Various methods used	Yes	Yes	Yes	Yes	2	13, 27, 29, 40, 54, 56
Palaemonidae	*Palaemon*	sp.	N	PCR	No	No	No	No	3	40
Palaemonidae	*Palaemon*	*adspersus*	E/I	PCR/TEM/ISH/dot blot	Yes	Yes	Yes	Yes	2	12
Palaemonidae	*Palaemon*	*macrodactylus*	N	PCR/qPCR	No	No	No	No	3	45

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Palaemonidae	*Palaemon*	*ritteri*	E (per os)	PCR/sequencing	Yes	No	Yes	No	1	59
Palaemonidae	*Palaemonetes*	*pugio*	N/I	qPCR	No	No	Yes	No	3	48
Palinuridae	*Panulirus*	*homarus*	I	EM in *P. vannamei.* No PCR or sequence	Yes	Yes	Yes	Yes	2	54
Palinuridae	*Panulirus*	*longipes*	E (per os)	EM in *P. vannamei*. No PCR or sequence	Yes	Yes	Yes	Yes	3	54, 66
Palinuridae	*Panulirus*	*ornatus*	E (per os)	EM in *P. vannamei*. No PCR or sequence	Yes	Yes	Yes	Yes	3	54, 66
Palinuridae	*Panulirus*	*penicillatus*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	1	6, 7, 66
Palinuridae	*Panulirus*	*polyphagus*	E (per os)	EM in *P. vannamei*. No PCR or sequence	Yes	Yes	Yes	Yes	2	54
Palinuridae	*Panulirus*	*versicolor*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	1	6, 7, 66
Parastacidae	*Cherax*	*destructor*	I	dot blot	Yes	No	Yes	Yes	2	20
Parastacidae	*Cherax*	*quadricarinatus*	E (per os)/I	PCR/qPCR/IHC	Yes	Yes	Yes	Yes	1	24, 61
Parthenopidae	*Parthenope*	*prensor*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Penaeidae	*Artemesia*	*longinaris*	N	PCR/qPCR	No	No	No	No	3	45
Penaeidae	*Metapenaeus*	*affinis*	N	PCR	No	No	No	No	3	25
Penaeidae	*Metapenaeus*	*brevicornis*	N	PCR	No	No	No	No	3	30
Penaeidae	*Metapenaeus*	*dobsoni*	N/E (per os)	PCR	Yes	Yes	Yes	Yes	2	29, 54
Penaeidae	*Metapenaeus*	*ensis*	N/E (per os)	PCR/ISH/dot blot/ISH	Yes	No	Yes	Yes	1	6, 7, 66, 67, 76
Penaeidae	*Metapenaeus*	*monoceros*	N/E (per os)	PCR	Yes	Yes	Yes	Yes	2	34, 54, 70
Penaeidae	*Parapenaeopsis*	*stylifera*	N	PCR/gene probes	No	No	No	No	3	25, 29
Penaeidae	*Penaeus*	*californiensis*	N	PCR/sequencing	No	No	No	No	3	43
Penaeidae	*Penaeus*	*paulensis*	N	PCR/sequencing	Yes	No	Yes	Yes	1	4

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Penaeidae	*Penaeus*	*aztecus*	E (per os)	Inoculum not characterised; typical histopathology only	Yes	No	Yes	Yes	2	37
Penaeidae	*Penaeus*	*chinensis*	N/I	qPCR/TEM/dot blot/ISH	Yes	Yes	Yes	Yes	1	23, 31, 32, 73, 76
Penaeidae	*Penaeus*	*duorarum*	E (per os)	Inoculum not characterised; typical histopathology only	Yes	No	Yes	Yes	2	37
Penaeidae	*Penaeus*	*indicus*	N	PCR/sequencing	Yes	No	Yes	Yes	1	34, 53, 54, 56, 64
Penaeidae	*Penaeus*	*japonicus*	N/E (per os)	PCR	Yes	Yes	Yes	Yes	1	11, 21, 40, 67, 71, 73, 74
Penaeidae	*Penaeus*	*merguiensis*	N/E	PCR/TEM/IFA	Yes	Yes	Yes	Yes	2	22, 68
Penaeidae	*Penaeus*	*monodon*	N	PCR/ISH/TEM/dot blot/ISH	Yes	Yes	Yes	Yes	1	34, 40, 54, 56, 66, 67, 73, 76
Penaeidae	*Penaeus*	*penicillatus*	N/E (per os)	PCR	No	No	No	No	3	11, 40, 66
Penaeidae	*Penaeus*	*semisulcatus*	N/E (per os)	PCR	No	No	No	No	3	40, 54, 66
Penaeidae	*Penaeus*	*setiferus*	E (per os)	Inoculum not characterised; typical histopathology only	Yes	Yes	Yes	Yes	2	37
Penaeidae	*Penaeus*	*stylirostris*	E (per os)	Inoculum not characterised; typical histopathology only	Yes	Yes	Yes	Yes	2	37
Penaeidae	Penaeus	vannamei	N/E (per os)	PCR/ISH/Histology/dot blot	Yes	Yes	Yes	Yes	1	14, 37, 42, 67, 76
Penaeidae	*Trachysalambria*	*curvirostris*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	1	7, 66

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Polybiidae	*Liocarcinus*	*depurator*	E (per os)	TEM/ISH/dot blot	Yes	No	Yes	Yes	1	12
Polybiidae	*Necora (=Liocarcinus)*	*puber*	E (per os)	PCR/TEM/ISH/dot blot	Yes	No	Yes	Yes	1	12
Polychaeta	*Marphysa*	*gravelyi*	N/E (per os)	PCR	No	Yes	No	No	3	65
Portunidae	*Callinectus*	*arcuatus*	N	PCR/sequencing	No	No	No	No	3	43
Portunidae	*Callinectes*	*sapidus*	N	PCR/sequencing	No	Yes	No	No	3	51
Portunidae	*Charybdis*	*annulata*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Portunidae	*Charybdis*	*cruciata*	N	PCR	No	No	No	No	3	29
Portunidae	*Charybdis*	*granulata*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	1	7, 66
Portunidae	*Charybdis*	*feriata*	E (per os)	PCR/ISH	Yes	No	Yes	Yes	2	36, 40, 66
Portunidae	*Charybdis*	*japonica*	N	PCR	No	No	No	No	3	63
Portunidae	*Charybdis*	*lucifera*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Portunidae	*Charybdis*	*natator*	N/E (per os)	PCR	No	No	No	No	3	36, 58
Portunidae	*Podophthalmus*	*vigil*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Portunidae	*Portunus*	*trituberculatus*	N	qPCR	No	No	No	No	2	47
Portunidae	*Portunus*	*trituberculatus*	N/E (per os)/I	qPCR/TEM/histopathology	Yes	No	Yes	Yes	3	48, 75

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Portunidae	*Portunus*	*pelagicus*	N/E (per os)/I	PCR	Yes	No	Yes	Yes	2	36, 62
Portunidae	*Portunus*	*sanguinolentus*	N/E (per os)/I	PCR/ISH	Yes	No	Yes	Yes	1	6, 7, 36, 40, 41, 58, 67
Portunidae	*Scylla*	*olivacea*	I	qPCR	Yes	No	Yes	Yes	2	60
Portunidae	*Scylla*	*serrata*	N/E (per os)	PCR/ISH	Yes	Yes	Yes	Yes	1	10, 34, 35, 38, 39, 40, 41, 54, 50, 62
Portunidae	*Scylla*	*tranquebarica*	N/E (per os)/I	PCR (natural only)	Yes	Yes	Yes	Yes	2	34, 54
Portunidae	*Thalamita*	*danae*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58
Rotifera (phylum)	*Brachionus*	*urceus*	N	PCR	No	No	No	No	3	70
Scyllaridae	*Scyllarus*	*arctus*	E (per os)/I	TEM/dot blot	Yes	No	Yes	No	2	12
Sergestidae	*Acetes*	sp.	E (per os)/I	PCR	Yes	No	Yes	Yes	2	62
Sesarmidae	*Labuanium*	*rotundatum*	N	PCR	No	No	No	No	3	49
Sesarmidae	*Sesarma*	sp.	E (per os)/I	PCR	Yes	Yes	Yes	Yes	2	35, 54
Solenoceridae	*Solenocera*	*crassicornis*	N	PCR	No	No	No	No	3	29
Squillidae	*Squilla*	*mantis*	N	PCR	No	No	No	No	3	29
Varunidae	*Cyrtograpsus*	*angulatus*	N	PCR/qPCR	No	No	No	No	3	45
Varunidae	*Eriocheir*	*sinensis*	N/E (per os)/I	PCR/sequencing	Yes	Yes	Yes	Yes	1	2, 17

Annex 30 (contd)

Annex 4 (contd)

Family	**Genus**	**Species**	**Stage 1: Route of infection***	**Stage 2: Pathogen identification**	**Stage 3: Evidence for infection**				**Outcome****	**References**
					A	**B**	**C**	**D**		
Varunidae	*Helice*	*tridens*	N	PCR	No	No	No	No	3	36
Grapsidae	*Helice*	*tientsinensis*	N	dot blot/ISH	Yes	No	Yes	Yes	2	76
Varunidae	*Neohelice (=Chasmagnathus)*	*granulata*	N	PCR/sequencing	No	No	No	No	3	5, 44
Varunidae	*Pseudograpsus*	*intermedius*	N	PCR	No	No	No	No	3	29, 30
Xanthidae	*Atergatis*	*integerrimus*	E (per os)/I	PCR	No	No	No	No	3	58
Xanthidae	*Demania*	*splendida*	E (per os)/I	PCR	No	No	No	No	3	58
Xanthidae	*Liagore*	*rubronaculata*	E (per os)/I	PCR	Yes	No	Yes	Yes	2	58

Route of infection Key*

N: Natural infection
E (per os/bath): Experimental infection per os/bath
I: Injection
nd: not determined

Outcome Key**

Outcome 1: Host species proposed to be listed in Article 9.7.2. of the Aquatic Code*.*
Outcome 2: Host species proposed to be listed in Chapter 2.2.7. of the Aquatic Manual *under the revised Section 2.2.2. 'Species with incomplete evidence for susceptibility'.*
Outcome 3: Host species proposed to be listed in Chapter 2.2.7. of the Aquatic Manual *under the revised Section 2.2.2. 'Species with incomplete evidence for susceptibility' where pathogen-specific positive PCR results (but an active infection has not been demonstrated) have been reported.*

Annex 30 (contd)

Annex 4 (contd)

Additional information relevant to WSSV

Host species to be included in Article 9.7.2. of the *Aquatic Code*

The *ad hoc* Group proposed amendments to the list of susceptible host species to be included in Article 9.7.2. of the *Aquatic Code*. Refer to Annex 5.

Host species to be included in Chapter 2.2.7. of the *Aquatic Manual*

The *ad hoc* Group proposed amendments to the list of species included in the revised Section 2.2.2. of **Chapter 2.2.7. of** the *Aquatic Manual*. Refer to Annex 6.

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Annex 30 (contd)

Annex 5

CHAPTER 9.7.

INFECTION WITH WHITE SPOT SYNDROME VIRUS ~~DISEASE~~

Article 9.7.1.

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

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

Article 9.7.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5: ~~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.~~ ~~B~~blue-leg ~~swimming crab~~ swimcrab (*Liocarcinus depurator*), Chinese mitten crab (*Eriocheir sinensis*), Danube crayfish (*Astacus leptodactylus*), ~~E~~edible crab (*Cancer pagurus*), European lobster (*Homarus gammarus*), ~~F~~fleshy prawn (*Penaeus chinensis*), ~~G~~giant tiger prawn (*Penaeus monodon*), Indian white prawn (*Penaeus indicus*), Kuruma prawn (*Penaeus japonica~~e~~us*), ~~Giant mud~~ Indo-Pacific swamp crab (*Scylla serrata*), Norway lobster (*Nephrops norvegicus*), ~~P~~painted spiny lobster (*Panulirus versicolor*), ~~P~~pronghorn spiny lobster (*Panulirus penicillatus*), ~~R~~red claw crayfish (*Cherax quadricarinatus*), ~~Red spot swimmer~~ threespot swimming crab (*Portunus sanguinolentus*), red swamp crayfish (*Procambarus clarkii*), ~~Sand~~ greasyback shrimp (*Metapenaeus ensis*), ~~S~~signal crayfish (*Pacifastacus leniusculus*), southern rough shrimp (*Trachysalambria curvirostris*), ~~S~~spinycheek crayfish (*Orconectes limosus*), ~~W~~white-clawed crayfish (*Austropotamobius pallipes*), ~~W~~whiteleg shrimp (*Penaeus vannamei*), ~~V~~velvet swimcrab ~~swimming crab~~(*Necora ~~(=Liocarcinus)~~ puber*), *Calanus pacificus californicus, Charybdis granulata, Dendronereis sp.,* ridgetail prawn (*Exopalaemon carinicauda*), Oriental prawn (*Exopalaemon orientis*), *Pagurus minutus,* barred grass shrimp (*Palaemon ritteri*), Sao Paulo shrimp (*Penaeus paulensis*), *Procambarus zonangulus.*

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

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Annex 30 (contd)

Annex 6

CHAPTER 2.2.6.

WHITE SPOT DISEASE

1. Scope

For the purpose of this chapter, white spot disease (WSD) is considered to be infection with white spot syndrome virus (WSSV).

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

The recommendations in this chapter apply to the following susceptible species which meet the criteria for listing species as susceptible in Chapter 1.5: ~~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.~~ ~~B~~blue-leg ~~swimming crab~~ swimcrab (*Liocarcinus depurator*), Chinese mitten crab (*Eriocheir sinensis*), Danube crayfish (*Astacus leptodactylus*), ~~E~~edible crab (*Cancer pagurus*), European lobster (*Homarus gammarus*), ~~F~~fleshy prawn (*Penaeus chinensis*), ~~G~~giant tiger prawn (*Penaeus monodon*), Indian white prawn (*Penaeus indicus*), Kuruma prawn (*Penaeus japonica~~e~~us*), ~~Giant mud~~ Indo-Pacific swamp crab (*Scylla serrata*), Norway lobster (*Nephrops norvegicus*), ~~P~~painted spiny lobster (*Panulirus versicolor*), ~~P~~pronghorn spiny lobster (*Panulirus penicillatus*), ~~R~~red claw crayfish (*Cherax quadricarinatus*), ~~Red spot swimmer~~ threespot swimming crab (*Portunus sanguinolentus*), red swamp crayfish (*Procambarus clarkii*), ~~Sand~~ greasyback shrimp (*Metapenaeus ensis*), ~~S~~signal crayfish (*Pacifastacus leniusculus*), southern rough shrimp (*Trachysalambria curvirostris*), ~~S~~spinycheek crayfish (*Orconectes limosus*), ~~W~~white-clawed crayfish (*Austropotamobius pallipes*), ~~W~~whiteleg shrimp (*Penaeus vannamei*), ~~V~~velvet swimcrab ~~swimming crab~~(*Necora ~~(=Liocarcinus)~~ puber*), *Calanus pacificus californicus, Charybdis granulata, Dendronereis sp.,* ridgetail prawn (*Exopalaemon carinicauda*), Oriental prawn (*Exopalaemon orientis*), *Pagurus minutus,* barred grass shrimp (*Palaemon ritteri*), Sao Paulo shrimp (*Penaeus paulensis*), *Procambarus zonangulus.*

~~To date, no decapod (order Decapoda) crustacean from marine and brackish or freshwater sources has been reported to be resistant (Flegel, 1997; Lightner, 1996; Lo & Kou, 1998; Maeda *et al.*, 2000; Stentiford *et al.*, 2009).~~

2.2.2. Species with incomplete evidence for susceptibility

Evidence is lacking for the following species to either confirm that the identity of the pathogenic agent is WSSV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection:

2.2.2.1. Species for which there is incomplete evidence to fulfil the criteria for listing a species as susceptible to infection with WSSV according to Chapter 1.5. of the *Aquatic Code* include: Asian shore crab (*Hemigrapsus sanguineus*), ~~B~~banana prawn (*Penaeus merguiensis*), ~~B~~blue shrimp (*Penaeus stylirostris*), ~~B~~blue swimming crab (*Portunus pelagicus*), ~~Brachyuran~~ banded-legged swimming crab (*Charybdis annulata*), calico fiddler crab (*Uca (=Leptuca) pugilator*), ~~Common shore~~ green crab (*Carcinus maenas*), ~~C~~crucifix ~~swimming~~ crab (*Charybdis feriata~~u~~s*), ~~G~~giant ~~freshwater~~ river prawn (*Macrobrachium rosenbergii*), freshwater crab (*Paratelphusa (Barytelphusa) pulvinata*), freshwater field crab (*Paratelphusa hydrodomous*), Japanese ghost shrimp (*Callianassa ~~harmandi~~ japonica*), Kadal shrimp (*Metapenaeus dobsoni*), Krill (*Acetes sp.*), ~~L~~lesser slipper lobster (*Scyllarus arctus*), ~~M~~mangrove crab (*Sesarma* sp.), ~~Mediterranean grey shrimp~~Baltic prawn (*Palaemon adspersus*), ~~M~~mud spiny lobster (*Panulirus polyphagus*), ~~N~~northern brown shrimp (*Penaeus aztecus*) ~~N~~northern pink shrimp (*Penaeus duorarum*), ~~Northern~~ stone king crab (*Lithodes maja*), ~~N~~northern white shrimp (*Penaeus setiferus*), ~~S~~scalloped spiny lobster (*Panulirus homarus*), ~~Sentinel~~ periscope crab (*Podophthalmus vigil*), *teppo* ~~S~~snapping shrimp (*Alpheus brevicristatus*), ~~S~~speckled shrimp (*Metapenaeus monoceros*), ~~S~~swimming brachyuran crab (*Charybdis lucifera*), ~~Y~~yabby crayfish (*Cherax destructor*), *Ashtoret miersii,* spectacled box crab (*Calappa philari~~i~~gius*), *Doclea muricata (=hybrida), Ergasilus manicatus,* mottled crab (*Grapsus albolineatus*), *Halimede ochtodes, Helice tientsinensis, Liagore rubronaculata,* slender river prawn (*Macrobrachium idella*), *Kuncho river prawn (Macrobrachium lamerr~~a~~ei),* Oriental river prawn (*Macrobrachium nipponense*), *Macrophthalmus japonica~~a~~us, Metopograpsus sp., Paradorippe granulata, Parthenope prensor, Philyra syndactyla,* swimming crab (*Portunus trituberculatus*), orange mud crab (*Scylla olivacea*), purple mud crab (*Scylla tranquebarica*), *Thalamita danae*.

Annex 30 (contd)

Annex 6 (contd)

2.2.2.2. Pathogen-specific positive PCR results (without confirmation of an active infection) have been reported in the following organisms: ~~B~~blue crab (*Callinectes sapidus*), common ~~B~~box crab (*Calappa lophos*), Indian fiddler crab (*Uca [=Gelasimus] vocans [=marionis nitidus]*), swimming crab (*Portunus trituberculatus*), ~~G~~green tiger prawn (*Penaeus semisulcatus*), ~~Karikkadi~~kiddi shrimp (*Parapenaeopsis stylifera*), ~~L~~longlegged spiny lobster (*Panulirus longipes*), ~~M~~mangrove rock crab (*Metopograpsus messor*), flower ~~M~~moon crab (*Matuta planipes*), ~~N~~noble crayfish (*Astacus astacus*), ~~O~~ornate spiny lobster (*Panulirus ornatus*), ~~Red-tail~~ redtail prawn (*Penaeus penicillatus*), ~~Y~~yellow shrimp (*Metapenaeus brevicornis*), ~~Yellow-leg~~yellowleg shrimp (*Penaeus californiensis*), *Alpheus lobidens, Apocyclops royi,* Argentine stiletto shrimp (*Artemesia longinaris*),brine shrimp (*Artemia salina),* brine shrimps (*Artemia sp*), *Atergatis integerrimus, Balanus* sp., *Brachionus urceus, Cuata swimcrab (Callinecte~~us~~ arcuatus), Charybdis cruciata,* Japanese swimming crab (*Charybdis japonica*), ridged swimming crab (*Charybdis natator*), *Coleoptera, ~~(~~Ephydridae~~)~~,* Japanese sand shrimp (*Crangon affinis*), *Demania splendida, Diogenes nitidimanus, Helice tridens, Labuanium rotundatum, Macrophthalmus sulcatus, Marphysa gravelyi,* maroon stone crab (*Menippe rumphii*), Jinga shrimp (*Metapenaeus affinis*), *Neohelice (=Chasmagnathus) granulata, Nitocra sp., Orconectes punctimanus,* Palaemon shrimps (*Palaemon sp.*), migrant prawn (*Palaemon macrodactylus*), *Palaemonetes pugio, Pagurus angustus, Pseudograpsus intermedius,* coastal mud shrimp (*Solenocera crassicornis*), spottail mantis squillid (*Squilla mantis*).

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Annex 31

Original: English
April-August 2016

ELECTRONIC *AD HOC* GROUP ON SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS

April–August 2016

The *ad hoc* Group on Safety of Products Derived from Aquatic Animals (the *ad hoc* Group) worked remotely as an electronic *ad hoc* group between April and August 2016.

Details of participants and the adopted agenda are presented in Annexes 1 and 2.

The *ad hoc* Group was convened at the recommendation of the OIE Aquatic Animal Health Standards Commission (the Aquatic Animals Commission) to conduct assessments on a range of commodities commonly traded internationally against the criteria provided in Chapter 5.4. *Criteria to assess the safety of aquatic animal commodities* of the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) for acute hepatopancreatic necrosis disease (AHPND).

The *ad hoc* Group conducted assessments for a range of aquatic animal products against the 'Criteria to assess the safety of aquatic animal commodities for any purpose from a country, zone or compartment not declared free from disease X' (Article 5.3.1.) and against the 'Criteria to assess the safety of aquatic animals or aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from disease X' (Article 5.4.2.) for inclusion in the new draft chapter on acute hepatopancreatic necrosis disease (9.X.) for inclusion in the *Aquatic Code*.

The following aquatic animal products were assessed and did meet the criteria in Article 5.4.1.:

i) 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);

ii) cooked crustacean products that have been subjected to heat treatment at 100°C for at least 1 min (or to any time/temperature equivalent which has been demonstrated to inactivate VpAHPND);

iii) crustacean oil;

iv) crustacean meal

v) chemically extracted chitin.

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.2.:

i) pasteurised crustacean products.

Annex 31 (contd)

The following aquatic animal products were assessed and did meet the criteria in Article 5.4.2.:

i) frozen peeled shrimp (shell off, head off).

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.2.:

i) Frozen shrimp (shell on head on).

The individual product assessments are presented in Annex 3.

_____

…/Annexes

Annex 31 (contd)

Annex 1

ELECTRONIC *AD HOC* GROUP ON SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS

April–August 2016

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Annex 31 (contd)

Annex 2

ELECTRONIC *AD HOC* GROUP ON SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS

April–August 2016

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Terms of Reference

Background

The aquatic animal products listed in point 1 of Article X.X.3. and point 1 of Article X.X.11. of the crustacean disease-specific chapters of the Aquatic Code have been assessed against the criteria in Chapter 5.4. *Criteria to assess the safety of aquatic animal commodities*.

Acute hepatopancreatic necrosis disease (AHPND) was included as an OIE listed disease (Chapter 1.3.) in the 2015 edition of the *Aquatic Code* and a new draft chapter for AHPND for the Aquatic Code is under development. In order to list relevant crustacean products in point 1 of Article X.X.3. and point 1 of Article X.X.11. in the draft AHPND chapter, assessments needed to be conducted against the criteria in Chapter 5.4.

Purpose

The electronic ad hoc Group (eAHG) on safety of products derived from aquatic animals were tasked with undertaking assessments for a select list of crustacean products against the criteria in Chapter 5.4. for acute hepatopancreatic necrosis disease (AHPND).

Agreed Terms of Reference

1. Consider all available scientific literature to inform the assessment of the selected commodities against the criteria in Chapter 5.4. for AHPND.

2. Assess the following crustacean products against Criteria 5.4.1. *Criteria to assess the safety of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from disease AHPND* and determine eligibility for products to be listed in Article 9.X.3.:

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

    b) cooked crustacean products;

    c) pasteurised crustacean products;

    d) frozen crustacean products;

    e) crustacean oil;

    f) crustacean meal;

    g) chemically extracted chitin.

Annex 31 (contd)

Annex 2 (contd)

3. Assess the following aquatic animal products against Criteria 5.4.2. *Criteria to assess the safety of aquatic animals or aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from AHPND* and determine eligibility for products to be listed in Article 9.X.11.:

   a) frozen peeled shrimp (shell off, head off);

   b) frozen shrimp (shell on, head on).

Outputs of the *ad hoc* Group

1. Draft a report for consideration by the Aquatic Animals Commission at their September 2016 meeting, including a recommendation for listing or not listing specified commodities as safe for trade.

Annex 31 (contd)

Annex 3

Table I
Heat sterilised hermetically sealed crustacean products

Article 5.4.1. criteria	*Rationale*	*Assessment*
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	This commodity largely contains muscle (meat). The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015); examination of muscle tissue for presence of the bacterium has not been reported in the literature and there is possibility of contamination of muscle by gut-associated tissue.	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	Heat treatment is 121°C for 3.6 min or equivalent (e.g. 111°C for 36 min) (Ababouch, 1999, 2002). *Vibrio parahaemolyticus* is inactivated when heated to 100°C for 1 min (Vanderzant and Nickelson, 1972; Zhang *et al*., 2014).	Yes
AND/OR		
b) Chemical (e.g. iodine, pH, salt, smoke);		
AND/OR		
c) Biological (e.g. fermentation).		
CONCLUSION **VpAHPND is highly likely to be inactivated by this process. Therefore, heat sterilised hermetically sealed crustacean products (i.e. a heat treatment at 121°C for at least 3.6 min or any time/temperature equivalent) are eligible for inclusion in Article 9.X.3. point 1.**		

Annex 31 (contd)

Annex 3 (contd)

Table II

Cooked crustacean products

Article 5.4.1. criteria	*Rationale*	*Assessment*
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	This commodity largely contains muscle (meat), but could contain other organs depending on the product. The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015); examination of muscle tissue for presence of the bacterium has not been reported in the literature.	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	*Vibrio parahaemolyticus* is inactivated when heated to 100°C for 1 min (Vanderzant and Nickelson, 1972; Zhang *et al*., 2014)	Yes
AND/OR		
b) Chemical (e.g. iodine, pH, salt, smoke);		
AND/OR		
c) Biological (e.g. fermentation).		

CONCLUSION

VpAHPND is likely to be inactivated by this process. Therefore, cooked crustacean products that have been subjected to heat treatment at 100°C for at least 1 min (or to any time/temperature equivalent which has been demonstrated to inactivate VpAHPND) are eligible for inclusion in Article 9.X.3. point 1.

Annex 31 (contd)

Annex 3 (contd)

Table III

Pasteurised crustacean products

Article 5.4.1. criteria	***Rationale***	***Assessment***
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	This commodity largely contains muscle (meat). The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015); examination of muscle tissue for presence of the bacterium has not been reported in the literature and there is possibility of contamination of muscle by gut-associated tissue.	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	There are reports of low temperature treatment regimes resulting in inactivation of *Vibrio* species bacteria, including *V. parahaemolyticus*. Andrews *et al.* (2000) indicated a 5D reduction in *Vibrio parahaemolyticus* in raw oysters subject to 50°C for 5 minutes. Andrews *et al.* (2003) reported that oysters contaminated with up to 10^6 cfu/g of *Vibrio parahaemolyticus* were successfully pasteurised by a 52°C treatment for 22 minutes. Zhang *et al.* (2014) reported complete inactivation of *Vibrio parahaemolyticus* in alkaline peptone water-salt broth under conditions of 60°C, 5 minutes or 70°C, 2 minutes or ≥80°C, 1 minute, although it is unclear if this was a 4 $\log_{10}$ reduction or a 6 $\log_{10}$ reduction. Johnston & Brown (2002) claimed that a 70°C, 2 minute treatment was 100% effective against *Vibrio* species in artificial seawater.	No

Annex 31 (contd)

Annex 3 (contd)

	However, none of these studies used shrimp or prawn tissue as the matrix for studying inactivation. There is evidence in Johnston & Brown (2002) that the matrix homogenate used has an effect on the D-value obtained. Whilst an older reference, Vanderzant & Nickelson (1972) used shrimp tissue for their experiments and report that treatments of 60°C or 80°C for 15 minutes were not effective in producing a 6 $\log_{10}$ reduction in *Vibrio parahaemolyticus* in shrimp tissue and bacteria were recoverable on direct plating and enrichment. Data suggests that neither 63°C, 17 minutes or 72°C, 1 minute has enough evidence to support an assessment of rendering the product safe. Whilst 90°C for 10 minutes is probably effective, there is still enough uncertainty to require further data before considering pasteurised products safe.
AND/OR b) *Chemical (e.g. iodine, pH, salt, smoke);* *AND/OR* c) *Biological (e.g. fermentation).*	
CONCLUSION **VpAHPND may not be inactivated by this process. Therefore, pasteurised crustacean products that have been subjected to heat treatment at 90°C for at least 10 min (or 72°C for 1 minute, or 63°C for 17 minutes) are not at this time eligible for inclusion in Article 9.X.3. point 1. Further data is required.**	

Note: Pasteurisation is a food treatment process that is well defined for milk products, but is not well defined for aquatic animal products. There are a number of time/temperature combinations that may be used depending on the product. Officially specified conditions will tend to be determined by the requirement to inactivate bacteria of concern to food safety. As such both the United States of America Food and Drug Administration (FDA, 2001) and Gould (1999) indicate that 90°C for 10 min is required to achieve a 6D reduction in *Clostridium botulinum*. Inactivation standards for *Listeria monocytogenes* are considerably lower. It is proposed therefore, that in the first instance a standard of 90°C, 10 min is used. The amount of heat applied during a heat treatment process will determine which of the identified hazards will be eliminated at that point (FOA Fisheries Technical Paper 334, Assurance of Seafood Quality, 1994). *Listeria monocytogenes* is often identified as the target pathogen as it is regarded as the most heat tolerant, food-borne pathogen that does not form spores (U.S. Food and Drug Administration [USFDA] Centre for Food Safety and Applied Nutrition, Fish and Fisheries Products Hazards and Controls Guidance, Third Edition, June 2001). When seafood processors in the US implement HACCP systems to eliminate *L. monocytogenes* contamination, the USFDA guideline recommends minimum internal product temperature/time treatments that include 63°C for 17 minutes and 72°C for 1 minute.

Annex 31 (contd)

Annex 3 (contd)

Table IV

Crustacean oil

Article 5.4.1. criteria.	***Rationale***	***Assessment***
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	Raw material for this process is likely to contain most tissues. The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015).	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	Raw material is cooked (may be pre-heated to 50–60°C before cooking at temperatures of 95–100°C for 15–20 minutes. For energy cost reasons and nutritional content, some processors use 80–85°C for 20 minutes). Cooked material is pressed to produce press liquor, and press liquor is heated to 90–95°C, which produces oil. Oil is purified with hot water (at 90°C) (FAO, 1986). *Vibrio parahaemolyticus* is inactivated when heated to 100 °C for 1 min (Vanderzant and Nickelson, 1972; Zhang *et al.*, 2014).	Yes
AND/OR		
b) Chemical (e.g. iodine, pH, salt, smoke);		
AND/OR		
c) Biological (e.g. fermentation).		
CONCLUSION **VpAHPND is highly likely to be inactivated by this process. Therefore, crustacean oil is eligible for inclusion in Article 9.X.3. point 1.**		

Annex 31 (contd)

Annex 3 (contd)

Table V

Crustacean meal

Article 5.4.1. criteria	***Rationale***	***Assessment***
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	Raw material for this process is likely to contain most tissues. The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015).	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	The process involves cooking, usually boiling at 100°C for at least 3 minutes, and a drying step at between 115 and 138°C (Velez *et al*., 1991). Raw material for this process is likely to contain most tissues. The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015).	Yes
AND/OR		
b) Chemical (e.g. iodine, pH, salt, smoke);		
AND/OR		
c) Biological (e.g. fermentation).		
CONCLUSION **VpAHPND is likely to be inactivated by this process. Therefore, crustacean meal is eligible for inclusion in Article 9.X.3. point 1.**		

Annex 31 (contd)

Annex 3 (contd)

Table VI

Chemically extracted chitin

Article 5.4.1. criteria	***Rationale***	***Assessment***
1. Absence of pathogenic agent in the traded commodity:		
a) There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.	Exoskeleton is used for this commodity. The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015). The bacterium is not therefore normally present in exoskeleton and associated cuticular epithelium. However, it is possible that remains of gut tissue may contaminate the exoskeleton.	No
AND		
b) The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.		
OR		
2. Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
a) Physical (e.g. temperature, drying, smoking);	The product is heated at 60–70°C for a few hours (Gagné, 1993) in a mild alkaline environment. Given a time temperature combination of 100°C, 1 minute is effective it is highly likely that several hours at 60–70°C will also result in inactivation of the *Vibrio parahaemolyticus*.	Yes
AND/OR		
b) Chemical (e.g. iodine, pH, salt, smoke);	Hydrochloric acid is used in the processing (Gagné, 1993). Vanderzant & Nickelson (1972) reported pH ≤5, 15 minutes is effective at inactivating *Vibrio parahaemolyticus*, therefore use of hydrochloric acid, especially following the previous long heat treatment would inactivate all *Vibrio parahaemolyticus* bacteria.	Yes
AND/OR		
c) Biological (e.g. fermentation).		

CONCLUSION

VpAHPND is likely to be inactivated by this process. Therefore, chemically extracted chitin is eligible for inclusion in Article 9.X.3. point 1.

Annex 31 (contd)

Annex 3 (contd)

B) Assessments using criteria in Article 5.4.2. (for Article 9.X.11. point 1)

1. The following aquatic animal products were assessed and did meet the criteria in Article 5.4.2.:

   i) frozen peeled shrimp (shell off, head off).

2. The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.2.:

   i) frozen shrimp (shell on head on).

Table I

Frozen peeled shrimp (shell off, head off)

Article 5.4.2. criteria		***Rationale***	***Assessment***
1.	***The aquatic animal product is prepared and packaged for retail trade for human consumption.***	It is part of the commodity definition.	Yes
AND/EITHER			
2.	***It includes only a small amount of raw waste tissues generated by the consumer.***	There are no waste tissues because the entire product is consumed.	Yes
OR			
3.	***The pathogenic agent is not normally found in the waste tissues generated by the consumer.***		

CONCLUSION
Frozen peeled shrimp (shell off, head off) that are prepared and packaged for retail trade for human consumption normally does not produce waste. Therefore, frozen shrimp (shell off, head off) is eligible for inclusion in Article 9.X.11.

Annex 31 (contd)

Annex 3 (contd)

Table II

Frozen shrimp (shell on, head on)

Article 5.4.2. criteria	*Rationale*	*Assessment*
1. The aquatic animal product is prepared and packaged for retail trade for human consumption.	It is part of the commodity definition.	Yes
AND/EITHER		
2. It includes only a small amount of raw waste tissues generated by the consumer.	Waste includes shell, cephalothorax, legs.	No
OR		
3. The pathogenic agent is not normally found in the waste tissues generated by the consumer.	The AHPND bacteria is present in gut-associated tissues (Tran *et al*., 2013; Soto-Rodriguez *et al*., 2015) which are part of the cephalothorax. While freezing appears to be effective at reducing bacterial numbers (*Vibrio parahaemolyticus*), 100% inactivation cannot be assured even after 10 weeks at low temperatures (Liu *et al*., 2009; Muntada-Garriga *et al*., 1995; Vasudevan *et al*., 2002).	No

CONCLUSION
Frozen shrimp (shell on, head on) that are prepared and packaged for retail trade for human consumption may produce amounts of wastes that cannot be considered small; the pathogenic agent may be found in the waste. Therefore, frozen shrimp (shell on, head on) is not eligible for inclusion in Article 9.X.11.

Annex 31 (contd)

Annex 3 (contd)

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Annex 32

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION WORK PLAN 2016–2017

Task	September 2016	February 2017
AQUATIC CODE		
Glossary	Amended some definitions and circulated for Member comments	Review Member comments
Criteria for the inclusion of diseases in the OIE list (Chapter 1.2.)	Reviewed Member comments and circulated for comment	Review Member comments
Diseases listed by the OIE (Chapter 1.3.)	Reviewed Member comments and circulated for comment. Reviewed assessment of tilapi lake virus for listing.	Review Member comments
Criteria for listing species as susceptible (Chapter 1.5.)	Developed a new Article 1.5.9. to address diseases with a wide host range.	Review Member comments
Disinfection of aquaculture establishments and equipment (Chapter 4.3.)	Reviewed Member comments and circulated for comment	Review Member comments
Recommendations for surface disinfection of salmonid eggs (Chapter 4.4.)	Reviewed Member comments and circulated for comment	Review Member comments
General obligations related to certification (Chapter 5.1.)	Reviewed Member comments and circulated for comment	Review Member comments
Crayfish plague *(Aphanomyces astaci)* (Chapter 9.1.)	Reviewed comments and circulated for comment	Review Member comments
Yellow head disease genotype 1 (Chapter 9.2.)	Reviewed comments and circulated for comment	Review Member comments
Infectious hypodermal and haematopoietic necrosis (Chapter 9.3.)	Reviewed comments and circulated for comment	Review Member comments
Infectious myonecrosis (Chapter 9.4.)	Reviewed comments and circulated for comment	Review Member comments
Necrotising hepatopancreatitis (Chapter 9.5.)	Reviewed comments and circulated for comment	Review Member comments
Taura syndrome (Chapter 9.6.)	Reviewed comments and circulated for comment	Review Members priorities
White spot disease (Chapter 9.7.)	Reviewed Member comments and circulated for comment	Review Member comments
White tail disease (Chapter 9.8.)	Reviewed Member comments and circulated for comment	Review Member comments
Acute hepatopancreatic necrosis disease (new Chapter 9.X.)	Reviewed Member comments and AHG report on safe commodities (Articles 9.X.3. and 9.X.11) and circulated for comment	Review Member comments
Revised Article X.X.8. (clean text and track changes text)	Reviewed Member comments and circulated for comment	Review Member comments

Annex 32 (contd)

Develop revised lists of susceptible species – all fish chapters	*Ad hoc* Group to meet 15-17 November to start work assessing susceptible species lists for OIE listed fish diseases	Review AHG report
Periods to claim/reclaim freedom (in relation to Chapter 1.4.) Develop principles for determining surveillance periods in disease-specific chapters and provide advice on amendments for Chapter 1.4.	Requested an ad hoc Group be convened in January 2017. Develop terms of reference for this work.	Review AHG report
New chapter on Biosecurity (Chapter 4.X.)	Developed ToR for a new *ad hoc* Group to develop text for this new chapter	*Ad hoc* Group to be convened early 2017 after Feb 2017 meeting
Possible development of chapters for other species where disinfection for eggs and larvae is practised and important for ensuring safe trade.	Requested priorities from Members re future work	Review Members priorities
Revision of Chapters 4.2. and 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
Develop concept for a possible guidance document on how to use the *Aquatic Code* to facilitate trade		Consider developing a concept note
AQUATIC MANUAL		
Crayfish plague *(Aphanomyces astaci)* (Chapter 2.2.1.)	Reviewed comments and circulated for comment	Review Member comments
Yellow head disease genotype 1 (Chapter 2.2.2.)	Amended text to align with other chapters	Review Member comments
Infectious hypodermal and haematopoietic necrosis (Chapter 2.2.3.)	Reviewed comments and circulated for comment	Review Member comments
Infectious myonecrosis (Chapter 2.2.4.)	Reviewed comments and circulated for comment	Review Member comments
Necrotising hepatopancreatitis (Chapter 2.2.5.)	Reviewed comments and circulated for comment	Review Member comments
Taura syndrome (Chapter 2.2.6.)	Reviewed comments and circulated for comment	Review Member comments
White spot disease (Chapter 2.2.7.)	Proposed changes to title and scope. Put on hold changes to section 2.2.2. pending outcomes of amendments to Chapter 1.5.	Review Member comments
White tail disease (Chapter 2.2.8.)	Reviewed comments and circulated for comment	Review Member comments
Acute hepatopancreatic necrosis disease (new Ch 2.2.X.)	Reviewed comments and circulated for comment	Review Member comments

Develop revised lists of susceptible species – all fish chapters	*Ad hoc* Group to meet 15-17 November to start work assessing susceptible species lists for OIE listed fish diseases	Review *ad hoc* Group report
Ad hoc Group on Aquatic Manual	A*d hoc* Group to be reconvened in January 2017 to continue their work	Review *ad hoc* Group report
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
Develop a strategic plan for the Ref. Lab. network system	Worked with the Lab Commission to develop a strategic plan	Work with the BSC to further develop and evolve a strategic plan for the future role of the network of aquatic animal Reference Labs
OTHER WORK		
Guidance document for assessments of new listings (Joint Commission activity)		Develop a guide and circulate before September 2017

EUROPEAN COMMISSION
DIRECTORATE-GENERAL FOR HEALTH AND FOOD SAFETY

Deputy Director-General for Food Safety

27. 07. 2016

Brussels
SANTE G2/PB/lp (2016) 3977986
Ares (2016)3961108

Dear Dr Url,

Subject: **Request for i) a scientific and technical assistance and ii) a scientific opinion concerning the risk of survival, establishment and spread of Batrachochytrium salamandrivorans (Bsal) in the EU**

I would like to submit a formal request to the European Food Safety Authority for a scientific and technical assistance and for a scientific opinion concerning the risk of entry, survival, establishment and spread of *Batrachochytrium salamandrivorans* (Bsal) into and in the EU, with particular emphasis on the relatively new European situation of occurrences of this pathogen in several EU Member States (Netherlands, Belgium, Germany, UK) both in kept and wild salamander populations.

EFSA is asked to provide a scientific and technical assistance by the end of 2016 and a scientific opinion by the end of 2017.

My services remain at your disposal for further information. On this matter, you can contact Mr P. Bernorio, who is responsible for the dossier in Unit G2, Animal Health and welfare unit and Ms M. Marini, who is the relevant contact point in the Unit D1 in charge of Science, stakeholders, enforcement. Their respective phones and e-mail addresses are indicated below.

Yours sincerely,

Ladislav Miko

Contact persons:
Ms. M. Marini (02.299.93307) Marina.Marini@ec.europa.eu
Mr. P. Bernorio (02.299.84882) Pierangelo.Bernorio@ec.europa.eu

Annex: Terms of Reference

C.c.: T. Bregeon, B. Van Goethem, M. Scannell, E. Zamora Escribano, L. Terzi, M. Marini, P. Bernorio, L. Kuster, B. Logar, (DG SANTE), A. Gervelmeyer (EFSA).

Dr Bernhard Url
Executive Director
European Food Safety Authority
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ITALY

ANNEX
TERMS OF REFERENCE

INTRODUCTION - BACKGROUND INFORMATION

Bsal was identified by scientists as recently as in 2013. Over the last couple of years Bsal occurred at least in certain parts of Europe either in wild population of salamanders and newts[1] (e.g. the Netherlands, Belgium in several locations) or in kept population (e.g. Germany, UK) or possibly in both populations. There is no data from other EU countries but similar cases either in wild or in kept salamanders cannot be excluded. In certain areas (e.g. the Netherlands) this fungus is said to have devastated local fire salamander populations. In other places the fungus is apparently present in susceptible species, but without increased mortalities. Many Asian salamander species seem to be immune or tolerant to this pathogen to various extents. Many European species seem to be susceptible.

This situation and state of knowledge is patchy, fragmented and is expected to change as more knowledge about this emerging pathogen and especially surveillance data becomes available, continually and gradually. Overall, scientific data on Bsal is still scarce with significant gaps. Currently the disease is not listed under OIE standards or in the EU rules.

A few affected countries, or those which anticipate that Bsal could affect them, adopted diverse control policies or consider various possible measures against the disease to cope with its feared short- and long-term consequences in wild animals and in kept salamanders and their trade. One of such examples is an import ban of certain salamander species introduced by the USA, where Bsal is either absent or not yet detected. Other measures are of non-legislative nature, such as rising of awareness among stakeholders on risks, guidelines for improved biosecurity or survey salamander populations, changes thereof, with the emphasis on increased mortalities and/or occurrence of Bsal. To date, these have been done under environmental policies.

Some actors have recently called for, *inter alia*, EU animal health policy and legislative measures to be adopted, in particular an immediate ban on import of many species of salamanders from Asia into the EU. It has been shown by phylogenetic analysis that the fungus, indeed, originates in certain parts of Asia. Therefore it has been speculated by some that trade in Asian salamanders may play a role in its spread into and within the EU, although there is no proof that this pathogen entered the EU via this route. And if so, when and under what circumstances. In general, details are missing on its actual spread into or in the EU or between kept and wild animals.

The Commission therefore needs a quick but comprehensive compilation, scrutiny of available data and assessment to determine if Bsal is a disease with the potential to harm kept and wild salamanders in the Union and various risk factors associated with:

- imports of Asian salamanders into the EU and their trade within the EU;
- movements of European salamanders (both caught from the wild or kept ones) within the EU, and

[1] The term "salamanders" is wide. All newts are salamanders but they are usually differentiated. The taxonomical term Salamandroidae does not cover all salamanders however, there are other sub-orders too, also called salamander in common speech. All belong to the Order Urodela. The term Caudata is also often used (amphibians with tails both when larvae and adult). In this mandate salamanders = Urodela = Caudata, for simplification.

- imports and movements of animal by-products obtained from Asian and European salamanders (both caught from the wild or kept ones).

Such assessment would be essential for the consideration of potential safeguard measures in relation to imports from Asia or for movements from infected to non-infected EU areas.

In the past, EFSA has produced scientific opinions dealing with various aspects of emerging pathogens, including those where environmental aspects or wild animals play increased role or are affected (such as e.g. on small hive beetle). Therefore, a similar opinion is necessary to understand better possible scenarios for the evolution of this new disease, the current epidemiological situation, the experience gained so far from the implementation of the various control policies and possible alternative methods to diminish negative effects on wild salamander populations and to ensure safe trade of kept animals and their products. Identification of gaps and uncertainties is also very important for this emerging disease.

Furthermore, EFSA has already been made aware of the adoption and publication of the Regulation on transmissible animal diseases (Animal Health Law[2]), hereinafter referred to as AHL. As Bsal is not included in the list of diseases in Annex II to the AHL (or on the list of any other existing EU animal health legislation), environmental actors asked the Commission to place Bsal onto that list. Therefore, the review of this list will be necessary in accordance with a set of criteria provided for in the AHL before it comes into force, taking into account the transitional periods envisaged for its application (five years starting from April 2016). Hence the Commission needs scientific advice for the assessment of the significance of Bsal within the framework of this already known listing and categorisation according to the AHL, in the same manner it was requested previously, for another two groups of diseases (Ref. SANTE G2/BL/lp (2015) 4940871, SANTE G3/LPA/lp (2016) 3154863, respectively).

The criteria, provided for in Article 7 and 8 and Annex IV of the AHL, shall be used as a basis for this analytical assessment. The risk manager needs a scientific advice in order to:

1. assess if Bsal causes disease for which control measures at the EU level are justified;
2. proceed with the profiling of the disease in view to its categorisation; and
3. assign listed species to Bsal identified as eligible for EU intervention.

The Commission have identified the main issues for which concrete elements of science may provide good basis for formulating policies and/or adapt current approach. These are as follows:

- Provisions for safe trade (entry into the Union and trade within the Union) with Asian and European salamanders and animal by-products obtained threfrom;
- Identifying links between groups of salamanders in trade (i.e. in consignments being moved or in shops etc.) and kept ones (i.e. stationary, whether for hobby or else) and salamanders in wild (i.e. in their natural habitat) and possible routes and risks of spreading Bsal between the specimens belonging to the above three groups and locations;
- Effects of the respective infection of salamanders with Bsal, including aspects stemming from different susceptibility of various species to Bsal;

[2] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R0429&rid=8

- Measures to monitor occurrence of Bsal in those groups and mitigate mortality due to Bsal, whether regulatory measures or non-regulatory ones.

TERMS OF REFERENCE

Scientific and technical assistance

I. Scientific and technical assistance in accordance with Article 31 of Regulation (EC) No 178/2002

In view of the above, in accordance with Article 31 of Regulation (EC) No 178/2002, the Commission asks EFSA to provide scientific and technical assistance concerning:

- Assessment of the potential of Bsal to affect the health of wild and kept salamanders in the Union;
- effectiveness and feasibility of a movement (including intra-EU trade and introduction from non-EU countries) ban of traded salamanders, including both Asian and non-Asian species;
- the validity, reliability and robustness of the available diagnostic methods for the detection of Bsal;
- possible alternative methods and feasible risk mitigation measures to ensure safe international and EU trade of salamanders and their products.

II. Scientific opinion in accordance with Article 29 of Regulation (EC) No 178/2002

In accordance with Article 29 of Regulation (EC) No 178/2002, the Commission asks EFSA to provide a scientific opinion on the following:

1) **<u>As regards susceptibility, morbidity and mortality, assess:</u>**
   a) Susceptibility and morbidity of various Asian and European salamanders to Bsal;
   b) Nature of Bsal as facultative or not pathogen of European salamanders;
   c) If there are species of salamanders carrying Bsal without clinical symptoms and/or clinical and serological evidence and if so, which ones;
   d) Mortality rates of native European salamander species due to Bsal;
   e) Role of other factors (e.g. habitat degradation, etc.) in increased mortalities associated with Bsal.
2) **<u>As regards presence, absence, surveillance and eradication, assess:</u>**
   a) the risk of survival and establishment of Bsal in the environment in the EU under various meteorological conditions;
   b) possible identification of various areas (e.g. countries, zones, territories etc.) which may be considered infected with Bsal or free from it;
   c) definition of requirements for reliable detection of Bsal in the wild in affected areas or exclusion of its presence;
   d) suitability of surveillance methods to ensure reliable and robust demonstration of presence or absence of Bsal.

3) **As regards spread of Bsal in and from infected areas or via infected animals or fomites, assess:**
   a) the risk of survival, spread and establishment of Bsal within already infected areas and spread from infected areas into other parts of the EU under various scenarios:
      i) by natural movements of live salamanders taking into account especially relevant geographical, hydrographical and meteorological conditions;
      ii) by movements of traded live salamanders and their traded products, body parts etc. from infected areas, both under identified risk mitigation measures or without;
   b) risk mitigating factors that could potentially be effective in ensuring safe international or intra-EU trade of live salamanders (both captured in the wild and bred) and their products and by-products as regards the transmission of Bsal, including diagnosis and potential treatment(s);
   c) the role of live, silent carriers of Bsal in spreading it as vectors and those of fomites (e.g. waste water, animal by-products, feed etc.) and risk mitigating measures concerning those;
   d) the possible routes of spread between kept salamanders, originating from international trade and the autochthonous salamanders living in wild, i.e. their natural habitat.
4) **As regards on-site protection from Bsal, assess:**
   a) Potential and feasible risk mitigating factors and methods in kept salamanders;
   b) Risk mitigating factors and methods for salamanders in their natural habitat.
5) **Listing and categorisation of Bsal in the framework of the Animal Health Law.**
   a) Assess, following the criteria laid down in Article 7 of the AHL, its eligibility of being listed for Union intervention as laid down in Article 5(3) of the AHL;
   b) If found eligible to be listed for Union intervention, provide:
      i) an assessment of its compliance with each of the criteria in Annex IV to the AHL for the purpose of categorisation of diseases in accordance with Article 9 of the AHL;
      ii) a list of animal species that should be considered candidates for listing in accordance with Article 8 of the AHL.