Organisation Mondiale de la Santé Animale

World Organisation for Animal Health

Organización Mundial de Sanidad Animal

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

Paris (France), 29 September–3 October 2014

EU comments

The EU would like to commend the OIE for its work and thank in particular the Aquatic Animals Commission for having taken into consideration EU comments on the Aquatic Code and Manual submitted previously.

A number of general and specific comments on this report of the September/October 2014 meeting of the Aquatic Animals Commission are inserted in the text below as well as in the text of the respective annexes of the report.

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

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The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at the OIE Headquarters from 29 September to 3 October 2014.

Details of participants and the adopted agenda are given at Annexes 1 and 2.

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts circulated after the Commission's February meeting: Australia, Canada, Chinese Taipei, Japan, New Zealand, Switzerland, the United States of America, the Member States of the European Union (EU), the African Union–Interafrican Bureau for Animal Resources (AU-IBAR) on behalf of OIE Delegates of Africa.

The Aquatic Animals Commission reviewed the documents identified in the agenda, addressing comments that Member Countries' had submitted on the February 2014 report and amended texts in the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) and the 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 the report. The Code Commission considered all Member Countries' comments.

Member Countries should note that, unless stated otherwise, texts submitted for comment may be proposed for adoption at the 82nd OIE General Session in May 2015. Depending on the comments received on each text, the Commission will identify the texts proposed for adoption in May 2015 in the report of its March 2015 meeting.

The Aquatic Animals Commission strongly encourages Member Countries to participate in the development of the OIE's international standards by submitting comments on this report, and prepare to participate in the process

of adoption at the General Session. Comments should be submitted as specific proposed text changes, supported by a structured 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.

The table below summarises the texts presented in the Annexes. Annexes 3 to 20 are presented for Member Country comment; Annexes 21 to 23 are presented for Member Countries' information.

Comments on this report must reach OIE Headquarters **by 30ᵗʰ January 2015** to be considered at the March 2015 meeting of the Aquatic Animals Commission. All comments should be sent to the OIE International Trade Department at: trade.dept@oie.int.

Texts for Member Countries' comments	Annex number
Aquatic Code:	
User's guide	Annex 3
Glossary	Annex 4
Notification of diseases and epidemiological information (Chapter 1.1.)	Annex 5
Diseases listed by the OIE (Chapter 1.3.)	Annex 6
Import risk analysis (Chapter 2.1.)	Annex 7
Recommendations for disinfection of salmonid eggs (new Chapter 4.X.)	Annex 8
Control of hazards in aquatic animal feed (Chapter 4.7.)	Annex 9
General obligations related to certification (Chapter 5.1.)	Annex 10
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Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals (Chapter 6.6.)	Annex 12
Amphibian disease-specific chapters (8.1. and 8.2.)	Annex 13A and 13B
Articles X.X.7. and X.X.11. of disease-specific chapters	Annex 14
Corrections in Articles 10.4.4. and 10.4.6.	Annex 15
Aquatic Manual:	
Infectious hypodermal and haematopoietic necrosis (Chapters 2.2.2)	Annex 16
Necrotising hepatopancreatitis (Chapter 2.2.4.)	Annex 17
Taura syndrome (Chapter 2.2.5.)	Annex 18
Yellow head disease (Chapter 2.2.8.)	Annex 19
Infection with *Perkinsus olseni* (Section 2.2.1. of Chapter 2.4.6.)	Annex 20
Annexes for Member Countries' information	
Assessment for acute hepatopancreatic necrosis disease in accordance with Article 1.2.2.	Annex 21
Infection with *Perkinsus olseni* (Chapter 11.6.)	Annex 22
Aquatic Animal Health Standards Commission Work Plan for 2014/2015	Annex 23

Meeting with the Director General

Dr Vallat welcomed the Aquatic Animals Commission and thanked them for their support and commitment to achieving OIE objectives. He noted that it is very important that the Commission have a broad vision for standard setting and other OIE objectives regarding aquatic animal health. He reiterated his support for any *ad hoc* Groups that the Commission may wish to convene to support their work.

Dr Vallat commented that the upcoming Global Conference on Aquatic Animal Health, to be held in in January 2015 in Vietnam, was an important event for the Commission that will provide an opportunity to highlight the work of the OIE and also to develop recommendations to guide the future work of the Aquatic Animals Commission and the OIE.

Dr Vallat noted that the election of all Specialist Commissions will be held in May 2015. He commented that the OIE Council is developing criteria for eligibility of members of the Specialist Commissions that will be applied to the 2015 elections to ensure continuing excellence of all members.

Dr Vallat explained that a questionnaire had been sent to a selected list of Delegates to try to find out why the Aquatic PVS Tool has only been requested by a small number of Delegates in comparison to the great success achieved with the Terrestrial PVS Tool. The responses to this questionnaire will be presented at the Global Aquatic Conference in Vietnam. He also reiterated that the OIE respects the independence of the Aquatic Animal Health Services (AAHS) in a Member Country and that the PVS Tool is designed to assist a country to improve its AAHS irrespective of whether the AAHS is within the Veterinary Services or another service of ministry.

Dr Vallat noted that the OIE is continuing to conduct very important work on antimicrobial resistance and that the OIE is increasingly receiving requests from many sectors requesting work on this topic. He noted that the OIE has been requested to undertake work to develop prioritisation criteria to guide vaccine (or other alternatives) development in aquatic and terrestrial animals with the goal of decreasing the use of antimicrobials in animals. Dr Vallat requested that the Commission contribute to this work.

1. **OIE** *Aquatic Animal Health Code*

   1.1. **Guide to the Use of the** *Aquatic Animal Health Code*

   The Aquatic Animals Commission considered Member Countries' comments and made relevant amendments while ensuring alignment, as far as possible, with the User's Guide in the 2014 edition of the *Terrestrial Code*.

   In response to several Member Countries' comments, the Commission deleted the words 'Veterinary Authority and other' when it appeared in front of 'Competent Authority' as they agreed this was a duplication of terms.

   The Aquatic Animals Commission did not agree with a Member Country comment to reinstate point 3 of the Introduction ('The *Aquatic Code* currently does not encompass any zoonotic disease, however, veterinary public health is part of the mandate of the OIE, including in the field of aquatic animal health.') as they considered that guidance to user's was not necessary on zoonotic diseases because the *Aquatic Code* does not currently address any zoonotic diseases. The Commission agreed that this point was more suitable for inclusion in the Foreword and suggested that this be included in the Foreword of the 2015 edition of the *Aquatic Code.*

   The revised User's guide is presented at Annex 3 for Member Countries' comments.

EU comment

The EU thanks the OIE and in general supports the proposed changes to the user's guide. A specific comment is inserted in the text of Annex 3.

   1.2. **Glossary**

The Aquatic Animals Commission proposed to amend the definitions for 'disinfectant' and 'disinfection' following a recommendation from the *ad hoc* Group on Disinfection (see also Item 1.6.). The Commission agreed that the revised definitions will more suitably reflect the use of these terms in the *Aquatic Code*.

DISINFECTANT
means a chemical compound <u>or a physical process</u> capable of destroying *pathogenic <u>agents</u>* ~~microorganisms~~ or inhibiting their growth ~~or survival ability~~.

DISINFECTION
~~means the application, after thorough cleansing, of procedures intended to destroy the infectious or parasitic agents of~~ *~~diseases~~* ~~of~~ *~~aquatic animals~~*~~, including zoonoses; this applies to~~ *~~aquaculture establishments~~* ~~(i.e. hatcheries, fish farms, oyster farms, shrimp farms, nurseries, etc.),~~ *~~vehicles~~*~~, and different equipment/objects that may have been directly or indirectly contaminated.~~

<u>means the process of cleaning and applying</u> *<u>disinfectants</u>* <u>to inactivate</u> *<u>pathogenic agents</u>* <u>on potentially contaminated items</u>

The Aquatic Animals Commission proposed to delete the definition for 'Infective period' because following the proposed amendments to Chapter 1.1. (see Item 1.6.) this definition will only appear in Chapter 4.5. and its use in the context of this chapter does not require a specific definition.

~~INFECTIVE PERIOD~~
~~means the longest period during which an affected~~ *~~aquatic animal~~* ~~can be a source of~~ *~~infection~~*~~.~~

In addition, the Aquatic Animals Commission reviewed amendments proposed by the Code Commission to the *Terrestrial Code* Glossary and agreed to make similar amendments in the *Aquatic Code* glossary to ensure alignment between the two *Codes*.

The Aquatic Animals Commission noted that the Glossary does not currently include a definition for 'biosecurity' and agreed with the Code Commission to develop a definition which could be proposed for adoption in both *Codes*.

<u>BIOSECURITY</u>
<u>means the set of management and physical measures designed to reduce the</u> *<u>risk</u>* <u>of introduction, establishment and spread of</u> *<u>pathogenic agents</u>* <u>to, from and within an</u> *<u>aquatic animal</u>* <u>population.</u>

The Aquatic Animals Commission agreed with the Code Commission that the current definition of 'hazard identification' adds little to the existing definition of *hazard* and could therefore be deleted from the glossary.

~~HAZARD IDENTIFICATION~~
~~means the process of identifying the pathogenic agent(s) which could potentially be introduced in the commodity considered for importation.~~

RISK ANALYSIS
means the ~~complete~~ process composed of *hazard ~~identification~~* identification, *risk assessment*, *risk management* and *risk communication*.

The Aquatic Animals Commission noted that risk assessment is applied more broadly than within the context of imports and therefore agreed with the Code Commission that "within the territory of an importing country" be deleted from the definition for 'risk assessment'.

RISK ASSESSMENT
means the scientific evaluation of the likelihood and the biological and economic consequences of entry, establishment and spread of a *hazard* ~~within the~~ *~~territory~~* ~~of an~~ *~~importing country~~*.

The revised Glossary is presented at <u>Annex 4</u> for Member Countries' comments.

> **EU comment**
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> **The EU in general supports the proposed changes to the glossary, in particular the new definition of biosecurity. However, a specific comment is inserted in the text of Annex 4. Furthermore, a change is suggested in the definition of "Veterinary Authority" to explicitly mention veterinary public health.**

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

In response to a Member Country comment, the Aquatic Animals Commission amended point 2 of Article 1.1.5. to remove reference to infective period because this information is not specified in the *Aquatic Code*. The Commission amended the text to refer to recommendations for 'claiming freedom from disease' that are included in Chapter 1.4. and the disease-specific chapters in Sections 8-11.

The revised Chapter 1.1. is presented at <u>Annex 5</u> for Member Countries' comments.

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

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

Acute hepatopancreatic necrosis disease

At their February 2014 meeting, the Aquatic Animals Commission had considered the possible listing of acute hepatopancreatic necrosis disease (AHPND) in accordance with Article 1.2.2. Criteria for listing an aquatic animal disease. The Commission had concluded that due to the lack of specific diagnostic methods for the causative agent of AHPND, the disease could not be proposed for listing at that time.

At this meeting, the Aquatic Animals Commission recognised the significance of AHPND to many countries and, in light of the development of new diagnostic methods, the Commission reconsidered the AHPND for listing.

The Commission developed an assessment for AHPND, in accordance with Article 1.2.2., and concluded that it now meets the relevant criteria for listing.

In light of the Aquatic Animals Commission's proposal to list AHPND and recognising that it is essential to distinguish the causative agent of AHPND from other forms of the bacterium, the Commission recommended that an *ad hoc* Group be convened to develop a chapter on AHPND for inclusion in the *Aquatic Manual*.

In addition, the Aquatic Animals Commission updated the Technical Disease Card for AHPND which is available on the OIE website at:

http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Aquatic_Commission/AHPND_DEC_2013.pdf

The assessment for AHPND developed by the Commission is presented at <u>Annex 21</u> for Member Countries' information.

The revised Chapter 1.3. is presented at <u>Annex 6</u> for Member Countries' comments.

> **EU comment**
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> **The EU in general supports the proposed changes to this chapter. However, we would like to point out that there is an important discrimination to be made between the significant production losses associated with the syndromic condition currently/previously refered to as 'EMS' and the specific disease AHPND. Indeed, recent presentations by Asian colleagues at the annual meeting of the EU Reference**

Laboratory for crustacean diseases proposed that a relatively low proportion of outbreaks of 'EMS' are actually shown to be caused by the agent responsible for AHPND (i.e. variant *V. parahaemoyticus* as described by Tran *et al*. and subsequent papers). Overall, we would recommend that the issue of EMS vs AHPND be carefully considered and that wider causes for EMS are investigated in parallel with moves to list AHPND (e.g. 60% of cases were associated with infection with the emergent microsporidian *Enterocytozoon hepatopenaeii* and other hepatopancreatic pathologies).

Furthermore, given recent reports of another chytrid fungus (*Batrachochytrium salamandrivorans*), (Martel *et al*., 2014) causing disease and rapid declines in wild amphibians, the EU suggests that the OIE AAHC include in their workplan a review of emerging amphibian diseases against the listing criteria, with a view to possible listing additional pathogens. In this connection the EU notes that there is currently no OIE Reference Laboratory for amphibian diseases and hence it is very difficult to obtain positive samples for purposes related to laboratory diagnostics.

References:

Martel, A., M. Blooi, C. Adriaensen, P. Van Rooij, W. Beukema, M. C. Fisher, R. A. Farrer, B. R. Schmidt, U. Tobler, K. Goka, K. R. Lips, C. Muletz, K. R. Zamudio, J. Bosch, S. Lötters, E. Wombwell, T. W. J. Garner, A. A. Cunningham, A. Spitzen-van der Sluijs, S. Salvidio, R. Ducatelle, K. Nishikawa, T. T. Nguyen, J. E. Kolby, I. Van Bocxlaer, F. Bossuyt and F. Pasmans, 2014: Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science,* 346, 630-631.

Martel, A., A. Spitzen-van der Sluijs, M. Blooi, W. Bert, R. Ducatelle, M. C. Fisher, A. Woeltjes, W. Bosman, K. Chiers, F. Bossuyt and F. Pasmans, 2013: *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences,* 110, 15325-15329.

1.5. Import risk analysis (Chapter 2.1.)

The Aquatic Animals Commission reviewed amendments in the corresponding chapter of the *Terrestrial Code,* adopted by the World Assembly of Delegates in May 2014, where text had been removed because it was not directly pertinent to an import risk analysis. The Commission agreed to make the same amendments in the *Aquatic Code* chapter as they considered it important that such horizontal chapters in the two *Codes* aligned. The Commission also proposed to delete 'potential' from the term 'potential hazard' throughout the chapter because this word is inaccurate as a qualifier of hazard, an amendment also adopted in the *Terrestrial Code*.

The revised Chapter 2.1. is presented at Annex 7 for Member Countries' comments.

EU comment

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

1.6. General recommendations on disinfection (Chapter 4.3.) and Recommendations for surface disinfection of salmonid eggs (new Chapter 4.X.)

General recommendations on disinfection (Chapter 4.3.)

The Aquatic Animals Commission reiterated that during their February 2014 meeting they had reviewed Chapter 1.1.3. 'Methods for disinfection of aquaculture establishments' in the OIE *Aquatic Manual* and had agreed that this chapter was misplaced in the *Aquatic Manual.* The Commission also agreed that Chapter 4.3. 'Methods for disinfection of aquaculture establishments' in the *Aquatic Code*

should be revised to better address this topic. The Commission noted that Chapter 1.1.3. in the *Aquatic Manual* will be deleted in anticipation of the revised *Aquatic Code* chapter.

The Aquatic Animals Commission reviewed the preliminary draft Chapter 4.3. developed by the *ad hoc* Group on Disinfection and agreed that it provided a good framework for the revised chapter. The Commission recommended that the *ad hoc* Group continue work on the development of this chapter.

<u>Recommendations for surface disinfection of salmonid eggs (new Chapter 4.X.)</u>

The Aquatic Animals Commission reviewed the draft document developed by the *ad hoc* Group on Disinfection on 'Recommendations for surface disinfection of salmonid eggs'. The Commission agreed that this text should be proposed as a new Chapter 4.X. 'Recommendations for surface disinfection of salmonid eggs' in Section 4 'Disease prevention and control' of the *Aquatic Code*.

The Aquatic Animals Commission noted that once the proposed draft Chapter 4.X. is adopted, the cross reference to a protocol on disinfection of salmonid eggs that currently appears in Chapters (10.4., 10.5., 10.6. and 10.10) would be amended as shown below:

'a) the eggs should be disinfected prior to importing, according to the methods described in Chapter <u>4.X.</u> ~~1.1.3.~~ of the *Aquatic <u>Code</u> ~~Manual~~* ~~(under study)~~ or those specified by the Competent Authority of the importing country;'

The Aquatic Animals Commission recognised the need to expand recommendations for egg surface infection to other aquatic animal species, as appropriate, and requested that the *ad hoc* Group on Disinfection address this in their future work.

The new draft Chapter 4.X. is presented at <u>Annex 8</u> for Member Countries' comments.

EU comment

The EU in general supports the proposed new chapter. A specific comment is inserted in the text of Annex 8.

1.7. Control of hazards in aquatic animal feed (Chapter 4.7.)

The Aquatic Animals Commission reminded Member Countries' that it had proposed to revise Chapter 4.7. 'Control of hazards in aquatic animal feed'. The Commission prepared a revised draft chapter in line with the purpose and scope that had been agreed at their October 2013 meeting. The revised chapter exclusively addresses the risk of transmission of infectious diseases of aquatic animals via feed and the prevention of entry of pathogenic agents of concern via feed. The revised chapter aims at assisting Member Countries to identify risk pathways and assess the risks related to pathogenic agents in feed.

In view of the significant number of changes being proposed the revised chapter is provided as clean text only.

The revised Chapter 4.7. is presented at <u>Annex 9</u> for Member Countries' comments.

EU comment

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

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

In response to Member Countries' comments the Aquatic Animals Commission amended Article 5.1.2 points 1 and 2 to avoid use of the term "appropriate level of protection" in the *Aquatic Code* except when directly referring to the SPS Agreement, and to replace the phrase "more trade restrictive" with "stricter". The Aquatic Animals Commission noted that these amendments were aligned with those being proposed by the Code Commission in the corresponding chapter in the *Terrestrial Code*.

The revised Chapter 5.1. is presented at <u>Annex 10</u> for Member Countries' comments.

EU comment

> **The EU thanks the OIE for having taken some of its comments into consideration and supports the proposed changes to this chapter.**

1.9. Certification procedures (Chapter 5.2.)

The Aquatic Animals Commission accepted Member Countries' suggestions to replace the word "documentation" with "exchange of data" in the introductory clause of Article 5.2.4 point 1, to amend the reference for guidance on electronic certification in Article 5.2.4 point 1b, and to introduce a new point 1c on secure methods of electronic data exchange. The Aquatic Animals Commission noted that these amendments were aligned with those being proposed by the Code Commission in the corresponding chapter in the *Terrestrial Code*.

The revised Chapter 5.2. is presented at Annex 11 for Member Countries' comments.

> **EU comment**
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> **The EU thanks the OIE for having taken some of its comments into consideration and in general supports the proposed changes to this chapter. However, a comment is inserted in the text of Annex 11.**

1.10. Draft chapter 6.6. 'Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals'

The Aquatic Animals Commission reviewed the draft Chapter 6.6. 'Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals' that had been developed by the *ad hoc* Group on Antibiotic Use in Aquatic Animals. The Commission noted that the draft chapter includes Article 6.6.2. 'Special considerations for conducting antimicrobial resistance risk analysis in aquaculture' to highlight the differences between risk analysis for antimicrobial resistance in terrestrial and aquatic animals.

The new draft Chapter 6.6. is presented at Annex 12 for Member Countries' comments.

> **EU comment**
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> **The EU in general supports the proposed new chapter, which we note is largely based on the corresponding chapter of the Terrestrial Code. However, the wording of some points which have been extensively discussed in the framework of the Terrestrisal Code chapter do not reflect the results of these discussions. For the sake of consistency and clarity, the EU therefore suggests aligning these points with the wording in the Terrestrial Code (Chapter 6.10.). Comments to this effect are inserted in the text of Annex12.**

1.11. Amphibian disease-specific chapters (8.1. and 8.2.)

The Aquatic Animals Commission recognised that Articles 8.1.10. and 8.2.10. of amphibian disease-specific chapters needed amending as they currently include reference to live aquatic animals intended for use in laboratories, zoos and the pet trade which reflect particular aspects of international trade in amphibians. The Commission noted that the importation of live amphibians to be kept in laboratories or zoos or as pets carries a different level of risk compared to agricultural, industrial or pharmaceutical use.

The Aquatic Animals Commission therefore proposed new Articles 8.1.13. and 8.2.13. to address live amphibians intended for use in laboratories and zoos. In addition, the Commission proposed amending Articles 8.1.10. and 8.2.10. to specifically address the different level of risk for animals intended for use in laboratories and zoos compared with those intended for agricultural, industrial or pharmaceutical uses.

For Infection with *Batrachochytrium dendrobatidis* (Chapter 8.1.) the Aquatic Animals Commission recognised the inconsistency between the *Aquatic Code* and *Aquatic Manual*, in the recommendations for treatment prior to importation of amphibians intended for the pet trade. The Commission agreed,

as described in the *Aquatic Manual*, that treatment of live animals prior to importation is not considered an adequate risk management measure. The Commission therefore proposed the removal of the provision for treatment of live aquatic animals to eradicate infection from Articles 8.1.8. and 8.1.10.

The Aquatic Animals Commission acknowledged that the aquatic animal pet trade is an important importation pathway and should be addressed in the future.

The revised Articles 8.1.8., 8.1.10., and 8.1.13.; and 8.2.10., and 8.2.13. are presented at Annex 13A and 13B for Member Countries' comments.

EU comment

The EU supports the proposed changes to these chapters.

1.12. Articles X.X.7. and X.X.11. of disease-specific chapters

The Aquatic Animals Commission recognised that the text in Articles X.X.7. and X.X.11. in disease-specific chapters is almost identical. These articles apply to importation of live aquatic animals (Article X.X.7.) and importation of aquatic animal products (Article X.X.11.) from a country, zone or compartment declared free from Disease X. [Note: this issue applies to Articles 10.4.10., 10.4.15., 10.4.11. and 10.4.16. in Chapter 10.4.]

The Aquatic Animals Commission proposed to merge these two Articles to improve readability.

The revised model Article X.X.7 and X.X.11 are presented at Annex 14 for Member Countries' comments.

EU comment

The EU supports the proposed changes in these model articles.

1.13. Corrections in Articles 10.4.4. and 10.4.6.

The Aquatic Animals Commission recognised that the text in point 2 of Articles 10.4.4. and 10.4.6. in Chapter 10.4. is incorrect. The Commission proposed a correction of the relevant text.

The revised Articles 10.4.4. and 10.4.6. are presented at Annex 15 for Member Countries' information.

EU comment

The EU supports the proposed changes to this chapter.

1.14. Infection with *Perkinsus olseni* (Chapter 11.6.)

The Aquatic Animals Commission noted that the need to amend Article 11.6.2. as a consequence of the proposed change in the corresponding *Aquatic Manual* chapter (see also Item 2.2.3.). The Commission noted that this amendment would be proposed for adoption in 2015 along with adoption of the corresponding amended *Aquatic Manual* chapter.

The revised Article 11.6.2. is presented at Annex 22 for Member Countries' information.

1.15. Criteria for listing species as susceptible to infection with a specific pathogen

Following adoption of a new Chapter 1.5. 'Criteria for listing species as susceptible to infection with a specific pathogen' at the 2014 General Session the Aquatic Animals Commission discussed the next steps to apply the criteria progressively to each OIE listed disease. The Commission agreed that an *ad hoc* Group should be convened to commence assessments for all OIE listed crustacean diseases starting with Yellow Head Disease as a pilot.

The Aquatic Animals Commission recommended that a new *ad hoc* Group be convened to commence this work.

2. *Manual of Diagnostic Tests for Aquatic Animals*

    2.1. **Review of the *Manual of Diagnostic Tests for Aquatic Animals* chapters**

        2.2.1. **Four crustacean chapters**

        In response to a Member Country's comments on Chapters 2.2.2. Infectious hypodermal and haematopoietic necrosis; 2.2.4. Necrotising hepatopancreatitis; 2.2.5. Taura syndrome, and 2.2.8. Yellow head disease, the Aquatic Animals Commission, in consultation with the chapters, authors, reviewed the comments and amended the text.

        The revised Chapters 2.2.2., 2.2.4., 2.2.8. and 2.2.5. are presented at Annex 16-19 for Member Countries' comment.

EU comment

The EU supports the proposed changes to these chapters.

    2.2. **Other Aquatic Manual issues**

        2.2.1. **Validation of diagnostic tests**

        The Aquatic Animals Commission noted that information of validation and performance of assays in the *Aquatic Manual* is inconsistent between chapters and absent in some cases. The Commission requested that Reference Laboratory experts be asked to update this information following a standard format.

        2.2.2. **Information on agent stability**

        The Aquatic Animals Commission agreed with a recommendation from the *ad hoc* Group on Disinfection (see also Item 1.6.) that the *Aquatic Manual* should provide more consistent information on agent stability, including the efficacy of disinfectants, where information is available. The Commission requested that Reference Laboratory experts be asked to review and update this information as appropriate.

        2.2.3. **Listing of *Crassostrea gigas* as susceptible to infection with *Perkinsus olseni* (Chapter 2.4.6.)**

        The Aquatic Animals Commission considered a Member Country's comment regarding the justification for the listing of *Crassostrea gigas* as a susceptible host species in both the *Aquatic Code* and *Aquatic Manual* chapters on infection with *Perkinsus olseni*. The Commission consulted the Reference Laboratory expert who confirmed that there is no information to substantiate that *Crassostrea gigas* is susceptible to *P. olseni*. The Commission therefore agreed to remove *Crassostrea gigas* from the list of susceptible species in the *Aquatic Code* (Chapter 11.6.) (see also Item 1.14.) and the *Aquatic Manual* Chapter 2.4.6.

        The revised section 2.2.1. of Chapter 2.4.6. is presented as Annex 20 for Member Countries' comment.

EU comment

The EU supports the proposed changes to this chapter.

        2.2.4. **Infection with Xenohaliotis califormiensis (Chapter 2.4.7.)**

The Aquatic Animals Commission reviewed a Member Country submission requesting that Chapter 2.4.7. Infection with *Xenohaliotis californiensis* be updated to include a diagnostic test that has been recently published. The Commission requested that this proposal be forwarded to the Reference Laboratory expert for their consideration.

2.2.5. Disease specific guidance documents on surveillance for a crustacean disease

The Aquatic Animals Commission reviewed the document on 'Surveillance for white spot virus' and requested that this be uploaded onto the Commission's webpages.

The Aquatic Animals Commission reminded Member Countries that documents on 'Surveillance for infection with *Bonamia ostreae*'and 'Surveillance for viral haemorrhagic septicaemia are also available on the Commission's webpages and are available at:

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

'The Aquatic Animals Commission noted that these documents provide valuable information to Member Countries and practical guidance on the implementation of the OIE standards for surveillance.

3. OIE Reference Centres

The Aquatic Animals Commission was updated on proposals from the Biological Standards Commission regarding Reference Centres. In view of the growing number of OIE Reference Laboratory applications and designations, the importance of assessing and monitoring the performance of the laboratories is growing. The Biological Standards Commission has proposed that all future applicants should already be a national reference laboratory for the disease in question, before submitting an application for OIE Reference Laboratory status.

Also, given that quality management systems are essential, the Biological Standards Commission agreed that all OIE Reference Laboratories must be accredited to ISO 17025 or equivalent. This requirement would apply to all new applicants. Existing OIE Reference Laboratories that are not yet accredited would be given 3-years to achieve this standard. Laboratories would be asked to upload a copy of their accreditation certificates in their annual report.

The Aquatic Animals Commission noted these proposals and encouraged Member Countries to consider the implications for OIE Reference Laboratories for aquatic animal diseases and to submit comments for consideration by the Aquatic Animals Commission.

EU comment

The EU supports the proposals from the Biological Standards Commission.

4. Twinning Projects

The Aquatic Animals Commission was updated on the status of aquatic animal disease twinning projects. As of September 2014, one project has been completed (Canada with Chile for infectious salmon anaemia), 2 are underway (USA with China for infectious haematopoietic necrosis; Norway with Brazil for infectious salmon anaemia) and 2 are approved and due to start (USA with Indonesia for shrimp diseases; Japan with Indonesia for koi herpesvirus disease). They noted that Chile became an OIE Reference Laboratory for Infection with infectious salmon anaemia virus in May 2014, following completion of a twinning project.

The Aquatic Animals Commission reviewed a twinning project proposal for viral encephalopathy and retinopathy, and provided technical comments.

5. Aquatic Animals Commission Work Plan for 2014/2015

The Aquatic Animals Commission reviewed and updated their work plan. The work plan provides Member Countries an overview of current and upcoming activities.

The detailed Aquatic Animals Commission's Work Plan for 2014/15 is presented at <u>Annex 23</u> for Member Countries information.

6. Third Global Conference of the OIE Reference Centres, Seoul (Rep. of Korea), 14–16 October 2014

The Aquatic Animals Commission noted that the draft programme for the Third Global Conference of the OIE Reference Centres includes a parallel session on aquatic animal disease. The Commission noted that this dedicated session will provide an opportunity to better inform the OIE designated experts on the most recent advances in the work of the Commission. They noted also that this session will address specific issues of importance such as validation of diagnostic tests, consistency in ranking diagnostic methods with regards to their purpose, and quality assurance in OIE Reference Centers

7. Third OIE Global Conference on Aquatic Animal Health: 'Riding the wave to the future', Ho Chi Minh City (Vietnam), 20-22 January 2015

The Aquatic Animals Commission highlighted that the upcoming Global Conference on Aquatic Animal Health (20-22 January, 2015 in Ho Chi Minh City, Vietnam) is an important event for the Commission because it will provide a forum for discussing important concepts in aquatic animal health and the recommendations will be important to guide the future work of the Commission and the OIE. All relevant information about the Conference can be found at: http://oie.int/eng/A_AAHRWF2015/introduction.htm

8. Other Business

8.1. Disease prioritisation for vaccine development

The Aquatic Animals Commission was informed of a proposal to prioritise diseases to guide vaccine development in aquatic and terrestrial animals with the goal of decreasing the use of antimicrobials in animals. The Commission requested to be kept informed of progress on this proposal and indicated their willingness to participate as required.

9. Next meeting

The next meeting is scheduled 2 to 6 March 2015.

_____.../*Annexes*

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

Paris, 29 September–3 October 2014

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OIE Aquatic Animal Health Standards Commission/September 2014

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

Paris, 29 September–3 October 2014

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Adopted agenda

1. OIE *Aquatic Animal Health Code*

    1.1.   User's guide

    1.2.   Glossary

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

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

    1.5.   Import risk analysis (Chapter 2.1.)

    1.6.   General recommendations of disinfection (Chapter 4.3.) and Recommendations for disinfection of salmonid eggs (new Chapter 4.X.)

    1.7.   Control of hazards in aquatic animal feed (Chapter 4.7.)

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

    1.9.   Certification procedures (Chapter 5.2.)

    1.10. Draft chapter 6.6. 'Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals'

    1.11. Amphibian disease-specific chapters (8.1. and 8.2.)

    1.12. Articles X.X.7. and X.X.11. of disease-specific chapters

    1.13. Corrections in Articles 10.4.4. and 10.4.6.

    1.14. Infection with *Perkinsus olseni* (Chapter 11.6.)

    1.15. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents

2. *Manual of Diagnostic Tests for Aquatic Animals*

    2.1.   Review of the *Manual of Diagnostic Tests for Aquatic Animals* chapters

        2.1.1. Four crustacean chapters

Annex 2 (contd)

2.2.   Other *Aquatic Manual* issues

2.2.1. Validation of diagnostic tests

2.2.2. Information on agent stability

2.2.3. Listing of *Crassostrea gigas* as susceptible to infection with *Perkinsus olseni* (Chapter 2.4.6.)

2.2.4. Infection with *Xenohaliotis califormiensis* (Chapter 2.4.7.)

2.2.5.   Disease specific guidance documents on surveillance for a crustacean disease

3. OIE Reference Centres

4. Twinning projects

5. Aquatic Animals Commission Work Plan for 2014/2015

6. Third Global Conference of the OIE Reference Centres, Seoul (Rep. of Korea), 14–16 October 2014

7. OIE Global Conference on Aquatic Animal Health: 'Riding the wave to the future'

8. Other Business

8.1.   Disease prioritisation for the vaccine manufacturing

9. Next meeting

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~~GUIDE TO THE~~ USER'S GUIDE ~~OF THE~~
AQUATIC ANIMAL HEALTH CODE

EU comment

The EU thanks the OIE and in general supports the proposed changes to the user's guide. A specific comment is inserted in the text below.

A. Introduction

1) The OIE *Aquatic Animal Health Code* (hereafter referred to as the *Aquatic Code*) ~~sets out~~ <u>provides</u> standards for the improvement of aquatic animal health worldwide. ~~More recently, t~~<u>T</u>he *Aquatic Code* ~~has~~ also include<u>s</u>~~d~~ standards for the welfare of farmed fish <u>and antimicrobial use in aquatic animals</u>. The purpose of this guide is to advise the ~~Veterinary Authorities and other~~ Competent Authorities in OIE Member Countries on how to use the *Aquatic Code.*

2) ~~Veterinary Authorities and other~~ Competent Authorities should use the standards in the *Aquatic Code* to ~~set up~~ <u>develop</u> measures ~~providing~~ for early detection, internal reporting, notification and control of pathogenic agents in aquatic animals (amphibians, crustaceans, fish and molluscs) and preventing their spread via international trade in aquatic animals and aquatic animal products, while avoiding unjustified sanitary barriers to trade.

3) ~~The *Aquatic Code* currently does not encompass any zoonotic disease, however, veterinary public health is part of the mandate of the OIE, including in the field of aquatic animal health.~~

<u>3</u>4) The OIE standards are based on the most recent scientific and technical information. Correctly applied, they protect aquatic animal health <u>during the production and trade in aquatic animals and aquatic animal products as well as the</u> ~~and~~ welfare of farmed fish ~~during production and trade in aquatic animals and aquatic animal products~~.

<u>4</u>5) The absence of chapters, articles or recommendations on particular pathogenic agents or commodities does not mean that ~~Veterinary Authorities and other~~ Competent Authorities may not apply appropriate *aquatic animal* health and welfare measures <u>based on risk analysis conducted in accordance with the *Aquatic Code*.</u> ~~However, such measures should be based on sound scientific justification according to the principles of the WTO SPS Agreement.~~

<u>5</u>6) The complete text of the *Aquatic Code* is available on the OIE website and may be downloaded from: http://www.oie.int.

B. *Aquatic Code* content

1) Key terms and expressions used in more than one chapter in the *Aquatic Code* ~~with a contextual meaning~~ are defined in the Glossary. The reader should be aware of ~~the contextual~~ definitions given in the Glossary when reading and using the *Aquatic Code.* Defined terms appear in italics. In the on-line version of the *Aquatic Code*, a hyperlink leads to the relevant definition.

2) The term '(under study)' is found in some rare instances, with reference to an article or part of an article. This means that this part of the text has not been adopted by the World Assembly of OIE Delegates and the particular provisions are thus not part of the *Aquatic Code*.

3) The standards in the chapters of Section 1 are designed for the implementation of measures for the ~~diagnosis,~~ surveillance and notification of pathogenic agents. The <u>section</u> ~~standards~~ include<u>s</u> the criteria for listing aquatic animal diseases, <u>the</u> diseases <u>which are</u> listed by the OIE, procedures for notification to the OIE<u>, and criteria for listing species as susceptible to infection with a specific pathogen</u>.

4) The standards in the chapters of Section 2 are designed to guide the importing country in conducting import risk analysis in the absence of OIE trade standards. The importing country may also use these standards to justify import measures which are ~~more trade restrictive~~ stricter than existing OIE trade standards.

5) The standards in the chapters of Section 3 are designed for the establishment, maintenance and evaluation of Aquatic Animal Health Services, including communication. These standards are intended to assist the Veterinary Services and Aquatic Animal Health Services of Member Countries to meet their objectives of improving aquatic animal health and welfare of farmed fish, as well as to establish and maintain confidence in their international aquatic animal health certificates.

6) The standards in the chapters of Section 4 are designed for the implementation of measures for the prevention and control of pathogenic agents. Measures in this section include zoning, compartmentalisation, disinfection, contingency planning, fallowing, ~~and~~ disposal of aquatic animal waste and control of hazards in aquatic animal feed.

7) The standards in the chapters of Section 5 are designed for the implementation of general sanitary measures for trade. ~~In particular,~~ They ~~chapters~~ address certification and the measures applicable by the exporting, transit and importing countries. ~~Section 5 also includes Aa~~ range of model health certificates are provided for consistent documentation ~~to be used as a harmonised basis~~ for international trade.

8) The standards in the chapters of Section 6 are designed to ensure the responsible and prudent use of antimicrobial agents in aquatic animals.

9) The standards in the chapters of Section 7 are designed for the implementation of welfare measures for farmed fish. The standards cover the general principles for welfare of farmed fish, including ~~their welfare~~ during ~~their~~ transport, ~~at the time of~~ stunning and killing for human consumption, and when ~~as well as in the situation of~~ killing for disease control purposes.

10) The standards in each of the chapters of Sections 8 to 11 are designed to prevent the ~~aetiological~~ pathogenic agents of OIE listed diseases from being introduced into an importing country. Each disease chapter includes a list of currently known susceptible species. The standards take into account the nature of the traded commodity, the aquatic animal health status of the exporting country, zone or compartment, and the risk reduction measures applicable to each commodity.

These standards assume that the agent is either not present in the importing country or is the subject of a control or eradication programme. Sections 8 to 11 each relate to amphibian, crustacean, fish and molluscan hosts, respectively. ~~Chapters include specific measures to prevent and control the infections of global concern.~~

C. Specific issues

1) **Notification**

Chapter 1.1. describes Member Countries' obligations under the OIE Organic Statutes. Listed ~~as well as emerging~~ diseases, as prescribed in Chapter 1.1., are compulsorily notifiable. Member Countries are encouraged ~~also~~ to also provide information to the OIE on other aquatic animal health events of epidemiological significance, including occurrence of emerging diseases.

Chapter 1.2. describes the criteria for the inclusion of a disease listed by the OIE.

Chapter 1.3. ~~provides~~ specifies the diseases that are listed by the OIE. Diseases are divided into four sections corresponding to amphibian, crustacean, fish and molluscan hosts, respectively.

2) **Pathogen differentiation**

Some pathogens have one or more variants. Existence of highly pathogenic variants and the need to differentiate them from more benign variants is recognised in the *Aquatic Code*. When pathogenic agents have strains that are stable, possess characteristics that can be used for diagnostic purposes, and display different levels of pathogenicity, different standards providing protection ~~should be~~ proportionate to the risk posed by the different strains ~~of the pathogenic agent~~ should be applied. Infection with infectious salmon anaemia virus is a ~~The first~~ listed disease for which ~~offering~~ risk

management options based on strain differentiation ~~are provided~~ is Infection with infectious salmon anaemia virus.

3) **Determining the susceptibility of species**

The *Aquatic Code* proposes the use of criteria to assess the susceptibility of host species to the pathogenic agents of diseases listed in the *Aquatic Code*. This is ~~of particular~~ importan<u>ce</u> in the ~~context of~~ aquaculture <u>context,</u> given the large number~~s~~ of <u>existing</u> ~~species,~~ and ~~the number of~~ new <u>aquaculture</u> species ~~existing in~~ <u>being introduced to</u> aquaculture.

4) **Trade requirements**

~~International a~~<u>A</u>quatic animal health <u>measures elated to international</u> trade ~~measures~~ should be based on OIE standards. A Member Country may authorise the importation of aquatic animals or aquatic animal products into its territory under conditions more or less restrictive than those recommended by the *Aquatic Code*. ~~To scientifically justify~~ <u>If measures are</u> more trade restrictive <u>than OIE standards</u> ~~measures~~ the importing country should <u>provide scientific justification by</u> conduct<u>ing</u> a risk analysis in accordance with OIE standards, as described in Chapter 2.1. Members of the WTO should refer to the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement).

Chapters 5.1. to 5.3. describe the obligations and ~~ethics~~ <u>ethical responsibilities of importing and exporting countries</u> in international trade. ~~Veterinary Authorities and other~~ Competent Authorities and all veterinarians ~~or~~ <u>and</u> certifying officials directly involved in international trade should be familiar with these chapters. ~~These~~ <u>c</u>Chapter~~s~~ 5.3. provide<u>s</u> guidance for informal mediation by the OIE.

<u>Disease-specific chapters in the *Aquatic Code* include articles listing the commodities that are considered safe for trade without the imposition of disease-specific sanitary measures, regardless of the status of the country or zone for the pathogenic agent in question. Where such a list is present, importing countries should not apply trade restrictions to the listed commodities with respect to the agent in question.</u>

EU comment

The EU notes that the above description of the concept of safe commodities has been applied for many years in the context of the Aquatic Code, and that it is proposed to apply this approach now also in the Terrestrial Code, for which the Aquatic Code could serve as model. Reference is made to the EU comments on the new concept of safe commodities in the report of the Code Commission (Annexes IV, VI and XXIV).

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5) **Trade in aquatic animal commodities**

Chapter 5.4. describes the criteria used to assess the safety of aquatic animal commodities.

Based on assessments using criteria in Article 5.4.1., in all disease chapters, point 1 of Article X.X.3. lists aquatic animal ~~products~~ <u>commodities</u> that may be imported for any purpose from a country, zone or compartment not declared free from the disease in question. The criteria for inclusion of aquatic animal ~~products~~ <u>commodities</u> in point 1 of Article X.X.3. are based on the absence of the pathogenic agent ~~in the traded aquatic animals~~ <u>commodity</u> ~~and aquatic animal products~~ or inactivation of the pathogenic agent by treatment or processing.

Based on assessments using criteria in Article 5.4.2, in all disease chapters, point 1 of Article X.X.12. <u>(for Chapter 10.4. the relevant Article is 10.4.17.)</u> ~~(amphibian and fish disease chapters) and Article X.X.11. (crustacean and mollusc disease chapters)~~ lists aquatic animal~~s~~ ~~or aquatic animal products~~ <u>commodities</u> for retail trade for human consumption <u>from a country, zone or compartment not declared free from the disease in question</u>. The criteria for inclusion of aquatic animal~~s~~ <u>commodities</u> ~~or aquatic animal products~~ in point 1 of Article X.X.12. ~~(amphibian and fish disease chapters) and Article X.X.11. (crustacean and mollusc disease chapters)~~ include consideration of the form and presentation of the product, the expected volume of waste tissues generated by the consumer and the likely presence of viable pathogenic agent in the waste.

~~Disease-specific chapters in the *Aquatic Code* reflect the reality of trade and include traded commodities, accounting for their diversity, and propose a list of safe commodities for trade facilitation. The disease-specific chapters of the Aquatic Code include an article listing the commodities that are considered safe for trade without the imposition of sanitary measures, regardless of the status of the country or zone for the agent in question. This is a work in progress and some chapters do not yet contain articles listing safe commodities. Where such a list is present, importing countries should not apply trade restrictions to the listed commodities with respect to the agent in question.~~

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— Text deeted.

GLOSSARY

EU comment

The EU in general supports the proposed changes to the glossary, in particular the new definition of biosecurity. However, a specific comment is inserted in the text below.

Furthermore, the EU would like to suggest a change in the definition of "Veterinary Authority" to explicitly mention veterinary public health, as follows:

"means the Governmental Authority of a Member Country, comprising *veterinarians*, other professionals and para-professionals, having the responsibility and competence for ensuring or supervising the implementation of *aquatic animal* health and welfare measures, international *aquatic animal* health certification and other standards, guidelines and recommendations in the veterinary public health domains as referred to in the *Aquatic Code* in the whole *territory*.".

Indeed, the OIE standards which are to be implemented by the veterinary authorities of OIE Members go beyond animal health and welfare, and encompass veterinary public health including animal production food safety, as indicated in Section 6 of the OIE Aquatic Code. Similar comments are made on the glossary of the Terrrestrial Code in the EU comments on the TAHSC September meeting report.

DISINFECTANTS

> means chemical compounds <u>or physical processes</u> capable of destroying *pathogenic <u>agents</u>* ~~microorganisms~~ or inhibiting their growth ~~or survival ability~~.

DISINFECTION

> ~~means the application, after thorough cleansing, of procedures intended to destroy the infectious or parasitic agents of *diseases* of *aquatic animals*, including zoonoses; this applies to *aquaculture establishments* (i.e. hatcheries, fish farms, oyster farms, shrimp farms, nurseries, etc.), *vehicles*, and different equipment/objects that may have been directly or indirectly contaminated.~~

> <u>means the process of cleaning and applying *disinfectants* to inactivate *pathogenic agents* on potentially contaminated items.</u>

EU comment

While understanding the rationale for the proposals of the relevant ad hoc group as regards the definitions of disinfectants and disinfection above, the EU has concerns as to possible confusion, especially when in the Aquatic Code physical processes will be regarded as disinfectants, and cleaning will be included in the definition of disinfection. Indeed, this is contrary to common understanding and dictionary definitions of these terms, and their use in the Terrestrial Code.

An alternative could be to define disinfection as a two-stage process, changing the order in the sentence, i.e. to mention disinfecton first, as follows:

"means the process of ~~cleaning and~~ applying *disinfectants*, after thorough cleansing, to inactivate *pathogenic agents* on potentially contaminated items".

> **Indeed, disinfection is a separate process to cleaning. Cleaning is undertaken to ensure the removal of all organic matter and is normally conducted prior to disinfection. Adequate cleaning is essential to help ensure appropriate disinfection.**

BIOSECURITY

means the set of management and physical measures designed to reduce the *risk* of introduction, establishment and spread of *pathogenic agents* to, from and within an *aquatic animal* population.

HAZARD IDENTIFICATION

means the process of identifying the pathogenic agent(s) which could potentially be introduced in the commodity considered for importation.

INFECTIVE PERIOD

means the longest period during which an affected *aquatic animal* can be a source of *infection*.

RISK ANALYSIS

means the complete process composed of *hazard identification* identification, *risk assessment*, *risk management* and *risk communication*.

RISK ASSESSMENT

means the scientific evaluation of the likelihood and the biological and economic consequences of entry, establishment and spread of a *hazard* within the *territory* of an *importing country*.

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— Text deleted.

C H A P T E R 1 . 1 .

NOTIFICATION OF DISEASES, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

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

[…]

Article 1.1.5.

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

2) An *infected zone* or *compartment* for a particular *disease* shall be considered as such until <u>freedom from the *disease* has been demonstrated in accordance with recommendations in Chapter 1.4. and the relevant recommendations described in the *disease*-specific chapters in Sections 8 to 11.</u> ~~a period exceeding the *infective period* specified in the *Aquatic Code* has elapsed after the last reported *case* and when full prophylactic and appropriate *aquatic animal* health measures have been applied to prevent possible reappearance or spread of the *disease*. These measures will be found in detail in various *disease* specific chapters of the *Aquatic Code*.~~

3) A Member Country may be considered to regain freedom from a specific *disease* when all relevant conditions given in the *Aquatic Code* have been fulfilled.

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

[…]

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— Text deleted.

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

EU comment

The EU in general supports the proposed changes to this chapter. Reference is made to general comments included on this point in item 1.4. of the introduction part of this report.

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 1.3.1.

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

– Epizootic haematopoietic necrosis

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

– Infection with *Gyrodactylus salaris*

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

– Infection with salmonid alphavirus

– Infectious haematopoietic necrosis

– Koi herpesvirus disease

– Red sea bream iridoviral disease

– Spring viraemia of carp

– Viral haemorrhagic septicaemia.

Article 1.3.2.

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

– Infection with abalone herpesvirus

– Infection with *Bonamia ostreae*

– Infection with *Bonamia exitiosa*

– Infection with *Marteilia refringens*

– Infection with *Perkinsus marinus*

– Infection with *Perkinsus olseni*

– Infection with *Xenohaliotis californiensis*.

Article 1.3.3.

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

= <u>Acute hepatopancreatic necrosis disease</u>

– Crayfish plague (*Aphanomyces astaci*)

– Infection with yellow head virus

– Infectious hypodermal and haematopoietic necrosis

– Infectious myonecrosis

– Necrotising hepatopancreatitis

– Taura syndrome

– White spot disease

– White tail disease.

Article 1.3.4.

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

– Infection with *Batrachochytrium dendrobatidis*

– Infection with ranavirus.

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CHAPTER 2.1.

IMPORT RISK ANALYSIS

EU comment

The EU supports the proposed changes to this chapter.

We understand that further to the adoption of the changes proposed in the glossary as regards the definitions related to risk analysis, the consequential changes as to the italisising of those terms in this chapter will be done at a later stage.

Article 2.1.1.

Introduction

The importation of *aquatic animals* and *aquatic animal products* involves a degree of *disease risk* to the *importing country*. This *risk* may be represented by one or several *diseases* or *infections*.

The principal aim of import *risk analysis* is to provide *importing countries* with an objective and defensible method of assessing the *disease risks* associated with the importation of *aquatic animals*, *aquatic animal products*, *aquatic animal* genetic material, feedstuffs, *biological products* and *pathological material*. The principles and methods are the same whether the *commodities* are derived from aquatic and/or terrestrial animal sources. The analysis should be transparent. This is necessary so that the *exporting country* is provided with clear reasons for the imposition of import conditions or refusal to import.

Transparency is also essential because data are often uncertain or incomplete and, without full documentation, the distinction between facts and the analyst's value judgements may blur.

This chapter provides recommendations and principles for conducting transparent, objective and defensible *risk analyses* for *international trade*. However, it cannot provide details on the means by which a *risk analysis* is carried out as the purpose of the *Aquatic Code* is simply to outline the necessary basic steps. The components of *risk analysis* ~~described in this chapter~~ are *hazard identification*, *risk assessment*, *risk management* and *risk communication* (Figure 1).
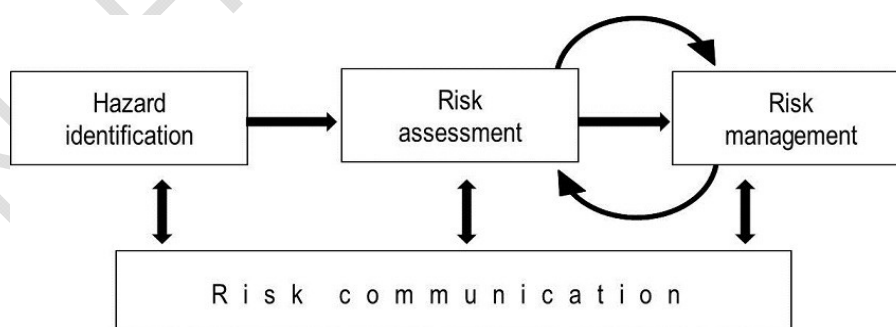

Fig. 1. The four components of risk analysis

The *risk assessment* is the component of the analysis that estimates the *risks* associated with a *hazard*. *Risk assessments* may be qualitative or quantitative. For many *diseases*, particularly for those *diseases* listed in the *Aquatic Code* where there are well developed internationally agreed standards, there is broad agreement concerning the likely *risks*. In such cases it is more likely that a qualitative assessment is all that is required. Qualitative assessment does not require mathematical modelling skills to carry out and so is

often the type of assessment used for routine decision-making. No single method of import *risk assessment* has proven applicable in all situations, and different methods may be appropriate in different circumstances.

The process of import *risk analysis* on *aquatic animals* and *aquatic animal products* usually needs to take into consideration the results of an evaluation of the *Aquatic Animal Health Services*, zoning and compartmentalisation, and *surveillance* systems that are in place for monitoring *aquatic animal* health in the *exporting country*. These are described in separate chapters in the *Aquatic Code*.

<div align="center">Article 2.1.2.</div>

Hazard identification

Hazard identification involves identifying the *pathogenic agents* that could potentially produce adverse consequences associated with the importation of a *commodity*.

The ~~potential~~ *hazards* identified would be those appropriate to the species being imported, or from which the *commodity* is derived, and which may be present in the *exporting country*. It is then necessary to identify whether each *hazard* is already present in the *importing country*, and whether it is a *listed disease* or is subject to control or eradication in that country and to ensure that import measures are not more trade restrictive than those applied within the country.

Hazard identification is a categorisation step, identifying biological agents dichotomously as ~~potential~~ *hazards* or not *hazards*. The *risk assessment* should be concluded if *hazard identification* fails to identify *hazards* associated with the importation.

The evaluation of the *Aquatic Animal Health Services*, *surveillance* and control programmes, and zoning and compartmentalisation systems are important inputs for assessing the likelihood of *hazards* being present in the *aquatic animal* population of the *exporting country*.

An *importing country* may decide to permit the importation using the appropriate sanitary standards recommended in the *Aquatic Code*, thus eliminating the need for a *risk assessment*.

<div align="center">Article 2.1.3.</div>

Principles of risk assessment

1) *Risk assessment* should be flexible in order to deal with the complexity of real-life situations. No single method is applicable in all cases. *Risk assessment* should be able to accommodate the variety of *aquatic animal commodities*, the multiple *hazards* that may be identified with an importation and the specificity of each *disease*, detection and *surveillance* systems, exposure scenarios and types and amounts of data and information.

2) Both qualitative *risk assessment* and quantitative *risk assessment* methods are valid.

3) The *risk assessment* should be based on the best available information that is in accord with current scientific thinking. The assessment should be well documented and supported with references to the scientific literature and other sources, including expert opinion.

4) Consistency in *risk assessment* methods should be encouraged and transparency is essential in order to ensure fairness and rationality, consistency in decision-making and ease of understanding by all the interested parties.

5) *Risk assessments* should document the uncertainties, the assumptions made, and the effect of these on the final *risk* estimate.

6) *Risk* increases with increasing volume of *commodity* imported.

7) The *risk assessment* should be amenable to updating when additional information becomes available.

Article 2.1.4.

Risk assessment steps

1. Entry assessment

    Entry assessment consists of describing the biological pathway(s) necessary for an importation activity to introduce a *pathogenic agent* into a particular environment, and estimating the probability of that complete process occurring, either qualitatively (in words) or quantitatively (as a numerical estimate). The entry assessment describes the probability of the entry of each of the ~~potential~~ *hazards* (the *pathogenic agents*) or under each specified set of conditions with respect to amounts and timing, and how these might change as a result of various actions, events or measures. Examples of the kind of inputs that may be required in the entry assessment are:

    a) Biological factors

        –   Species, strain or genotype, and age of *aquatic animal*

        –   Strain of agent

        –   Tissue sites of *infection* and/or contamination

        –   Vaccination, testing, treatment and *quarantine*.

    b) Country factors

        –   *Incidence* or *prevalence*

        –   Evaluation of *Aquatic Animal Health Services*, *surveillance* and control programmes, and zoning and compartmentalisation systems of the *exporting country*.

    c) Commodity factors

        –   Whether the *commodity* is alive or dead

        –   Quantity of *commodity* to be imported

        –   Ease of contamination

        –   Effect of the various processing methods on the *pathogenic agent* in the *commodity*

        –   Effect of storage and transport on the *pathogenic agent* in the *commodity*.

    If the entry assessment demonstrates no significant *risk*, the *risk assessment* does not need to continue.

2. Exposure assessment

    Exposure assessment consists of describing the biological pathway(s) necessary for exposure of animals and humans in the *importing country* to the *hazards* (in this case the *pathogenic agents*) from a given *risk* source, and estimating the probability of these exposure(s) occurring, either qualitatively (in words) or quantitatively (as a numerical estimate).

Annex 7 (contd)

The probability of exposure to the identified *hazards* is estimated for specified exposure conditions with respect to amounts, timing, frequency, duration of exposure, routes of exposure, and the number, species and other characteristics of the animal and human populations exposed. Examples of the kind of inputs that may be required in the exposure assessment are:

a) Biological factors

   –   Properties of the agent (e.g. virulence, pathogenicity and survival parameters)

   –   Genotype of host.

b) Country factors

   –   Presence of potential vectors or intermediate hosts

   –   *Aquatic animal* demographics (e.g. presence of known susceptible and carrier species, distribution)

   –   Human and terrestrial animal demographics (e.g. possibility of scavengers, presence of piscivorous birds)

   –   Customs and cultural practices

   –   Geographical and environmental characteristics (e.g. hydrographic data, temperature ranges, water courses).

c) Commodity factors

   –   Whether the *commodity* is alive or dead

   –   Quantity of *commodity* to be imported

   –   Intended use of the imported *aquatic animals* or *products* (e.g. domestic consumption, restocking, incorporation in or use as *aquaculture feed* or bait)

   –   Waste disposal practices.

If the exposure assessment demonstrates no significant *risk*, the *risk assessment* may conclude at this step.

3. Consequence assessment

Consequence assessment consists of describing the relationship between specified exposures to a biological agent and the consequences of those exposures. A causal process should exist by which exposures produce adverse health or environmental consequences, which may in turn lead to socio-economic consequences. The consequence assessment describes the potential consequences of a given exposure and estimates the probability of them occurring. This estimate may be either qualitative (in words) or quantitative (a numerical estimate). Examples of consequences include:

a) Direct consequences

   –   *Aquatic animal infection*, *disease*, production losses and facility closures

   –   Public health consequences.

    b)   Indirect consequences

        –   *Surveillance* and control costs

        –   Compensation costs

        –   Potential trade losses

        –   Adverse, and possibly irreversible, consequences to the environment.

4.   <u>Risk estimation</u>

Risk estimation consists of integrating the results of the entry assessment, exposure assessment, and consequence assessment to produce overall measures of *risks* associated with the *hazards* identified at the outset. Thus *risk* estimation takes into account the whole of the *risk* pathway from *hazard* identified to unwanted outcome.

For a quantitative assessment, the final outputs may include:

–   The various populations of *aquatic animals* and/or estimated numbers of *aquaculture establishments* or people likely to experience health impacts of various degrees of severity over time

–   Probability distributions, confidence intervals, and other means for expressing the uncertainties in these estimates

–   Portrayal of the variance of all model inputs

–   A sensitivity analysis to rank the inputs as to their contribution to the variance of the *risk* estimation output

–   Analysis of the dependence and correlation between model inputs.

<p align="center">Article 2.1.5.</p>

Principles of risk management

1)   *Risk management* is the process of deciding upon and implementing measures to <u>address the *risks* identified in the *risk assessment*</u> ~~achieve the Member Country's appropriate level of protection~~, whilst at the same time ensuring that negative effects on trade are minimised. The objective is to manage *risk* appropriately to ensure that a balance is achieved between a country's desire to minimise the likelihood or frequency of *disease* incursions and their consequences and its desire to import *commodities* and fulfil its obligations under international trade agreements.

2)   The international standards of the OIE are the preferred choice of *sanitary measures* for *risk management*. The application of these *sanitary measures* should be in accordance with the intentions of the standards.

<p align="center">Article 2.1.6.</p>

Risk management components

1)   *Risk* evaluation - the process of comparing the *risk* estimated in the *risk assessment* with the <u>reduction in *risk* expected from the proposed *risk management* measures</u> ~~Member Country's appropriate level of protection~~.

Annex 7 (contd)

2) Option evaluation - the process of identifying, evaluating the efficacy and feasibility of, and selecting measures to reduce the *risk* associated with an importation ~~in order to bring in line with the Member Country's appropriate level of protection~~. The efficacy is the degree to which an option reduces the likelihood or magnitude of adverse health and economic consequences. Evaluating the efficacy of the options selected is an iterative process that involves their incorporation into the *risk assessment* and then comparing the resulting level of *risk* with that considered acceptable. The evaluation for feasibility normally focuses on technical, operational and economic factors affecting the implementation of the *risk management* options.

3) Implementation - the process of following through with the *risk management* decision and ensuring that the *risk management* measures are in place.

4) Monitoring and review - the ongoing process by which the *risk management* measures are continuously audited to ensure that they are achieving the results intended.

Article 2.1.7.

Principles of risk communication

1) *Risk communication* is the process by which information and opinions regarding *hazards* and *risks* are gathered from potentially affected and interested parties during a *risk analysis*, and by which the results of the *risk assessment* and proposed *risk management* measures are communicated to the decision-makers and interested parties in the *importing* and *exporting countries*. It is a multidimensional and iterative process and should ideally begin at the start of the *risk analysis* process and continue throughout.

2) A *risk communication* strategy should be put in place at the start of each *risk analysis*.

3) The *communication of risk* should be an open, interactive, iterative and transparent exchange of information that may continue after the decision on importation.

4) The principal participants in *risk communication* include the authorities in the *exporting country* and other stakeholders such as domestic aquaculturists, recreational and commercial fishermen, conservation and wildlife groups, consumer groups, and domestic and foreign industry groups.

5) The assumptions and uncertainty in the model, model inputs and the *risk* estimates of the *risk assessment* should be communicated.

6) Peer review of *risk analyses* is an essential component of *risk communication* in order to obtain a scientific critique and to ensure that the data, information, methods and assumptions are the best available.

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— Text deleted.

CHAPTER 4.X.

RECOMMENDATIONS FOR SURFACE DISINFECTION OF SALMONID EGGS

> **EU comment**
>
> **The EU in general supports the proposed new chapter. A specific comment is inserted in the text below.**

Article 4.X.1.

Introduction

The practice of disinfecting salmonid *eggs* at hatcheries is an essential part of ensuring that endemic *diseases* are not transferred to incubators with *eggs* and ultimately between facilities and forms a part of normal hatchery hygiene protocols. The *disinfection* process is also important when trading salmonid *eggs* between *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, povodine-iodine. Different protocols should be applied depending on the stage of *egg* development.

Iodophores are commonly used *disinfectants* for treating salmonid *eggs*. They 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. Povodine-iodine solutions are the most commonly used iodophore because of their low toxicity and neutral pH under most circumstances. If other iodine based agents are used for *disinfection* it is essential that they are adequately buffered.

Article 4.X.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.

Salmonid *eggs* should undergo the following *disinfection* protocol:

1)   rinsed in 0.9% saline (30–60 seconds) to remove organic matter; then

> **EU comment**
>
> **The EU suggests specifying in the point above (and throughout the article) that the saline used for rinsing should be pathogen free, as follows:**
>
> **"1) rinsed in 0.9% <u>pathogen free</u> saline (30-60 seconds) […]".**
>
> **Indeed, this seems necessary in order to avoid possible unintentional contamination of salmonid eggs.**

2)   immersed in a iodophor solution containing 100 ppm available iodine for a minimum of 10 minutes. The iodophore solution should be used only once. The ratio of *eggs* to iodophor solution should be a minimum of 1:4; then

3)   rinsed again in 0.9% saline for 30–60 seconds; then

4)   held in pathogen free water.

Solutions may be buffered using 100 mg sodium bicarbonate ($NaHCO_3$) per litre of diluted iodophore solution if the pH is low.

C H A P T E R   4 . 7 .

CONTROL OF PATHOGENIC AGENTS IN AQUATIC ANIMAL FEED

EU comment

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

Article 4.7.1.

Introduction

Feed can be a source of infectious *disease* in *aquatic animals*.

Because *aquatic animals* are often a principle ingredient in *feeds* for *aquatic animals*, and because the use of unprocessed or semi-processed *feed* continues to be a common practice, the risk of *disease* transmission via *feed* needs to be addressed.

Article 4.7.2.

Purpose and scope

The purpose of this chapter is to address transmission of infectious *diseases* of *aquatic animals* via *feed* to prevent entry and spread into a country, *zone* or *compartment* free from *pathogenic agents* of concern.

This chapter applies to the production and use of all products destined for *feed* and *feed ingredients* whether produced commercially or on farm.

Risk analysis principles (in accordance with Chapter 2.1.) should be applied to determine the risks associated with the production and use of *feed* in *aquatic animals*.

This chapter is complementary to guidance provided by the Codex Code of Practice on Good Animal Feeding (CAC/RCP 54-2004).

Article 4.7.3.

Responsibilities

The responsibilities of the *Competent Authority* include setting and enforcing regulatory requirements related to *animal feed*, and verifying that these requirements are met. This also includes raising awareness on risks related to use of unprocessed or semi-processed *feed* in *aquaculture*.

Feed producers have the responsibility to ensure that production of *feed* meets regulatory requirements. Records and contingency plans should be in place, as appropriate, to enable the tracing, recall, or destruction of non-compliant products. All personnel involved in the harvest, manufacture, transport, storage and handling of *feed* and *feed ingredients* should be adequately trained and aware of their role and responsibility in preventing the spread of infectious *diseases* of *aquatic animals*. Equipment for producing, storing and transporting *feed* and *feed ingredients* should be kept clean and maintained in good working order.

Owners and managers of *aquaculture establishments* should adhere to regulatory requirements and implement health programmes on their farms in order to manage risks related to the use of unprocessed or

semi-processed *feed*. This can be done through record keeping of sources of *feed* for traceability purposes, implementation of on farm risk mitigation measures, and early detection of infectious *diseases*.

Private veterinarians and other *aquatic animal health professionals* providing specialist services to producers and to the *feed* industry may be required to meet specific regulatory requirements pertaining to the services they provide (e.g. disease reporting, quality standards, transparency).

<div align="center">Article 4.7.4.</div>

Hazards associated with aquatic animal feed

Biological hazards that may occur in *feed* and *feed ingredients* include *pathogenic agents* such as bacteria, viruses, fungi, and parasites. The scope of these recommendations covers listed *diseases* and other *pathogenic agents* that cause an adverse effect on *aquatic animal* health.

Chemical and physical hazards associated with feed and feed ingredients are not addressed in this Chapter.

Antimicrobial resistance arising from the use of *antimicrobial agents* in *feed* is addressed in Section 6.

<div align="center">Article 4.7.5.</div>

Risk pathways and exposure

Feed may be contaminated with *pathogenic agents* present at the time of harvesting, transport, storage, and processing of *commodities* used as *feed ingredients*. Contamination may also occur during manufacture, transport, storage, and use of *feed*. Poor hygienic practices during processing and manufacture, transport and storage are potential sources of contamination with *pathogenic agents*.

Aquatic animals can be directly exposed to *pathogenic agents* in *feed*. *Aquatic animals* can also be indirectly exposed through contamination of the environment by *feed*.

<div align="center">Article 4.7.6.</div>

Risk management

1. Use of feed and feed ingredients from any source

    Some *commodities* undergo significant processing such as heat treatment, acidification, extrusion and extraction. There may be a negligible risk that *pathogenic agents* will survive in such products if they have been produced in accordance with Good Manufacturing Practice.

    Criteria provided in Chapter 5.4. may be used to assess the safety of *commodities* to be used as *feed ingredients*.

    Articles X.X.3. of all *disease*-specific chapters in Sections 8 to 11 lists *commodities* considered safe for any purpose including use as *feed* or *feed ingredients*.

    Competent Authorities should also consider sourcing *feed* and *feed ingredients* from a country, *zone* or *compartment* free from *pathogenic agents* of concern.

2. Use of feed and feed ingredients from sources that may not be free from pathogenic agents of concern

    When using feed and feed ingredients from sources that may not be free from *pathogenic agents* of concern, *Competent Authorities* should consider the following risk mitigation measures:

    a) treatment (e.g. by heating or acidification) of the *commodity* using a method approved by the *Competent Authority* to inactivate *pathogenic agent(s)* as per Articles X.X.10. (for Chapter 10.4. the relevant Article is 10.4.17.) of all disease-specific chapters in Sections 8 to 11; or

> **EU comment**
>
> **The point 2. a) above refers to referencing Article 10.4.17., however this should be Article 10.4.14.**

b)   confirmation (e.g. by testing) that *pathogenic agents* are not present in the *commodity*; or

c)   use of *feed* only in populations that are not susceptible to the *pathogenic agent(s)* in question and where *susceptible species* will not come into contact with the *feed* or its waste products.

3.   Feed production

To prevent contamination by *pathogenic agents* during processing, manufacture, storage and transport of *feed* and *feed ingredients*, the following is recommended:

a)   flushing, sequencing or physical clean-out of manufacturing lines and storage facilities should be performed between batches as appropriate;

b)   buildings and equipment for processing and transporting *feed* and *feed ingredients* should be constructed in a manner that facilitates hygienic operation, maintenance and cleaning and prevents contamination;

c)   *feed* manufacturing plants should be designed and operated to avoid cross-contamination between batches;

d)   processed *feed* and *feed ingredients* should be stored separately from unprocessed *feed ingredients*, under appropriate storage conditions;

e)   *feed* and *feed ingredients*, manufacturing equipment, storage facilities and their immediate surroundings should be kept clean;

f)   measures to inactivate *pathogenic agents*, such as heat treatment, should be used where appropriate;

g)   labelling should provide for the identification of *feed* and *feed ingredients* as to the batch, place and date of production to assist in tracing *feed* and *feed ingredients*.

———————————

CHAPTER 5.1.

GENERAL OBLIGATIONS RELATED TO CERTIFICATION

EU comment

The EU thanks the OIE for having taken some of its comments into consideration and supports the proposed changes to this chapter.

Article 5.1.1.

A combination of factors should be taken into account to facilitate *international trade* in *aquatic animals* and *aquatic animal products*, without incurring unacceptable *risks* to human and *aquatic animal* health.

Because of differences between countries in their *aquatic animal* health situations, various options are offered by the *Aquatic Code*. The *aquatic animal* health situation in the *exporting country*, in the *transit country* or *countries* and in the *importing country* should be considered before determining the requirements for trade. To maximise harmonisation of the *aquatic animal* health aspects of *international trade*, *Competent Authorities* of Member Countries should base their import requirements on the OIE standards.

These requirements should be included in the certificates drawn up in accordance with the model *international aquatic animal health certificates* provided for in Chapter 5.11.

Certification should be exact and concise, and should clearly address the requirements of the *importing country*. For this purpose, prior consultation between *Competent Authorities* of *importing* and *exporting countries* may be necessary. This consultation helps to determine the exact requirements of the certification.

Certificates should be issued and signed by a single competent official authorized by the *Competent Authority* to perform inspections, and endorsed through signature and/or official stamp of the *Competent Authority*. The certification requirements should not include conditions for *diseases* that are not transmitted by the *commodity* concerned. The certificate should be signed in accordance with the provisions of Chapter 5.2.

When officials of a *Competent Authority* wish to visit another country for matters of professional interest to the *Competent Authority* of the other country, the latter should be informed prior to any such visit. This visit should be mutually agreed upon between *Competent Authorities*.

Article 5.1.2.

`Responsibilities of the importing country`

1) The import requirements included in the *international veterinary certificate* should assure that *commodities* introduced into the *importing country* comply with the standards of the OIE. *Importing countries* should <u>align</u> ~~restrict~~ their requirements <u>with</u> ~~to~~ those <u>recommended in the relevant standards of the OIE</u> ~~necessary to achieve the national appropriate level of protection~~. If <u>there are no such standards or if the country chooses a level of protection requiring measures</u> ~~these are~~ stricter than the standards of the OIE, <u>these</u> ~~they~~ should be based on an import *risk analysis*.

2) The *international aquatic animal health certificate* should not include requirements for the exclusion of *pathogenic agents* or *aquatic animal diseases* that are present in the *importing country* and are not subject to any official control programme~~, except when the strain of the *pathogenic agent* in the *exporting country* is of significantly higher pathogenicity and/or has a larger host range~~. The measures imposed on imports to manage the *risks* posed by a *pathogenic agent* or *aquatic animal disease* should not <u>be stricter</u> ~~require a higher level of protection~~ than <u>those</u> ~~that provided by measures~~ applied as part of the official control programme operating within the *importing country*.

3) The *international aquatic animal health certificate* should not include measures against *pathogenic agents* or *diseases* that are not OIE listed, unless the *importing country* has demonstrated through an import *risk analysis*, carried out in accordance with Section 2, that the *pathogenic agent* or *disease* poses a significant *risk* to the *importing country*.

4) The transmission of the requirements of the *importing country* or certificates from the *Competent Authority* of the *importing country* and the communication of import requirements to persons other than the *Competent Authority* of another country necessitates that copies of these documents be also sent to the *Competent Authority* of the *exporting country*. This important procedure avoids delays and difficulties that may arise between traders and *Competent Authorities* when the authenticity of the certificates or permits is not established.

The transmission of this information is the responsibility of *Competent Authorities* of the *exporting country*. However, it can be issued by private sector *veterinarians* at the place of origin of the *commodities* when this practice is the subject of appropriate approval and authentication by *Competent Authorities*.

5) Situations may arise that result in changes to the consignee, identification of the means of transportation, or *frontier post* after a certificate is issued. If it is determined that these do not change the *aquatic animal* health or public health status of the consignment, then they should not prevent the acceptance of the certificate.

<center>Article 5.1.3.</center>

Responsibilities of the exporting country

1) An *exporting country* should, on request, supply the following to *importing countries*:

   a) information on the aquatic animal health situation and national aquatic animal health information systems to determine whether that country is free or has *zones* or *compartments* free from *listed diseases*, and on the pathway followed to achieve *disease* freedom e.g. historical freedom, absence of *susceptible species* or *targeted surveillance*, including the regulations and procedures in force to maintain the free status;

   b) regular and prompt information on the occurrence of *listed diseases*;

   c) details of the country's ability to apply measures to control and prevent *listed diseases*;

   d) information on the structure of the *Competent Authority* and the authority that they exercise;

   e) technical information, particularly on biological tests and vaccines applied in all or part of the country.

2) *Competent Authorities* of *exporting countries* should:

   a) have official procedures for the authorisation of *certifying officials*, defining their functions and duties as well as conditions of oversight and accountability, including possible suspension and termination of the authorisation;

   b) ensure that relevant instructions and training are provided to *certifying officials*;

   c) monitor the activities of the *certifying officials* to verify their integrity and impartiality.

3) The *Competent Authority* of the *exporting country* is ultimately accountable for certification used in *international trade*.

<center>Article 5.1.4.</center>

Responsibilities in case of an incident related to importation

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

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

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

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

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— Text deleted.

CHAPTER 5.2.

CERTIFICATION PROCEDURES

EU comment

The EU thanks the OIE for having taken some of its comments into consideration and in general supports the proposed changes to this chapter. However, a comment is inserted in the text below.

Article 5.2.1.

Protection of the professional integrity of the certifying official

Certification should be based on the highest possible ethical standards, the most important of which is that the professional integrity of the *certifying official* should be respected and safeguarded.

It is essential to include in the certificate only those specific statements that can be accurately and honestly signed by a *certifying official*. For example, these requirements should not include certification of an area as being free from *diseases* that are not notifiable in that country, or the occurrence of which the signing *certifying official* is not necessarily informed about. It is unacceptable to ask for certification for events that will take place after the document is signed when these events are not under the direct control and supervision of the signing *certifying official*.

Article 5.2.2.

Certifying officials

Certifying officials should:

1) be authorised by the *Competent Authority* of the *exporting* country to sign *international aquatic animal health certificates*;

2) only certify matters that are within their own knowledge at the time of signing the certificate, or that have been separately attested by another competent party authorised by the *Competent Authority*;

3) sign only at the appropriate time certificates that have been completed fully and correctly; where a certificate is signed on the basis of supporting documentation, the *certifying official* should have verified or be in possession of that documentation before signing;

4) have no conflict of interest in the commercial aspects of the *aquatic animals* or *aquatic animal products* being certified and be independent from the commercial parties.

Article 5.2.3.

Preparation of international aquatic animal health certificates

Certificates should be drawn up in accordance with the following principles:

1) Certificates should be designed so as to minimise the potential for fraud including use of a unique identification number, or other appropriate means to ensure security. Paper certificates should bear the signature of the *certifying official* and the official identifier (stamp) of the issuing *Competent Authority*. Each page of a multiple page certificate should bear the unique certificate number and a number indicating the number of the page out of the total number of pages. Electronic certification procedures should include equivalent safeguards.

2) Certificates should be written using terms that are simple, unambiguous and as easy to understand as possible, without losing their legal meaning.

3) If so required, certificates should be written in the language of the *importing country*. In such circumstances, they should also be written in a language understood by the *certifying official*.

4) Certificates should require appropriate identification of *aquatic animals* and *aquatic animal products* except where this is impractical (e.g. eyed eggs).

5) Certificates should not require a *certifying official* to certify matters that are outside his/her knowledge or that he/she cannot ascertain and verify.

6) Where appropriate, when presented to the *certifying official*, certificates should be accompanied by notes of guidance indicating the extent of enquiries, tests or examinations expected to be carried out before the certificate is signed.

7) The text of a certificate should not be amended except by deletions that should be signed and stamped by the *certifying official*.

8) The signature and stamp should be in a colour different to that of the printing of the certificate. The stamp may be embossed instead of being a different colour.

9) Only original certificates should be accepted by the *importing country*.

10) Replacement certificates may be issued by a *Competent Authority* to replace original certificates that have been, for example, lost, damaged, contain errors, or where the original information is no longer correct. These replacements should be provided by the issuing authority and be clearly marked to indicate that they are replacing the original certificate. A replacement certificate should reference the number and the issue date of the certificate that it supersedes. The superseded certificate should be cancelled and where possible, returned to the issuing authority.

Article 5.2.4.

Electronic certification

1) Certification may be provided by electronic <u>exchange of data</u> ~~documentation~~ sent directly from the *Competent Authority* of the *exporting country* to the *Competent Authority* of the *importing country*.

   a) Systems providing electronic certificates normally provide an interface with the commercial organisation marketing the commodity for provision of information to the certifying authority. The certifying official should have access to all necessary information such as origin of aquatic animals and laboratory results.

   b) When exchanging electronic certificates and in order to fully utilise electronic data exchange the Competent Authorities should use internationally standardised language, message structure and exchange protocols. Guidance for electronic certification in standardised World Wide Web Consortium (WC3) Extensible Markup Language (XML schemas) as well as secure exchange mechanisms between Competent Authorities is provided by the United Nations Centre for Trade Facilitation and Electronic Business (UN/CEFACT).

EU comment

The EU notes that not all changes proposed in the corresponding chapter of the Terrestrial Code have been taken over in this Article. Indeed, in the point above, the words "World Wide Web Consortium (WC3)" and "schemas" should be deleted, in order to fully align with the proposed wording in the Terrestrial Code.

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   c) <u>A secure method of electronic data exchange should be ensured by digital authentication of the certificates, encryption, non-repudiation mechanisms, controlled and audited access and firewalls.</u>

2) Electronic certificates should carry the same information as conventional certificates.

3) The *Competent Authority* should have in place systems for the security of electronic certificates against access by unauthorised persons or organisations.

4) The *certifying official* should be officially responsible for the secure use of his/her electronic signature.

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—   Text deleted.

C H A P T E R   6 . 5 .

RISK ANALYSIS FOR ANTIMICROBIAL RESISTANCE ARISING FROM THE USE OF ANTIMICROBIAL AGENTS IN AQUATIC ANIMALS

EU comment

The EU in general supports the proposed new chapter, which we note is largely based on the corresponding chapter of the Terrestrial Code. However, the wording of some points which have been extensively discussed in the framework of the Terrestrisal Code chapter do not reflect the results of these discussions. For the sake of consistency and clarity, the EU therefore suggests aligning these points with the wording in the Terrestrial Code (Chapter 6.10.). Comments to this effect are inserted in the text below.

Article 6.5.1.

Recommendations for analysing the risks to aquatic animal and human health from antimicrobial resistant microorganisms of aquatic animal origin

1. Introduction

    Antimicrobial resistance is a naturally occurring phenomenon influenced by many factors. However, the main driving force for the selection of antimicrobial resistance is the use of *antimicrobial agents* in any situation, including human, animal and other usages (under study).

    Antimicrobial resistance associated with the use of *antimicrobial agents* for therapeutic and non-therapeutic purposes has led to the selection and dissemination of antimicrobial resistance microorganisms, with a resulting loss of therapeutic efficacy in animal and human medicine of one or several *antimicrobial agents*.

2. Objective

    For the purpose of this chapter, the principal aim of *risk analysis* is to provide Member Countries with a transparent, objective and scientifically defensible method of assessing and managing the human and *aquatic animal* health *risks* associated with the selection and dissemination of resistance arising from the use of *antimicrobial agents* in *aquatic animals*.

    Guidance on the issue of foodborne antimicrobial resistance related to the non-human use of *antimicrobial agents* is covered by the Codex Guidelines for risk analysis of foodborne antimicrobial resistance (CAC/GL77-2011).

3. The risk analysis process

    The components of *risk analysis* described in this chapter are *hazard identification*, *risk assessment*, *risk management* and *risk communication*.

EU comment

As the terminology of the above paragraph is not in line with the components in the CODEX Guidelines mentioned under point 2, the EU suggests making a cross reference of both terminologies, to point out these differences.

    The chapter includes factors to be considered at various steps of the *risk analysis* process. These factors are not intended to be exhaustive and not all elements may be applicable in all situations.

4. Hazard identification

For the purpose of this chapter, the *hazard* is the resistant microorganism or resistance determinant that emerges as a result of the use of a specific *antimicrobial agent* in *aquatic animals*. This definition reflects the potential for resistant microorganisms to cause adverse health effects, as well as the potential for horizontal transfer of genetic determinants between microorganisms. The conditions under which the *hazard* might produce adverse consequences include any scenarios through which humans or *aquatic animals* could become exposed to an antimicrobial resistant pathogen, fall ill and then be treated with an *antimicrobial agent* that is no longer effective.

5. Risk assessment

The assessment of the *risk* to human and *aquatic animal* health from antimicrobial resistant microorganisms resulting from the use of *antimicrobial agents* in *aquatic animals* should examine:

a) the likelihood of emergence of resistant microorganisms arising from the use of an *antimicrobial agent*, or more particularly, dissemination of the resistance determinants if transmission is possible between microorganisms;

b) all pathways and their importance by which humans and *aquatic animals* could be exposed to these resistant microorganisms or resistance determinants, together with the likelihood of exposure;

c) the consequences of exposure in terms of *risks* to human and *aquatic animal* health.

The general principles of *risk assessment* as defined in Chapter 2.1. apply equally to both qualitative and quantitative *risk assessment*. At a minimum, a qualitative *risk assessment* should be undertaken.

Article 6.5.2.

Special considerations for conducting antimicrobial resistance risk analysis in aquaculture

1. Introduction

Antimicrobial resistance (AMR) *risk analysis* in *aquaculture* is challenged by a variety of factors that impact both *risk assessment* and *risk management*, including the diversity of *aquaculture*, relative lack of methods for culture and antimicrobial susceptibility testing (AST), relative lack of approved drugs, and potential for the development of a reservoir of resistant microorganisms and resistance determinants with a potential for horizontal transmission.

Nevertheless, the fundamental principles of *risk analysis* (*risk assessment*, *risk management*, *risk communication*) provide a framework just as valuable for *aquaculture* as for terrestrial animal production.

2. Definition of the risk

The definitions of *risk* used in this chapter are those associated with the use of *antimicrobial agents* within *aquaculture*.

Because many types of *aquaculture* operations (in particular, open systems) intersect with terrestrial animal production and human environments, it is especially important to clearly identify the *risk* to be assessed. The selection and dissemination of resistant microorganisms or resistant determinants may be associated with the use of *antimicrobial agents* on *aquatic animals* or it may be the result of antimicrobial use in nearby terrestrial animal production operations or the presence of *antimicrobial agents* in human waste water.

Special care is, therefore, required in design of data collection programmes for *risk assessment* to take account of these confounding factors.

3. Diversity of aquaculture

The range of species under culture, the number and type of different culture systems, and the range of *antimicrobial agents* and their routes of administration impact elements of the *risk assessment*, particularly the release assessment. Therefore, careful attention should be used when grouping seemingly similar sectors of the *aquaculture* industry.

Identification, selection and monitoring of *risk management* options are also influenced by the diversity of *aquaculture*.

4. Lack of methods for antimicrobial susceptibility testing (AST)

The current situation in *aquaculture* is that standardised methods for antimicrobial susceptibility testing for many relevant species are generally lacking resulting in a loss in the ability to quantify specific *risks* and an increase in attendant uncertainty. Standardised AST methods should be used where available; or when standardised methods are not available well-described, scientifically sound approaches should be applied.

EU comment

The EU suggests redrafting the paragraph above as it is not clear what exactly is being referred to (Disk diffusion or MIC, interpretative criteria).

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5. Lack of approved drugs

The small number of approved *antimicrobial agents* for use *aquaculture* challenges *risk analysis*, both in terms of *risk assessment* and *risk management*.

The collection and use of thorough information on the types and quantities of *antimicrobial agents* that are in use in *aquaculture* and relevant to the *risk assessment* is important. In some circumstances legal extra-/off-label and illegal uses may also need to be considered. See Chapter 6.3.

For *risk management*, the small number of approved drugs in combination with a range of regulatory and *aquatic animal* health infrastructure in countries engaged in *aquaculture* presents additional challenges. *Risk management* options should be practical and take into account the ability for enforcement and compliance.

For *monitoring* and *surveillance* programmes, a lack of approved drugs means systems for collection of data and information on the quantities of *antimicrobial agents* used may need to consider not only licensed distribution of approved drugs, but information on the use of unapproved drugs.

6. Potential for development of a reservoir (horizontal transmission)

Microorganisms inhabiting the environment represent the fundamental reservoir of resistant determinants in the biosphere. This reservoir represents the basic origin of all *antimicrobial agent* resistance determinants encountered in human and veterinary medicine. The frequency of resistance determinants in environmental microorganisms is maintained by intrinsic, non-anthropogenic factors; all human uses of *antimicrobial agents*, including *aquaculture*, have the potential to increase the size of the reservoir.

EU comment

There seems to be an inaccuracy with regard to the environment as a reservoir of resistance. Resistance can develop spontaneously in any bacterial population, and/or be selected for by antibiotic use (and other factors) also in any bacterial population – not just those present within the biosphere, i.e. it can occur within animals or human too.

Furthermore, it is not clear whether animals and humans are part of the environment or not.

Finally, the second sentence appears to be erroneous; at least it is not very clear (use of antimicrobial agents leading to increase of reservoir).

There is a *risk* that the use of *antimicrobial agents* in *aquaculture* will result in a rise in the frequency of determinants in environmental microbiome and that this may result in an increase in the frequency with which determinants are transferred to microorganisms capable of infecting humans, animals or *aquatic animals*. The assessment and management of this *risk* is extremely complex. The biological pathways both for the release assessment and the exposure assessment are myriad and at present no specific guidelines can be offered.

<div align="center">Article 6.5.3.</div>

Analysis of risks to human health

1. Definition of the risk

    The *infection* of humans with microorganisms that have acquired resistance due to *antimicrobial agent* usage in *aquatic animals*, and resulting in the loss of benefit of antimicrobial therapy used to manage the human *infection*.

2. Hazard identification

    –   Microorganisms that have acquired resistance, (including multiple resistance) arising from the use of an *antimicrobial agent* in *aquatic animals*.

    –   Microorganisms having obtained a resistance determinant from other microorganisms which have acquired resistance arising from the use of an *antimicrobial agent* in *aquatic animals*.

    The identification of the *hazard* should include consideration of the class or subclass of the *antimicrobial agent*. This definition should be read in conjunction with point 4 of Article 6.5.1.

3. Release assessment

    A release assessment describes the biological pathways necessary for the use of a specific *antimicrobial agent* in *aquatic animals* to lead to the release of resistant microorganisms or resistance determinants into a particular environment, and estimating either qualitatively or quantitatively the probability of that complete process occurring. The release assessment describes the probability of the entyr of each of the *hazards* under each specified set of conditions with respect to amounts and timing, and how these might change as a result of various actions, events or measures.

> **EU comment**
>
> **The EU suggests aligning the wording of this paragraph above to the corresponding one in the Terrestrial Code (Article 6.10.2 point 3), as follows:**
>
> **"A release assessment describes the biological pathways ~~necessary for the use of a specific antimicrobial agent in aquatic animals to~~ that may lead lead to the release of resistant microorganisms or resistance determinants into a particular environment <u>due to the use of a specific antimicrobial agent in aquatic animals</u> and estimating either [...]"**
>
> **Reference is made to the discussions that have previously taken place on this point in the context of the Terrestrial Code.**

    The following factors should be considered in the release assessment:

    –   species of *aquatic animals* treated with the *antimicrobial agent*(s) in question;

    –   *aquaculture* production system (intensive/extensive, net pens, tanks, raceways, ponds, other);

    –   number of *aquatic animals* treated, their age and their geographical distribution;

    –   prevalence of *disease* for which the *antimicrobial agent* is indicated or is used in the target *aquatic animal* population;

–   data on trends in *antimicrobial agent* use and changes in *aquaculture* production systems;

–   data on potential extra-label or off-label use;

–   methods and routes of administration of the *antimicrobial agent*;

–   dosage regimen (dose, dosing interval and duration of the treatment);

–   pharmacokinetics and relevant pharmacodynamics of the *antimicrobial agent*;

–   prevalence of *pathogenic agents* that are likely to develop resistance in an *aquatic animal* species;

EU comment

As in the Terrestrial Code (Article 6.10.2.), the EU suggests including an additional indent above re. prevalence of commensal bacteria, as follows:

"- prevalence of commensal bacteria which are able to transfer resistance to human pathogens;"

Indeed, commensal bacteria are also relevant for aquatic animals in this context.

–   mechanisms and pathways of direct or indirect transfer of resistance;

–   potential linkage of virulence attributes and resistance;

–   cross-resistance or co-resistance with other *antimicrobial agents*;

–   data on trends and occurrence of resistant microorganisms obtained through *surveillance* of *aquatic animals* and *aquatic animal products* and waste products.

The following confounding factors should be considered in the release assessment:

–   resistant microorganisms or resistant determinants associated with *aquatic animals* or *aquatic animal products* that are a result of terrestrial contamination of the aquatic environment, *feed* contamination or contamination during post-harvest processing.

EU comment

Again, to align with the Terrestrial Code Article 6.10.2., the EU suggests adding the following at the end of the point above:

", or contamination from sewage plants."

Indeed, contamination from human sewage, for example through sewage plants, is also relevant for aquatic animals.

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4.   Exposure assessment

An exposure assessment describes the biological pathways necessary for exposure of humans to the resistant microorganisms or resistance determinants released from a given *antimicrobial agent's* use in *aquatic animals*, and estimates the probability of exposures occurring. The probability of exposure to the identified *hazards* is estimated for specified exposure conditions with respect to amounts, timing, frequency, duration of exposure, routes of exposure, species and other characteristics of the human populations exposed.

The following factors should be considered in the exposure assessment:

–   human demographics, including population subgroups, and food consumption patterns, including traditions and cultural practices with respect to the preparation and storage of food;

- prevalence of resistant microorganisms in food at the point of consumption;

- microbial load in contaminated food at the point of consumption;

- environmental contamination with resistant microorganisms;

EU comment

As in the Terrestrial Code (Article 6.10.3.), the EU suggests including an additional indent above re. occurrence of antimicrobial resistant microorganism in feed, as follows:

"- Occurrence in feed of resistant microorganisms that have the capacity to become established in the *aquatic animals*, thus leading to contamination of the food thereof;"

Indeed, feed is also a relevant factor to be considered.

- transfer of resistant microorganisms between humans, *aquatic animals,* and the *environment*;

EU comment

As in the Terrestrial Code (Article 6.10.3.), the EU suggests adding the words "and their resistance determinants" after the word "microorganism" in the indent above.

Indeed, not only the microorganisms have to be considered as a hazard, but also the genes of resistance, i.e. resistance determinants.

- measures taken for microbial decontamination of food;

- survival capacity and dissemination of resistant microorganisms during the food production process (including slaughtering, processing, storage, transportation and retailing);

- disposal practices for waste products and the likelihood for human exposure to resistant microorganisms or resistance determinants through those waste products;

- capacity of resistant microorganisms to become established in humans;

- human-to-human transmission of the microorganisms under consideration;

- capacity of resistant microorganisms to transfer resistance to human commensal microorganisms and zoonotic agents;

- amount and type of *antimicrobial agents* used to treat humans;

- pharmacokinetics, such as metabolism, bioavailability, distribution to the gastrointestinal flora.

5. Consequence assessment

A consequence assessment describes the relationship between specified exposures to resistant microorganisms or resistance determinants and the consequences of those exposures. A causal process should exist by which exposures produce adverse health or environmental consequences, which may in turn lead to socio-economic consequences. The consequence assessment describes the potential consequences of a given exposure and estimates the probability of them occurring.

The following factors should be considered in the consequence assessment:

- microbial dose and subsequent host response interactions;

- variation in susceptibility of exposed populations or subgroups of the population;

- variation and frequency of human health effects resulting from loss of efficacy of *antimicrobial agents* and associated costs;

- potential linkage of virulence attributes and resistance;

–   changes in food consumption patterns due to loss of confidence in the safety of food products and any associated secondary *risks*;

–   interference with antimicrobial therapy in humans;

–   importance of the *antimicrobial agent* in human medicine;

–   prevalence of resistance in human bacterial pathogens under consideration.

6.   <u>Risk estimation</u>

A *risk* estimation integrates the results from the release assessment, exposure assessment and consequence assessment to produce overall estimates of *risks* associated with the *hazards*. Thus, *risk* estimation takes into account the whole of the *risk* pathway from *hazard identification* to the unwanted consequences.

The following factors should be considered in the *risk* estimation:

–   number of people falling ill and the proportion of that number infected with antimicrobial resistant microorganisms;

–   adverse effects on vulnerable human sub-population (children, immuno-compromised persons, elderly, pregnant, etc.);

–   increased severity or duration of infectious *disease*;

–   number of person/days of illness per year;

–   deaths (total per year; probability per year or reduced life expectancy for a random member of the population or a member of a specific more exposed sub-population) linked to antimicrobial resistant microorganisms when compared with deaths linked to sensitive microorganisms of the same species;

–   severity of the *disease* caused by the target resistant microorganisms;

–   availability of alternative antimicrobial therapy;

–   potential impact of switching to an alternative *antimicrobial agent* (e.g. alternatives with potential increased toxicity);

–   occurrence of antimicrobial resistance in target pathogens observed in humans.

7.   <u>Risk management</u>

The OIE defines *risk management* as consisting of the steps described below.

a)   Risk evaluation

Risk evaluation - the process of comparing the *risk* estimated in the *risk assessment* with the reduction in *risk* expected from the proposed *risk management* measures.

b)   Option evaluation

A range of *risk management* options is available to minimise the emergence and dissemination of antimicrobial resistance and these include both regulatory and non-regulatory options, such as the development of codes of practice for the use of *antimicrobial agents* in animal husbandry.

EU comment

For clarity reasons, the EU suggests adding the words "<u>aquaculture production systems or aquatic</u>" before the words "animal husbandry".

Risk management decisions need to consider fully the implications of these different options for human health and *aquatic animal* health and welfare and also take into account economic considerations and any associated environmental issues. Effective control of *aquatic animal*

diseases can have the dual benefits of reducing the *risks* to human health associated with both the bacterial pathogen under consideration and antimicrobial resistance.

c) Implementation

Risk managers should develop an implementation plan that describes how the decision will be implemented, by whom and when. *Competent Authorities* should ensure an appropriate regulatory framework and infrastructure.

d) Monitoring and review

Risk management options should be continuously monitored and reviewed in order to ensure that the objectives are being achieved.

8. Risk communication

Communication with all interested parties should be promoted at the earliest opportunity and integrated into all phases of *risk analysis*. This will provide all interested parties, including *risk* managers, with a better understanding of *risk management* approaches. *Risk communication* should be also well documented.

Article 6.5.4.

Analysis of risks to aquatic animal health

1. Definition of the risk

The *infection* of *aquatic animals* with microorganisms that have acquired resistance due to antimicrobial usage in *aquatic animals*, and resulting in the loss of benefit of antimicrobial therapy used to manage the *aquatic animal infection.*

2. Hazard identification

–   Microorganisms that have acquired resistance, (including multiple resistance) arising from the use of an *antimicrobial agent* in *aquatic animals*.

–   Microorganisms having obtained a resistance determinant from another micro-organism which has acquired resistance arising from the use of an *antimicrobial agent* in *aquatic animals*.

The *identification of the hazard* should include considerations of the class or subclass of the *antimicrobial agent*. This definition should be read in conjunction with point 4 of Article 6.5.1.

3. Release assessment

The following factors should be considered in the release assessment:

–   *aquatic animal* species treated with the *antimicrobial agent* in question;

–   *aquaculture* production system (intensive/extensive, net pens, tanks, raceways, ponds, other);

–   number of *aquatic animals* treated, and their age, geographical distribution, and where appropriate, sex;

–   prevalence of *disease* for which the *antimicrobial agent* is indicated or is used in the target *aquatic animal* population;

–   data on trends in *antimicrobial agent* use and changes in *aquaculture* production systems;

–   data on potential extra-label or off-label use;

–   dosage regimen (dose, dosing interval and duration of the treatment);

–   methods and routes of administration of the *antimicrobial agent*;

–   the pharmacokinetics and relevant pharmacodynamics of the *antimicrobial agent*;

– site and type of *infection*;

– development of resistant microorganisms;

– mechanisms and pathways of resistance transfer;

– cross-resistance or co-resistance with other *antimicrobial agents*;

– data on trends and occurrence of resistant microorganisms obtained through *surveillance* of *aquatic animals*, *aquatic animal products* and waste products.

The following confounding factors should be considered in the release assessment:

– resistant microorganisms or resistant determinants associated with *aquatic animals* or their products that are a result of terrestrial contamination of the aquatic environment, *feed* contamination or contamination during post-harvest processing.

EU comment

Again, to align with the relevant article of the Terrestrial Code, the EU suggests adding the following at the end of the point above:

", or contamination from sewage plants."

Indeed, contramination from human sewage, for example through sewage plants, is also relevant for aquatic animals.

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4.   Exposure assessment

The following factors should be considered in the exposure assessment:

– prevalence and trends of resistant microorganisms in clinically ill and clinically unaffected *aquatic animals*;

– prevalence of resistant microorganisms in *feed* and in the *aquatic animal* environment;

– animal-to-animal transmission of the resistant microorganisms and their resistance determinants (*aquatic animal* husbandry practices, movement of *aquatic animals*);

– number or percentage of *aquatic animals* treated;

– quantity and trends of *antimicrobial agent* used in *aquatic animals*;

– survival capacity and spread of resistant micro-organisms;

– exposure of wildlife to resistant microorganisms;

– disposal practices for waste products and the likelihood for *aquatic animal* exposure to resistant microorganisms or resistance determinants through those products;

– capacity of resistant microorganisms to become established in *aquatic animals*;

– exposure to resistance determinants from other sources such as water, effluent, waste pollution, etc.;

– pharmacokinetics, such as metabolism, bioavailability, distribution to relevant flora - considering the gastrointestinal flora of many aquatic species may be transient;

– transfer of resistant microorganisms and resistance determinants between humans, *aquatic animals*, and the environment.

5. Consequence assessment

    The following factors should be considered in the consequence assessment:

    –   microbial dose and subsequent host response interactions;

    –   variation in *disease* susceptibility of exposed populations and subgroups of the populations;

    –   variation and frequency of *aquatic animal* health effects resulting from loss of efficacy of *antimicrobial agents* and associated costs;

    –   potential linkage of virulence attributes and resistance;

    –   importance of the *antimicrobial agent* in *aquatic animal* health (see OIE list of *antimicrobial agents* of veterinary importance).

> **EU comment**
>
> **The EU suggests amending the above indent as follows:**
>
> **"- importance of the *antimicrobial agent* in *aquatic animal* <u>and human</u> health (see OIE list of antimicrobial agents of veterinary importance <u>and WHO list of Critically Important Antimicrobials</u>)."**
>
> **Indeed, while the proposed text is in line with the corresponding one in the Terrestrial Code, in this case it is not clear what criteria have been used to establish the list of important antimicrobial agents for aquatic animals.**
>
> **Furthermore, even if this chapter concerns the analysis of risk to aquatic animal health, it should not only consider animal health but also public health aspects; thus also the need to mention the list of CIA of the WHO.**

6. Risk estimation

    The following factors should be considered in the *risk* estimation:

    –   additional burden of *disease* due to antimicrobial resistant microorganisms;

    –   number of therapeutic failures due to antimicrobial resistant microorganisms;

    –   increased severity and duration of infectious *disease*;

    –   impact on aquatic animal welfare;

    –   estimation of the economic impact and cost on *aquatic animal* health and production;

    –   deaths (total per year; probability per year for a random member of the population or a member of a specific more exposed sub-population) linked to antimicrobial resistant microorganisms when compared with deaths linked to sensitive microorganisms of the same species;

    –   availability of alternative antimicrobial therapy;

    –   potential impact of switching to an alternative *antimicrobial agent* e.g. alternatives with potential increased toxicity.

7. Risk management

    The relevant provisions in point 7 of Article 6.5.3. apply.

8. Risk communication

    The relevant provisions in point 8 of Article 6.5.3. apply.

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— Text deleted.

CHAPTER 8.1.

INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

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

[…]

Article 8.1.8.

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

1) When importing live *aquatic animals* of species referred to in Article 8.1.2. from a country, *zone* or *compartment* not declared free from *infection* with *B. dendrobatidis*, the *Competent Authority* of the *importing country* should~~:~~

   a) ~~require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* attesting that the *aquatic animals* of the species referred to in Article 8.1.2. have been appropriately treated to eradicate *infection* and have been subsequently tested to confirm absence of the *disease* according to specifications provided in the relevant chapter in the *Aquatic Manual*;~~

   ~~OR~~

   a~~b~~) assess the *risk* and apply *risk* mitigation measures such as:

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

   ii) the treatment of water used in transport and of all effluent and waste materials in a manner that inactivates *B. dendrobatidis*.

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

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

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

   b) evaluate stock health/disease history;

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

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

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

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

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g) if *infection* with *B. dendrobatidis* 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 *infection* with *B. dendrobatidis* free or specific pathogen free (SPF) for *infection* with *B. dendrobatidis*;

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

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

This Article does not apply to *aquatic animals* referred to in point 1 of Article 8.1.3.

[…]

Article 8.1.10.

Importation of live aquatic animals intended for use in animal feed, or for agricultural, ~~laboratory, zoo, pet trade,~~ industrial or pharmaceutical use, from a country, zone or compartment not declared free from infection with *B.dendrobatidis*

When importing, for use in animal *feed*, or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of the species referred to in Article 8.1.2. from a country, *zone* or *compartment* not declared free from *infection* with *B. dendrobatidis*, 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 to 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 *B. dendrobatidis.*

~~When importing live *aquatic animals* of species referred to in Article 8.1.2. from a country, *zone* or *compartment* not declared free from *B. dendrobatidis*, the *Competent Authority* of the *importing country* should:~~

~~1) require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* attesting that the *aquatic animals* have been appropriately treated to eradicate *infection* and have been subsequently tested to confirm absence of the *disease* according to specifications provided in the relevant chapter in the *Aquatic Manual*;~~

~~OR~~

~~2) assess the *risk* and apply *risk* mitigation measures such as:~~

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

~~b) the treatment of water used in transport and all effluent and waste materials in a manner that inactivates *B. dendrobatidis.*~~

This Article does not apply to *commodities* referred to in point 1 of Article 8.1.3.

[…]

<u>Article 8.1.13.</u>

<u>Importation of live aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with *B.dendrobatidis*</u>

<u>When importing, for use in laboratory and zoo</u>**,** <u>live *aquatic animals* of species referred to in Article 8.1.2. from a country, *zone* or *compartment* not declared free from *infection* with *B. dendrobatidis*, the *Competent Authority* of the *importing country* should ensure:</u>

1) <u>the direct delivery to and lifelong holding of the consignment in *quarantine* facilities authorised by the *Competent Authority;* and</u>

2) <u>the treatment of water used in transport in a manner that inactivates *B. dendrobatidis*; and</u>

3) <u>carcasses are disposed of in accordance with Chapter 4.6.</u>

[…]

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— Text deleted.

CHAPTER 8.2.

INFECTION WITH RANAVIRUS

EU comment

The EU supports the proposed changes to this chapter.

[…]

Article 8.2.10.

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

When importing, for use in animal *feed,* or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of the species referred to in Article 8.2.2. from a country, *zone* or *compartment* not declared free from *infection* with ranavirus, 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 to 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 ranavirus.

~~When importing live *aquatic animals* of species referred to in Article 8.2.2. from a country, *zone* or *compartment* not declared free from ranavirus, the *Competent Authority* of the *importing country* should assess the *risk* and apply *risk* mitigation measures such as:~~

~~1) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment;~~

~~2) the treatment of all effluent and waste materials in a manner that inactivates ranavirus.~~

This Article does not apply to *commodities* referred to in point 1 of Article 8.2.3.

[…]

Article 8.2.13

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

When importing, for use in laboratory and zoo, live *aquatic animals* of species referred to in Article 8.2.2. from a country, *zone* or *compartment* not declared free from *infection* with ranavirus, the *Competent Authority* of the *importing country* should ensure:

1) the direct delivery to and lifelong holding of the consignment in *quarantine* facilities authorised by the *Competent Authority;* and

2) the treatment of water used in transport in a manner that inactivates ranavirus; and

3) carcasses are disposed of in accordance with Chapter 4.6.

Articles X.X.7. and X.X.11.

(Note: In Chapter 10.4. these amendments apply to Articles 10.4.10., 10.4.15., 10.4.11. and 10.4.16.)

EU comment

The EU supports the proposed changes in these model articles.

Article X.X.7.

Importation of live aquatic animals <u>and aquatic animal products</u> from a country, zone or compartment declared free from disease X

When importing live *aquatic animals* and *aquatic animal products* of species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from disease X, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles X.XX. or X.X.X. (as applicable) and X.X.X., the place of production of the live *aquatic animals* and *aquatic animal products* is a country, *zone* or *compartment* declared free from disease X.

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

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

~~Article X.X.11.~~

~~**Importation of aquatic animal products from a country, zone or compartment declared free from disease X**~~

~~When importing *aquatic animal products* of species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from disease X, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* certifying that, on the basis of the procedures described in Articles X.X.X. or X.X.X. (as applicable) and X.X.X., the place of production of the *aquatic animal products* is a country, *zone* or *compartment* declared free from disease X.~~

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

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

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— Text deleted.

OIE Aquatic Animal Health Standards Commission/September 2014

CHAPTER 10.4.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

EU comment

The EU supports the proposed changes to this chapter.

[…]

Article 10.4.4.

Country free from infection with infectious salmon anaemia virus

In this article, all statements referring to a country free from *infection* with ISAV are for any detectable ISAV, including HPR0 ISAV.

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from *infection* with ISAV if all the areas covered by the shared water bodies are declared countries or *zones* free from *infection* with ISAV (see Article 10.4.6.).

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

1) none of the *susceptible species* referred to in Article 10.4.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) the *disease* status prior to *targeted surveillance* is unknown ~~any of the *susceptible species* referred to in Article 10.4.2. are present and there has been no detectable occurrence of *infection* with ISAV~~ 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 ISAV;

OR

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

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

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

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

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

In the meantime, part or all of the non-affected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 10.4.6.

The pathway for *self-declaration of freedom* from *infection* with ISAV HPR0 based on absence of clinical disease (referred to as historical freedom in Article 1.4.6.) cannot be achieved because *infection* with ISAV HPR0 is unlikely to cause any clinical signs.

[…]

Article 10.4.6.

Zone or compartment free from infection with infectious salmon anaemia virus

In this article, all statements referring to a *zone* or *compartment* free from *infection* with ISAV are for any detectable ISAV, including HPR0 ISAV.

If a *zone* or *compartment* extends over more than one country, it can only be declared a *zone* or *compartment* free from *infection* with ISAV 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 ISAV 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 10.5.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) the *disease* status prior to *targeted surveillance* is unknown ~~any of the *susceptible species* referred to in Article 10.4.2. are present and there has been no detectable occurrence of *infection* with ISAV~~ 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 ISAV;

OR

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

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

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

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

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

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CHAPTER 2.2.2.

INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS

EU comment

The EU supports the proposed changes to this chapter.

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1. Scope

Infectious hypodermal and haematopoietic necrosis (IHHN) disease is caused by infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Bonami & Lightner, 1991; Bonami *et al.,* 1990; Lightner, 1996a; 2011; Lightner *et al.,* 1983a; 1983b; Lotz *et al.,* 1995; Tang & Lightner, 2002). A large portion of the IHHNV genome has been found to be inserted in the genome of some genetic lines of *Penaeus monodon.* There is no evidence that this variant of IHHNV is infectious (Tang & Lightner, 2002; 2006).

Synonyms: the International Committee on the Taxonomy 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) (Fauquet *et al.,* 2005). 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 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 three distinct genotypes of IHHNV have been identified (Tang & Lightner, 2002; Tang *et al.,* 2003b): Type 1) from the Americas and East Asia (principally the Philippines); Type 2) from South-East Asia; Type 3A) East Africa, India and Australia; and Type 3B) the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al.,* 2007). The first two genotypes are infectious to the representative penaeids, *P. vannamei* and *P. monodon*, while the latter two genetic variants are not infectious to these species (Tang & Lightner, 2002; Tang *et al.,* 2003b; 2007). IHHNV type 3A and type 3B related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.,* 2007). The putative IHHNV sequences in the *P. monodon* genome are not infectious to the representative host species *P. vannamei* and *P. monodon* (Lightner *et al.,* 2009; Tang & Lightner, 2006; Tang *et al.,* 2007).

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 stable virus of the known penaeid shrimp viruses. Infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996a; Lightner *et al.,* 1987; 2009).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

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. Susceptible stages of the host

IHHNV has been demonstrated in all life stages (i.e. eggs, larvae, postlarvae [PL], 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 IHHNV infection (Motte *et al.,* 2003).

2.2.3. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1 and 2.2.2.

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

2.2.5. Persistent infection with lifelong carriers

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infections and/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. Vectors

No vectors are known in natural infections.

2.2.7. 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; Nunan *et al.,* 2001).

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 has been demonstrated (Lightner, 1996a; Lightner *et al.,* 1983a; 1983b; 1985), as has vertical transmission via infected eggs (Motte *et al.,* 2003).

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 IHHNV has been reported from cultured *P. vannamei* and *P. stylirostris* in most of the shrimp-culturing regions of the Western Hemisphere and in wild penaeids throughout their range along the Pacific coast of the Americas (Peru to northern Mexico), the virus has not been reported in wild penaeid shrimp on the Atlantic coast of the Americas (Bondad-Reantaso *et al.,* 2001; Brock & Main, 1994; Lightner, 1996a; 1996b; Lightner *et al.,* 1992a; Lightner & Redman, 1998a). IHHNV has also been reported in cultured penaeid shrimp from Pacific islands including the Hawaiian Islands, French Polynesia, Guam, and New Caledonia. In the Indo-Pacific region, the virus has been reported from cultured and wild penaeid shrimp in East Asia, South-East Asia, and the Middle East (Bondad-Reantaso *et al.,* 2001; Lightner, 1996a). An IHHN-like virus has been reported from Australia (Krabsetsve *et al.,* 2004; Owens *et al.,* 1992), and the presence of IHHN in farmed prawns in Australia was reported to the OIE in 2008. As discussed in Section 2.1.1, IHHNV-related sequences have been found inserted into the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.,* 2007).

2.3.4. Mortality and morbidity

Depending on the host species and the genotype of the virus, IHHN may take three distinct forms: in unselected *P. stylirostris*, infection by IHHNV results in an acute, usually catastrophic disease with mortalities approaching 100%. In contrast, in *P. vannamei*, some selected lines of *P. stylirostris*, and in *P. monodon* under some conditions, infection by IHHNV results in a more subtle, chronic disease, RDS, in which high mortalities are unusual, but significant 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 no evidence that this variant of IHHNV is infectious (Tang & Lightner, 2002; 2006).

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 lower viral load than shrimp held at 24°C. However, even at the higher temperature, significant (up to 10^5 virus copies 50 ng^{-1} of shrimp DNA) IHHNV replication still occurred in shrimp held at 32°C (Montgomery-Brock *et al.,* 2007).

2.4. Control and prevention

2.4.1.   Vaccination

No effective vaccination methods for IHHNV have been developed.

2.4.2.   Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3.   Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4.   Resistance breeding

Selected stocks of *P. stylirostris* that are resistant to IHHN 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 lines of *P. stylirostris* that were bred for IHHN disease resistance, were found to be refractory to infection (Tang *et al.,* 2000). However, such stocks have no increased resistance to diseases such as white spot syndrome virus (WSSV), and, hence, their use has been limited, although with some stocks a genetic basis for IHHN susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.,* 1997).

2.4.5.   Restocking with resistant species

There has been some limited application and success with IHHNV-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarin-Herzberg & Ascencio 2001). The relative resistance of *P. vannamei* to IHHN disease, despite infection by IHHNV, is considered to be among the principal factors that led to *P.vannamei* being the principal shrimp species farmed in the Western Hemisphere and, since 2004, globally (Lightner, 2005; Lightner *et al.,* 2009; Rosenberry, 2004).

2.4.6.   Blocking agents

There are reports of shrimp with high viral loads of IHHNV being resistant to infection by WSSV (Bonnichon *et al.,* 2006; Tang *et al.,* 2003a). However, there are no reports to date for IHHNV blocking agents.

2.4.7.   Disinfection of eggs and larvae

IHHNV has been demonstrated to be 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 transmission of IHHNV (Motte *et al.,* 2003).

2.4.8.   General husbandry practices

Some husbandry practices have been successfully applied to the prevention of IHHNV infections and disease. Among these has been the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001; Motte *et al.,* 2003), as well as the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Lotz *et al.,* 1995; Pruder *et al.,* 1995; Wyban 1992). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (Jaenike *et al.,* 1992; Lightner, 2005; Pruder *et al.,* 1995). Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status (Lightner *et al.,* 2009). The development of SPF *P. vannamei* that were free not only of IHHNV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Lightner *et al.,* 2009; Rosenberry, 2004).

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by IHHNV are all life stages (eggs, larvae, PL, juveniles and adults) (Motte *et al.,* 2003). While IHHNV 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 IHHNV detection or certification for IHHN disease freedom.

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs and tissues

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 PLs) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

Haemolymph or excised pleopods may be collected and used for testing (usually for PCR, or dot-blot hybridisation with specific probes) when non-lethal testing of valuable broodstock is necessary (Lightner, 1996a; Lightner & Redman, 1998a).

3.5. Samples/tissues that are not suitable

IHHNV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection by IHHNV (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Certain cuticular deformities, specifically a deformed rostrum bent to the left or right, which may be presented by *P. vannamei* and *P. stylirostris* with RDS, are pathognomonic for infection 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 IHHN 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. IHHN disease in Penaeus stylirostris

IHHNV often causes an acute disease with very high mortalities in juveniles of this species. Vertically infected larvae and early PL do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size and/or age dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Bondad-Reantaso *et al.,* 2001; Brock *et al.,* 1983; Brock & Main, 1994; Lightner, 1983; 1988; 1993; 1996a; 2011; Lightner *et al.,* 1983a, 1983b). Gross signs are not IHHN specific, but juvenile *P. stylirostris* with acute IHHN show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species with acute IHHN have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. *Penaeus stylirostris* at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *P. stylirostris* as such individuals become more bluish. In *P. stylirostris* and *P. monodon* with terminal-phase IHHNV infections, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Bondad-Reantaso *et al.,* 2001; Lightner, 1983; 1988; 1993; 1996a; 2011; Lightner *et al.,* 1983a; 1983b).

4.2.1.2. IHHN disease in Penaeus vannamei

RDS, a chronic form of IHHN 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 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* usually show CVs of 10–30% (Bray *et al.,* 1994; Brock & Lightner, 1990; Brock *et al.,* 1983; Brock & Main, 1994; Browdy *et al.,* 1993; Carr *et al.,* 1996; Lightner, 1996a; Primavera & Quinitio, 2000; Pruder *et al.,* 1995).

4.2.2.   Clinical chemistry

Not applicable.

4.2.3.   Microscopic pathology

Acute IHHNV infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained histological methods (see Section 4.2.6). Chronic 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 IHHNV infection. These characteristic IHHN 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 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 specific DNA probe to IHHNV provides a definitive diagnosis of IHHNV infection (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4.2.4.   Wet mounts

No reliable methods have been developed for direct microscopic pathology.

4.2.5.   Smears

Not applicable.

4.2.6.   Fixed sections

Histopathology: histology may be used to provide a definitive diagnosis of IHHNV infection. Because 10% buffered formalin and other fixatives provide, at best, only fair fixation of the shrimp, the use of Davidson's fixative (containing 33% ethyl alcohol [95%], 22% formalin [approximately 37% formaldehyde], 11.5% glacial acetic acid and 33.5% distilled or tap water) is highly recommended for all routine histological studies of shrimp (Bell & Lightner, 1988; Lightner, 1996a). To obtain the best results, dead shrimp should not be used. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are killed by injection of fixative directly into the hepatopancreas; the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline is opened with fine-pointed surgical scissors to enhance fixative penetration (the abdomen may be removed and discarded), the whole shrimp (or cephalothorax less the abdomen) is immersed in fixative for from 24 to no more than 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported (via post or courier to the diagnostic laboratory) by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags (see Section 4.2.3).

In-situ hybridisation (see Section 4.3.1.2.3 below).

4.2.7.   Electron microscopy/cytopathology

Electron microscopy is not recommended for routine diagnosis of IHHNV.

4.3. Agent detection and identification methods

4.3.1.   Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See section 4.2.6.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

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

4.3.1.2.2. Antibody-based antigen detection methods

None has been successfully developed.

4.3.1.2.3. Molecular techniques

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

DNA probes for dot-blot and ISH applications: gene probe and PCR methods provide greater diagnostic 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 test.

Dot-blot hybridisation procedure for IHHNV: the probe is labelled with a non-radioactive label, digoxigenin-11-dUTP (DIG-11-dUTP). The system using DIG to label nucleic acid probes was developed by Boehringer Mannheim Biochemicals (this company is now owned by Roche Diagnostic Corporation), which is described in the Roche *DIG Nonradioactive Labeling and Detection Product Selection Guide* and *DIG Application Manual for Filter Hybridization^{TM} System User's Guide for Membrane Hybridization* and from Boehringer Mannheim's *Nonradioactive In Situ Hybridization Application Manual*[1] (2006a; 2006b). The protocols given below use a DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (Lightner, 1996a; Mari *et al.,* 1993). An alternative method for producing DIG-labelled probes uses specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit^{TM}.

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

i)   Prepare a positively charged nylon membrane (Roche Diagnostics Cat. No. 1-209-299 or equivalent): cut pieces to fit samples and controls and mark with 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 be assayed in TE (Tris/EDTA [ethylene diamine tetra-acetic acid]) buffer 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 can be haemolymph, tissues homogenised in TN (Tris/NaCl: 0.4 M NaCl and 20 mM Tris-HCl, pH 7.4) buffer, or extracted DNA in 10 mM Tris/HCl.

iii) Boil samples for 10 minutes and quench on ice for 5 minutes. Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein. Keep on ice until samples are dotted on to the membrane.

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

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

vi) 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 0.5– 1 hour.

vii) Boil the DIG-labelled probe for 10 minutes, quench 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.

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

viii) 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 (use 4 ml/filter and seal in bags)	3 ×	15 minutes at 68°C
Buffer I	1 ×	5 minutes at room temperature
Buffer II	1 ×	30 minutes at room temperature
Buffer I	1 ×	5 minutes at room temperature

(Buffers are prepared ahead of time).

ix) React the membrane in bags with anti-DIG AP conjugate (Roche Diagnostics 1-093-274) diluted 1/5000 in Buffer I. Use 3 ml per membrane; incubate for 30–45 minutes at room temperature on a shaker platform.

x) Wash membrane well with:

Buffer I	2 ×	15 minutes at room temperature
Buffer III	1 ×	5 minutes at room temperature

xi) Develop the membranes in bags using 3 ml per membrane of development solution (nitroblue tetrazolium salt [NBT]/X-phosphate in Buffer III) made up just prior to use. React in the dark at room temperature for 1–2 hours. Stop the reactions in Buffer IV and dry the membranes on 3MM filter paper.

xii) Photograph the results (colour fades over time).

xiii) Store dry membranes in heat-seal bags.

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

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

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

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

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

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

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

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

vii) Boil the DIG-labelled probe for 10 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 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 AP conjugate (Roche Applied Science cat. 10686322) 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 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$_2$O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

ii) 10 × Tris/NaCl/EDTA (TNE) buffer
   Tris base   60.57 g
   NaCl   5.84 g
   EDTA   3.72 g
   DD H$_2$O   900 ml (qs to 1 litre)
   pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 × TNE in 900 ml DD H$_2$O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

iii) Proteinase K, 100 µg ml^{-1} (prepare just prior to use)
   PBS   10 ml 1 × PBS
   Proteinase K   1 mg

iv) 0.4% formaldehyde
   37% formaldehyde   5.4 ml
   DD H$_2$O   500 ml

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

v) Prehybridisation buffer (50 ml final volume)
   4 × SSC   10 ml 20 × SSC
   50% formamide   25 ml 100% formamide
   1 × Denhardt's   2.5 ml 20 × Denhardt's
   5% dextran sulphate   10 ml 25% dextran sulphate
   Warm to 60°C

   Boil 2.5 ml of 10 mg ml^{-1} salmon sperm DNA and add to buffer for final concentration of 0.5 mg ml^{-1} salmon sperm DNA; store at 4°C.

vi) 20 × SSC buffer
   3M NaCl   175.32 g NaCl
   0.3 M Na$_3$C$_6$H$_5$O$_7$.2H$_2$O   88.23 g Na citrate.2H$_2$O
   DD H$_2$O   1000 ml (qs)
   pH to 7.0; autoclave; store at 4°C.

   To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H$_2$O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H$_2$O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H$_2$O. Filter solutions through a 0.45 µm filter; store at 4°C.

vii) 20 × Denhardt's solution
   BSA (Fraction V)   0.4 g bovine serum albumin
   Ficoll 400   0.4 g Ficoll
   PVP 360   0.4 g polyvinylpyrollidine
   DD H$_2$O   100 ml
   Filter solutions through a 0.45 µm filter; store at 4°C. Aliquot 2.5 ml into small tubes and store frozen.

viii) 25% dextran sulphate
   Dextran sulphate   25 g
   DD H$_2$O   100 ml
   Mix to dissolve; store frozen in 10 ml aliquots.

ix) Salmon sperm DNA (10 mg ml^{-1})
   Salmon sperm DNA   0.25 g
   DD H$_2$O   25 ml

Annex 16 (contd)

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) 10 × Buffer I
1 M Tris/HCl   121.1 g Tris base
1.5 M NaCl   87.7 g NaCl
DD H$_2$O   1000 ml (qs)
pH to 7.5 with HCl. Autoclave; store at 4°C.
To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H$_2$O. Filter through a 0.45 µm filter; store at 4°C.

xi) 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) Buffer III
100 mM Tris/HCl   1.21 g Tris base
100 mM NaCl   0.58 g NaCl
DD H$_2$O   100 ml (qs)
pH to 9.5 with HCl
Then add:
50 mM MgCl$_2$ 1.02 g MgCl$_2$.6H$_2$O
Filter through a 0.45 µm filter; store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)
Polyvinyl alcohol   10 g
DD H$_2$O   100 ml

To prepare, slowly add PVA to water while stirring on low heat. (It takes 2–3 hours for PVA to go into solution.) Dispense 10 ml per tube and store frozen at –20°C.

xiv) 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) Buffer IV

10 mM Tris/HCl   1.21 g Tris base
1 mM EDTA   0.37 g EDTA.2H$_2$O (disodium salt)
DD H$_2$O   1000 ml
pH to 8.0 with HCl. Filter through a 0.45 µm filter; store at 4°C.

xvi) 0.5% Bismarck Brown Y

Bismarck Brown Y   2.5 g
DD H$_2$O   500 ml
Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

Polymerase chain reaction for IHHNV: several single-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), including 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; Tang & Lightner, 2006; Tang *et al.,* 2007; Saksmerprome *et al.,* 2011). New PCR primers have been developed that can detect the IHHN viral sequence but do not react with IHHNV-related sequences present in the *P. monodon* stocks from Africa, Australia (Tang *et al.,* 2007), or Thailand (Saksmerprome *et al.,* 2011). Primer set 309F/R amplifies only a segment from 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 and/or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. PCR using primers from another region of the genome, real-time PCR, 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.,* 2000)
77012F	356 bp	5'-ATC-GGT-GCA-CTA-CTC-GGA-3'	50%/68°C	AF218266
77353R		5'-TCG-TAC-TGG-CTG-TTC-ATC-3'	55%/63°C	(Nunan *et al.,* ~~2000~~ 2001)
392F	392 bp	5'-GGG-CGA-ACC-AGA-ATC-ACT-TA-3'	50%/68°C	AF218266
392R		5'-ATC-CGG-AGG-AAT-CTG-ATG-TG-3'	50%/71°C	(Tang *et al.,* 2000; 2007)
309F	309 bp	5'-TCC-AAC-ACT-TAG-TCA-AAA-CCA-A-3'	36%/68°C	AF218266
309R		5'-TGT-CTG-CTA-CGA-TGA-TTA-TCC-A-3'	40%/69°C	(Tang *et al.,* 2007)
MG831F	831 bp	5'-TTG-GGG-ATG-CAG-CAA-TAT-CT-3'	45%/58°C	DQ228358
MG831R		5'-GTC-CAT-CCA-CTG-ATC-GGA-CT-3'	55%/62°C	(Tang *et al.,* 2007)

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region (ORF 1) of the IHHNV genome. Primers 77353/77012 are from a region in between the nonstructural and the structural (coat protein) protein-coding regions 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 77012/77353 or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.

General PCR method for IHHNV: the PCR method described below for IHHNV generally follows the methods outlined in Nunan *et al.* (2000). Cumulative experience with the technique has led to modifications with respect to template (DNA extraction of clinical specimens), choice of primers (Table 4.1), and volume of reaction.

i)   Use as a template, the DNA extracted from ground tissue homogenate (TN buffer, 0.4 M NaCl, 20 mM Tris, pH 7.4) or haemolymph (collected with a small amount of 10% sodium citrate) or from tissue or haemolymph that was fixed in 95% ethanol and then dried. A control consisting of tissue 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™ Nucelic Acid kits (Life Technologies), or Maxwell@ 16 Cell LEV DNA Purification Kit (Promega), or ~~reagents from Gibco Life Sciences~~ DNazol ~~Cat. No. 10503 027~~ (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 per 50 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 ng μl^{-1} 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 involves a 5-minute step 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 (35 cycles) and an extension programme. The programme is set as follows:

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, 10 × PCR buffer, the four dNTPs, the two primers, MgCl$_2$, AmpliTaq Gold and water (assume use of 1 μl of template; if using more, adjust water accordingly). Add mix to each tube. Use thin-walled tubes designed for PCR. Always run a positive and a negative control.

'Master Mix':

DD H$_2$O	32.5 μl × number of samples
10 × PCR buffer	5 μl × number of samples
10 mM dTTP	1 μl × number of samples
10 mM dATP	1 μl × number of samples
10 mM dCTP	1 μl × number of samples
10 mM dGTP	1 μl × number of samples
25 mM MgCl$_2$	4 μl × number of samples
Forward primer (100 ng μl^{-1})	1.5 μl × number of samples
Reverse primer (100 ng μl^{-1})	1.5 μl × number of samples
AmpliTaq Gold	0.5 μl × number of samples

Vortex this solution to mix all reagents well; keep on ice.

NOTE: The volume of the PCR reaction may be modified. Previously, the PCR reactions for IHHNV were run in 100 μl volumes, but it is not necessary to use that amount of reagents, therefore 50 μl volumes are described in this procedure. Likewise, the PCR reactions can also be run in volumes as small as 25 μl. To do this, increase or decrease the volume of the reagents accordingly.

vi) For a 50 μl reaction mix, add 49 μl Master Mix to each tube and then add 1 μl of the sample 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.

viii) If mineral oil was used, recover samples from under the mineral oil using a pipette set at 50 μl and transfer to a fresh tube. Using the long-tipped pipette tips (designed for loading gels) results in less oil being carried over with the sample.

ix) Run 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.

Real-time PCR (*qPCR*) *method for IHHNV:* qPCR methods have been developed for the detection of IHHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHHNV genome (Dhar *et al.,* 2001; Tang & Lightner, 2001).

The qPCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (2001).

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for non-structural protein. The primers and TaqMan probe are designed by the Primer Express software (Applied Biosystems). 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.

iii) The qPCR reaction mixture contains: TaqMan Universal PCR Master Mix (Applied Biosystems, part no. 4324018), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 or equivalent can also be used). The cycling profile is: activation of AmpliTaq Gold for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The levels of fluorescence are measured at the end of the annealing and extension step.

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 of PCR product. Samples will be defined as negative if there is no Ct (threshold cycle) value is after 40 cycles. the Ct (threshold cycle) values exceed 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive. To confirm the real-time PCR results, an aliquot of PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. An 81-bp DNA fragment can be visualised in the samples that are positive for IHHNV.

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 from IHHNV-infected tissue.

Sequencing: PCR products may be cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified product 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 of these IHHNV PCR assays also detected IHHNV-related sequences in *P. monodon* populations in Africa, Australia and Thailand (Tang & Lightner, 2006; Saksmerprome *et al.,* 2011). To discriminate the IHHNV-related sequences from the actual virus, PCR assays using primers that detect the IHHN viral sequence and do not react with IHHNV-related sequences present in the *P. monodon* stocks from Africa or Australia (Tang *et al.,* 2007), or Thailand (e.g. Saksmerprome *et al.,* 2011) have been developed.

PCR commercial kits are available for IHHNV diagnosis and can be acceptable provided they have been validated as fit for such purpose. The OIE validation procedure is described in Chapter 1.1.2 Principles and methods of validation of diagnostic assays for infectious diseases.

4.3.2. Serological methods

Shrimp are invertebrate animals and do not produce antibodies. Therefore, serological methods for IHHN are not available.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of 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. 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
DNA probes – *in situ*	d	d	b	b	a	a
PCR, qPCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; qPCR = real-time polymerase chain reaction.

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

Poor hatching success of eggs, and poor survival and culture performance of the larval and PL stages (Motte *et al.,* 2003) when broodstock are used from wild or farmed stocks where IHHNV is enzootic.

In 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 may show poor and highly disparate growth, poor overall culture performance, and cuticular deformities, including especially bent rostrums and deformed sixth abdominal segments.

Demonstration of eosinophilic to pale basophilic intranuclear inclusion bodies in the typical target tissues for IHHNV. As IHHNV intranuclear inclusion bodies are nearly identical in appearance to those occurring in the early stages of WSSV 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.

7.2. Definition of confirmed case

Any combination of at least two of the following four methods (with positive results):

• Positive dot-blot hybridisation test results for IHHNV.

• ISH positive histological signal to IHHNV-type lesions.

• PCR positive results for IHHNV.

• Sequencing of PCR specific products may be required when the purpose is to determine the genotype of IHHNV.

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CHAPTER 2.2.4.

NECROTISING HEPATOPANCREATITIS

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Necrotising hepatopancreatitis (NHP) disease is caused by infection with Gram-negative, pleomorphic intracellular alpha-proteobacterium (Frelier *et al.,* 1992; Lightner & Redman, 1994; Lightner *et al.,* 1992; Loy *et al.,* 1996a; 1996b). 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.*, 2011a).

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; 2011b; 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 is a pleomorphic, Gram-negative, intracytoplasmic bacterium. It is a member of the α-subclass of proteobacteria and remains unclassified (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 (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy *et al.,* 1996a; 1996b). Genetic analysis of the NHPB associated with North and South American outbreaks of NHP suggest that the isolates are either identical or very closely related subspecies (Loy *et al.,* 1996a; 1996b).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent

NHPB-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. NHPB frozen at –20°C –70°C and –80°C have been shown to retain infectivity in experimental transmission trials with *Penaeus vannamei* (Crabtree *et al.,* 2006; Frelier *et al.,* 1992).

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

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).

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.*, 2011b).

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).

2.2.2. Susceptible stages of the host

NHPB has been demonstrated in juveniles, adults and broodstock of *P. vannamei*.

2.2.3. Species or sub-population predilection

See Sections 2.2.1 and 2.2.2.

2.2.4. Target organs and infected tissue

The target tissue is the hepatopancreas, with NHPB infection reported in all hepatopancreatic cell types.

2.2.5. Persistent infection with lifelong carriers

Some members of *P. vannamei* populations that survive NHPB infections and/or epizootics may carry the intracellular bacteria for life and pass it on to other populations by horizontal transmission (Aranguren *et al.,* 2006; Lightner, 2005; Morales-Covarrubias, 2008; 2010; Vincent & Lotz, 2005).

Natural transmission of NHPB 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; 2011a; Vincent & Lotz, 2005).

2.2.6. 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

Transmission of NHPB can be horizontal by cannibalism; transmission by contaminated water has been demonstrated (Aranguren *et al.,* 2006; 2010; Frelier *et al.,* 1993; Gracia-Valenzuela *et al.*, 2011; Morales-Covarrubias *et al.*, 2011b; Vincent *et al.*, 2004).

2.3.2. Prevalence

Some reported mean values for NHPB prevalence in wild stocks are between 5.6 and 15% in *P. duorarum,* and between 5 and 17% in *P. aztecus* collected from Carrizal and Carbonera, Laguna Madre of Tamaulipas, Mexico (Aguirre-Guzman *et al.,* 2010); 0.77% in *P. vannamei,* and 0.43% in *P. stylirostris* collected from Tumbes Region, Peru (Lightner & Redman, 1994).

Some reported mean values for NHPB prevalence in shrimp farms are betwwen 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.*, 2011a).

2.3.3. Geographical distribution

NHPB 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 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.*, 2011a).

2.3.4. Mortality and morbidity

In *P. vannamei*, infection by NHPB results in an acute, usually catastrophic disease with mortalities approaching 100%.

2.3.5. Environmental factors

The replication rate of NHPB increases at lengthy periods of high temperatures (>29°C) and salinity changes (20–38%). In Mexico, NHPB has been detected at low prevalences (<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 prevalences and mortality (>20%) are observed (Morales-Covarrubias, 2010).

2.4. Control and prevention

Control

The use of the antibiotics, oxytetracycline and florfenicol 50%, in medicated feeds every 8 hours for 10 days is probably the best NHP treatment currently available, particularly if disease is detected in the initial phase (Frelier *et al.,* 1995; Morales-Covarrubias *et al.*, 2011b).

Prevention

a) Early detection (initial phase) of clinical NHP is important for successful treatment because of the potential for cannibalism to amplify and transmit the disease.

b) Shrimp starvation and cannibalism of shrimps with NHPB, as well as positive conditions for NHPB cultivation, are important factors for NHPB propagation in *P. vannamei*.

c) The use of hydrated lime (Ca(OH)$_2$) to treat pond bottoms during pond preparation before stocking can help reduce NHP incidence.

    d) Preventive measures can include raking, tilling and removing sediments from the bottom of the ponds, prolonged sun drying of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, and drying and extensive liming of ponds.

    e) The use of specific pathogen-free (SPF) and female broodstock is an effective preventive measure.

2.4.1. Vaccination

No scientifically confirmed reports.

2.4.2. Chemotherapy

No scientifically confirmed reports.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Resistance breeding

No scientifically confirmed reports.

2.4.5. Restocking with resistant species

No scientifically confirmed reports.

2.4.6. Blocking agents

No scientifically confirmed reports.

2.4.7. Disinfection of eggs and larvae

NHPB has been demonstrated to be transmitted horizontally by cannibalism (Frelier *et al.,* 1993; Gracia-Valenzuela *et al.*, 2011; Johnson, 1990; Jory, 1997; Lightner, 1996; Lightner & Redman, 1994; Loy *et al.,* 1996b; Morales-Covarrubias *et al.*, 2011b; Vincent & Lotz, 2005; 2007). Disinfection of eggs and larvae is, therefore, a good management practice (Lee & O'Bryen, 2003) and is recommended for its potential to reduce NHPB 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 infections and disease. Among these has been the application of polymerase chain reaction (PCR) to prescreening of wild or pond-reared broodstock.

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by NHPB are life stages (postlarvae [PL], juveniles and adults).

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method, see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, sub adults and adults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs or tissues

NHPB infects most enteric tissue. The principal target tissue for NHPB is hepatopancreas. Faeces may be collected and used for testing (usually by PCR, or dot-blot hybridisation with specific probes) when non-lethal testing of valuable broodstock is necessary (Bondad-Reantasco *et al.,* 2001; Bradley-Dunlop *et al.,* 2004; Briñez *et al.,* 2003; Frelier *et al.,* 1993; Lightner, 1996; Morales-Covarrubias *et al.*, 2011b).

3.5. Samples/tissues those are not suitable

NHPB are enteric bacteria and do not replicate in the midgut (enteric tissues), caeca (enteric tissues), 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 infections may be 'enhanced' in a contained population by rearing shrimps in relatively crowded or stressful conditions. The 'crowding stress' factors may include high stocking densities, ablated, and marginal water quality (i.e. low dissolved oxygen, elevated water temperature, or elevated ammonia or nitrite) in the holding tank water. These conditions may encourage expression of low-grade NHPB infections and the transmission of the agent from carriers to previously uninfected hosts in the population resulting 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 NHP. These include: lethargy, reduced food intake, atrophied hepatopancreas, anorexia and empty guts, noticeable 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.

4.1.2. Behavioural changes

In acute NHP disease, *P. vannamei* may present behavioural changes.

4.2. Clinical methods

4.2.1. Gross pathology

NHPB often causes an acute disease with very high mortalities in young juveniles, adult and broodstock. In horizontally infected in young juveniles, adult and broodstock, the incubation period and severity of the disease is somewhat size and/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.*, 2011b). Gross signs are not NHP specific, but acute NHP 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 NHP in *P. vannamei* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained histological methods (see Section 4.2.6).

4.2.3.1. Initial phase of necrotising hepatopancreatitis

Initial NHPB infection is more difficult to diagnose using routine H&E histological methods. For diagnosis of initial infections, the use of molecular methods are recommended for NHPB detection (e.g. by PCR or application of NHPB-specific DNA probes to dot-blot hybridisation tests or *in-situ* hybridisation (ISH) of histological sections).

4.2.3.2. The acute phase of necrotising hepatopancreatitis

The acute NHP disease is characterised by atrophied hepatopancreas with moderate atrophy of the tubule epithelia, presence of bacterial ~~form~~ <u>cells</u> and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations). Hypertrophic cells, individual ephitelial cells appeared to be separated from adjacent cells, undergo necrosis and desquamation in to tubular lumen and the tubular epithelial cell lipid content is variable.

4.2.3.3. Transition phase of necrotising hepatopancreatitis

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

4.2.3.4. Chronic phase of necrotising hepatopancreatitis

In the chronic phase of NHP, 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 haemocyte nodules.

4.2.4. Wet mounts

Wet-mount squash examination of hepatopancreas (HP) tissue is generally conducted to detect presumptive NHP disease. The hepatopancreas may be atrophied and have any of the following characteristics: soft and watery; fluid filled center; paled with black stripes (melanised tubules); pale center instead of the normal orange coloration. Elevated mortality rates reaching over 90% can occur within 30 days of onset of clinical signs if not treated. 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 is based on the deformation or tubular atrophy mainly of the apical region of the tubule.

NHP disease has four phases:

Phase Initial: low presence of tubular deformation (1–5 field^{-1} organism^{-1}) and cell detachment.

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

Transition phase: infiltration of haemocytes, increased numbers of deformed tubules (11–15 field^{-1} organism^{-1}), melanised tubules, necrotic tubules and a high level of encapsulation present in different regions of the sample. At this stage haemocyte nodules were observed in the presence of 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 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, NHP-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 NHP disease and unusual mortalities.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

NHPB 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 specific cDNA probes to NHP according to the methods described in Bradley-Dunlop *et al.* (2004) and Loy & Frelier (1996).

4.3.1.2.3. Molecular techniques

ISH and ~~reverse transcription (RT)-~~PCR tests for NHPB have been developed, and ~~RT-~~PCR kits for NHPB are commercially available. PCR tests for NHP have been developed and a number of methods and and commercial products using these methods are available (Loy & Frelier, 1996; Loy *et al.,* 1996b). Gene probes and PCR methods provide greater diagnostic sensitivity than do classic histological approaches to NHP diagnosis. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp.

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

Non-radioactive, DIG-labelled cDNA probes for NHPB 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 detection and diagnosis that employ classical histological methods (Johnson, 1990; Lightner, 1996; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2011b). 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, provides a definitive diagnosis of NHPB infection (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.*, 2006). Pathognomonic NHPB-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. (See Chapter 2.2.2 IHHN for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

4.3.1.2.3.2. ~~Reverse transcription (RT)-~~PCR method

Hepatopancreas and faeces may be assayed for NHPB 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 ~~ribosomal~~ 16S rRNA of NHPB~~, which amplify a 379 bp fragment~~ (Nunan *et al.*, 2008). ~~The primer concentration (F2/R2) used for each is 0.31 μM. The cycling parameters are: Step 1: 95°C for 2 minutes, 1 cycle; Step 2: 60°C for 30 seconds, 72°C for 30 seconds and 95°C for 30 seconds, 25 cycles; Step 3: 60°C for 1 minute, 72°C for 2 minutes, 1 cycle; 4°C infinite hold.~~ The ~~RT-~~PCR method outlined below ~~for NHPB~~ generally follows the method ~~used~~ described in ~~Nunan *et al.* (2008)~~ Aranguren *et al.* (2010) with modifications by an OIE Reference Laboratory in the USA.

i) *Preparation of ~~RNA~~ DNA template:* ~~RNA~~ DNA can be extracted from 25-50 mg of fresh, frozen and ethanol-preserved hepatopancreas. Extraction of ~~RNA~~ DNA should be performed using commercially available ~~RNA~~ DNA tissue extraction kits, such as the High Pure RNA Tissue Kit [2] (Roche, Germany) and following the manufacturer's procedures for production of quality RNA templates. Other DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucelic Acid kits (Life Technologies),or Maxwell@ 16 Cell LEV DNA Purification Kit (Promega).

ii) ~~The RT-PCR assay is carried out in solution, using final RNA concentration must be 10–1000 ng ml−1.~~

ii) The following controls should be included in every ~~RT-~~PCR assay for NHPB: a) known NHPB negative tissue sample; b) a known NHPB-positive sample (hepatopancreas); and c) a 'no template' control.

iii) The ~~GeneAmp® EZ rTth RNA PCR kit (Applied Bioscience, USA)~~ PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

iv) The optimised ~~RT-~~PCR conditions (final concentrations in ~~50~~ 25 μl total volume) for detection of NHPB in shrimp hepatopancreas samples are: primers (~~0.46~~ 0.2 μM each), dNTPs (~~300~~ 200 μM each), ~~rTth DNA~~ Taq polymerase (~~2.5 U 50~~ 0.1 U μl⁻¹), manganese ~~acetate~~ chloride (~~2.5~~ 1.5 mM)~~, in 5 × EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2)~~ in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.

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

v) If the thermal cycler does not have a heated lid, then light mineral oil (50 μl) is overlaid on the top of the 50 25 μl reaction mixtures to prevent condensation or evaporation during thermal cycling.

vii) ~~The RNA template and all the reagents are combined and reverse transcription was allowed to proceed at 60°C for 30 minutes, followed for 2 minutes.~~

vi) The cycling parameters are: Step 1: 95°C for 2 5 minutes, 1 cycle; Step 2: 60 95°C for 30 seconds, 72 60°C for 30 seconds and 95 72°C for 30 seconds, 25 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.

ix) ~~Details of the composition of the reagents and buffers used here may be found in Chapter 2.2.2 IHHN.~~

4.3.1.2.3.3. Real-time PCR method

Real-time PCR methods have been developed for the detection of NHPB. 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 genome (Aranguren *et al.,* 2010; Vincent & Lotz, 2005).

The real-time PCR method using TaqMan chemistry described below for NHPB 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 (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 ~~RNA~~ DNA template:* the extraction and purification of ~~RNA~~ DNA template from hepatopancreas, is the same as that described in the section for traditional ~~real time~~ 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 RNA, 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. 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. Samples will be defined as negative if there is no Ct (threshold cycle) value is after 40 cycles.

vi) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler. A positive control should also be included, and this can be an *in-vitro* transcribed RNA plasmid DNA containing the target sequence, purified bacteria, or ~~RNA~~ DNA extracted from NHPB-infected hepatopancreas.

Annex 17 (contd)

4.3.1.2.3.4. Sequencing

RT-PCR products may be cloned and sequenced <u>or sequenced directly</u> when necessary to confirm infection by NHPB 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 isolation and purification are available (Aranguren *et al.,* 2010; Vincent & Lotz, 2005), but these are not recommended for routine diagnosis of NHP.

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.

5.   Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of NHPB 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
qPCR	a	a	a	a	a	a
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;
qPCR = quantitative PCR.

6. Test(s) recommended for targeted surveillance to declare freedom from 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-induced lesions in the hepatopancreas by histology (with or without confirmation by ISH with NHPB-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is represented by:

- Sudden high mortalities in late PL, juvenile or subadult *P. vannamei* or *P. stylirostris* in regions where NHPB 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 NHP, 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 is enzootic.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- Histological demonstration of diagnostic acute-phase NHPB lesions in (especially) the atrophied hepatopancreas with moderate atrophy of the tubule mucosa, presence of bacterial form and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations).

- ISH positive histological signal to NHPB-type lesions.

- PCR positive results for NHPB.

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CHAPTER 2.2.5.

TAURA SYNDROME

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Taura syndrome (TS) is a virus disease of penaeid shrimp caused by infection with Taura syndrome virus (TSV) (Bonami *et al.,* 1997; Fauquet *et al.,* 2005; Lightner 1996a; Mari *et al.,* 1998).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is TSV, as described by Bonami *et al.* (1997) and Mari *et al.* (1998; 2002). At least four genotypes (strains) have been documented based on the gene sequence encoding VP1 (= CP2), the largest and presumably dominant of the three major structural proteins of the virus. Based on VP1 (= CP2) 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 to with MAb 1A1 (in the enzyme-linked immunosorbent assay [ELISA], Western blots and *in-situ* hybridisation [ISH] immunohistochemistry (IHC) with infected tissues) and those that do not. The MAB 1A1 non-reactors were subdivided into Types B (TSV 98 Sinaloa, Mexico) and Type C (TSV 02 Belize), based on host species and virulence. All TSV isolates of the Americas and most, if not all, South-East Asian genotypes react with MAb 1A1. In marked contrast, none of the Belize genotype group reacts with MAb 1A1 (Erickson *et al.,* 2002; 2005), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.

TSV particles are 32 nm in diameter, non-enveloped icosahedrons and have a buoyant density of 1.338 g ml^{-1}. The genome of TSV consists of a linear, positive-sense single-stranded RNA 10,205 nucleotides in length, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (Bonami *et al.,* 1997; Mari *et al.,* 1998; 2002; Robles-Sikisaka *et al.,* 2001).

TSV has been assigned to the genus Aparavirus in the Family Dicistroviridae in the 9th report of the International Committee on Taxonomy of Viruses (ICTV; King *et al.,* 2012).

Other reported causes of TS: Taura syndrome in Ecuador was initially linked to fungicide contamination of shrimp farms, a contention that was supported by litigation for ~ 16 years after the disease was scientifically shown to have a viral aetiology (Bonami *et al.,* 1997; Hasson *et al.,* 1995; Lightner, 2005). Hence, several papers in the literature propose a toxic aetiology for 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

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. Susceptible stages of the host

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

2.2.3. Species or subpopulation predilection (probability of detection)

No data.

2.2.4. Target organs and infected tissue

TSV infects and has been shown to replicate (using ISH with specific DNA probes) principally in the cuticular epithelium (or hypodermis) of the general exoskeleton, foregut, hindgut, gills and appendages, and often in the connective tissues, the haematopoietic tissues, the lymphoid organ (LO), and antennal gland. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, striated muscle, and the ventral nerve cord, its branches and its ganglia typically show no histological signs of infection by TSV and are usually negative for TSV by ISH (Bondad-Reantaso *et al.,* 2001; Hasson *et al.,* 1997; 1999a; 1999b; Jimenez *et al.,* 2000; Lightner, 1996a; Lightner & Redman 1998a; 1998b; Lightner *et al.,* 1995; Srisuvan *et al.,* 2005).

2.2.5. Persistent infection with lifelong carriers

Some members of populations of *P. vannamei* or *P. stylirostris* that survive TSV infections and/or epizootics may carry the virus for life (Hasson *et al.,* 1999a; 1999b) and, although not documented, assumed to pass the virus to their progeny by vertical transmission.

2.2.6. Vectors

Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV-infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus 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 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

TS 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), TS 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 have been reported from virtually every penaeid shrimp-growing country in the Americas and Hawaii (Aguirre Guzman & Ascencio Valle, 2000; Brock, 1997; Lightner, 2011; Lightner *et al.,* 2012; Robles-Sikisaka *et al.,* 2001). TSV is 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, TSV has also been associated with significant mortalities in *P. indicus* being farmed in Saudi Arabia (Wertheim *et al.,* 2009).

2.3.4.   Mortality and morbidity

In on-farm epizootics of TS involving unselected (i.e. not selected for TSV resistance) stocks of *P. vannamei*, the principal host species for TSV, typical cumulative mortalities range from 40 to >90% in cultured populations of PL, juvenile, and subadult life stages. TSV-resistant lines of *P. vannamei* are available which show survival rates of up to 100% in laboratory challenge with all four TSV genotypes (Lightner *et al.,* 2009; Moss *et al.,* 2001).

2.3.5.   Environmental factors

Outbreaks of TS are more frequent when salinities are below 30 ppt (Jimenez *et al.,* 2000).

2.4. Control and prevention

2.4.1.   Vaccination

No effective vaccines for TSV are available.

2.4.2.   Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3.   Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4.   Resistance breeding

After TS emerged in Ecuador in 1992–1994, *P stylirostris* were found that possessed resistance to TSV (genotype 1, MAb 1A1 Type A). Following from this discovery and due to TSV reaching Mexico in 1994 where it caused crop failures of *P. vannamei,* selected lines of TSV-resistant *P. stylirostris* became the dominant shrimp farmed in western Mexico from 1995. However, in 1998–1999, a new 'strain' of TSV (Type B; Erickson *et al.,* 2002; Fegan & Clifford, 2001; Lightner, 1999; 2005; Zarin-Herzberg & Ascencio, 2001) emerged and caused massive epizootics in *P. stylirostris.* The emergence of this new 'strain' of TSV was soon followed in late 1999 by the introduction of white spot syndrome virus (WSSV) into shrimp farms in western Mexico, to which *P. stylirostris* had no resistance, effectively ending any interest in the culture of *P. stylirostris* in Mexico.

TSV-resistant domesticated stocks of *P. vannamei* and *P. stylirostris* have been developed. Some domesticated lines of TSV-resistant *P. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (Clifford, 1998; Moss *et al.,* 2001; White *et al.,* 2002). After the appearance of TS in Central America, improved TS resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region (Laramore, 1997).

2.4.5.   Restocking with resistant species

Selected lines of TS resistant *P. vannamei* have been developed and are commercially available (Clifford, 1998; Laramore, 1997; Moss *et al.,* 2001; White *et al.,* 2002).

2.4.6.   Blocking agents

Resistance to TSV infection was reported by expression of the TSV coat protein antisense RNA in *P. vannamei* zygotes. Transgenic juveniles reared from zygotes protected in this manner showed improved resistance to TSV challenge by *per os* or intramuscular (IM) injection routes (Lu & Sun, 2005). Similar results have been produced by injection of short random double-stranded RNAi sequences into juvenile *P. vannamei* (Robalino *et al.,* 2004).

2.4.7. Disinfection of eggs and larvae

While TSV is believed to be transmitted vertically (transovarian transmission), there have been no published report documenting this route of transmission. Disinfection of eggs and larvae (Chen *et al.,* 1992) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry practices have been applied successfully to reduce the risks TSV infections and disease occurring during farm grow-out. These include the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Lotz *et al.,* 1995; Moss *et al.,* 2001; Pruder *et al.,* 1995; Wyban 1992; Wyban *et al.,* 2004). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of TS. Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF *P. vannamei* that were free not only of TSV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Rosenberry, 2004).

3. Sampling

3.1. Selection of individual specimens

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

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and PL pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs and tissues

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

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

3.5. Samples/tissues that are not suitable

TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection by TSV.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only acute-phase TS 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 TS disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase TS disease present behavioural changes. Typically, severely affected shrimp apparently become hypoxic and move to the pond edges or pond surface where dissolved oxygen levels are higher. Such shrimp may attract seabirds in large numbers. In many TS disease outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicate the presence of a serious disease outbreak (which is often either TS or WSD when sea birds are observed) to the farm manager.

4.2. Clinical methods

4.2.1. Gross pathology

TS disease has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b; 2011; Lightner *et al.,* 1995). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of TS are unique and provide a presumptive diagnosis of the disease.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase 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 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 TS epizootics, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of TSV infection. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites resolving 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 TS 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)

TS disease in the acute and chronic phases can be diagnosed most reliably using histological methods (Hasson *et al.,* 1999b; Lightner, 1996a). Pathognomonic TSV-induced pathology is unique in acute-phase infections (Brock *et al.,* 1995; Lightner, 1996a; 2011). In chronic TSV infections, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.,* 1999b; Lightner 2011), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When LOS are observed by routine histology and chronic TSV infection is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR [see Section 4.3.1.2.7]) is recommended for confirmation of TSV infection.

4.2.3.1. Acute phase of Taura syndrome

Diagnosis of TS 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 TS, 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 TS disease when there is no concurrent necrosis of the parenchymal cells of the LO tubules. The absence of necrosis of the LO in acute-phase TSV infections distinguishes TS disease from acute-phase yellowhead disease in which similar patterns of necrosis to those induced by 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 TS 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 TS, 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 TS 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 TS 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 TS) focal lesions of acute-phase TS in cuticular epithelial cells. Preparations presenting TS acute-phase lesions will contain numerous spherical structures (see the histopathological methods in Section 4.2.3 above), which are pyknotic and karyorrhectic nuclei and cytoplasmic remnants of necrotic cells.

4.2.5.   Smears

Not applicable.

4.2.6.   Fixed sections

See Section 4.2.3.

4.2.7.   Electron microscopy/cytopathology

Not currently applicable for diagnostic purposes.

4.3. Agent detection and identification methods

4.3.1.   Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

TSV has not been grown *in vitro*, as no crustacean cell lines exist (Lightner, 1996a; Pantoja *et al.,* 2004). Despite a publication that incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle *et al.,* 2003), two other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (Luo *et al.,* 2004; Pantoja *et al.,* 2004).

4.3.1.2.2. Antibody-based antigen detection methods

An MAb for detection of TSV may be used to assay samples of haemolymph, tissue homogenates, or Davidson's AFA-fixed tissue sections from shrimp (Erickson *et al.,* 2002; 2005; Poulos *et al.,* 1999). TSV MAb 1A1 may be used to distinguish some variants or 'strains' of TSV from other strains (Erickson *et al.,* 2002; 2005).

4.3.1.2.3. Bioassay method

Confirmation of TSV infection may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the virus (Brock *et al.,* 1997; Garza *et al.,* 1997; Hasson *et al.,* 1999b; 1995; Lightner, 1996a; Lotz, 1997; Overstreet *et al.,* 1997). Oral or injection protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped carcasses of suspect shrimp to SPF juvenile *P. vannamei* in small tanks (White *et al.,* 2002). The use of a negative control tank of indicator shrimp, which receive only SPF (TSV-free) tissue and normal shrimp feed is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, 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 TS disease and unusual mortalities (Hasson *et al.,* 1999b; Lightner, 1996a; White *et al.,* 2002).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during a TSV epizootic. Heads only should be used if shrimp display gross transition-phase lesions (multifocal melanised spots on the cuticle) or no clinical signs of infection (chronic phase) as the virus, if present, will be concentrated in the LO (Hasson *et al.,* 1999b; Lightner, 1996a). For non-lethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (Lightner, 1996a).

To perform the IM (injection) bioassay for TSV:

Note that tissues and the resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

i)   Prepare a 1:2 or 1:3 ratio of TSV-suspect shrimp heads or whole shrimp with TN buffer (see Chapter 2.2.2, infectious hypodermal and haematopoietic necrosis [IHHN], for the composition of this buffer) 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 for short-term [weeks] storage and –80°C for 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 material 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.

Annex 18 (contd)

x) If TSV was present in the inoculum, the indicator shrimp should begin to die within 24–48 hours post-injection. Lower doses of virus may take longer to establish a lethal infection and shrimp should be monitored for at least 10–15 days post-injection.

xi) The presence (or absence) of TSV in the indicator shrimp should be confirmed by histological analysis (and/or ISH by gene probe, if available) of Davidson's fixed moribund shrimp. If additional confirmation is needed beyond demonstration of pathognomonic TSV lesions, RT-PCR with sequencing of the resulting amplicon can be carried out.

4.3.1.2.4. Sentinel shrimp bioassay method

As a variation to the bioassay technique, a 'sentinel shrimp' system may be used. For example, TSV-sensitive stocks of small juvenile SPF *P. vannamei* may be held in net-pens in tanks, or in the same water system, with other shrimp of unknown TSV status to bioassay for the presence of infectious agents such as TSV.

4.3.1.2.5. Dot-blot immunoassay method

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore, South San Francisco, California [CA], USA)[3].

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 Tris-NaCl (100 mM each) buffer containing 50 mM MgCl$_2$, pH 9.5.

vi) Reactions are stopped with distilled water.

vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semi-purified TSV. A negative reaction is one in which no coloured spot is visible in the well.

4.3.1.2.6. Other antibody-based methods

The TSV MAb 1A1 may be applicable to other antibody-based test formats (i.e. indirect fluorescent antibody [IFAT] or immunohistochemistry [IHC] tests with tissue smears, frozen sections, or deparaffinised fixed tissues). MAb 1A1 is applicable for use in an IHC format using Davidson's AFA-fixed tissue sections (Erickson *et al.,* 2002; 2005).

It is recommended that unexpected results from MAb-based tests for TSV should be interpreted in the context of clinical signs, case history, and in conjunction with other test results (e.g. RT-PCR test results, or findings from histology or ISH with a TSV-specific DNA probe – see appropriate sections in this chapter).

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

4.3.1.2.7. Molecular techniques

ISH and RT-PCR tests for TSV have been developed, and kits of RT-PCR methods for TSV are commercially available. The dot-blot method for TSV detection is not available.

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

Non-radioactive, DIG-labelled cDNA probes for TSV may be produced in the laboratory. The ISH method provides greater diagnostic sensitivity than do more traditional methods for TSV detection and diagnosis that employ classic histological methods (Hasson *et al.,* 1999a; Lightner, 1996a; 1999; Lightner & Redman 1998b; Mari *et al.,* 1998). The ISH assay of routine histological sections of acute- and transition-phase lesions in the cuticular epithelium, other tissues, and of LOS in transition and chronic phase with a specific DIG-labelled cDNA probe to TSV, provides a definitive diagnosis of TSV infection (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b). Pathognomonic TSV-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. Not reacting to the probe are the prominent karyorrhectic nuclear fragments and pyknotic nuclei that contribute to the pathognomonic 'buckshot riddled' appearance of TS lesions (Lightner, 1996a; Mari *et al.,* 1998). (See Chapter 2.2.2 IHHN for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

False-negative ISH results may occur with Davidson's fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson's fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This hydrolysis can be avoided through the use of neutral fixatives, including an 'RNA-friendly' fixative developed for shrimp, or by the proper use (avoiding fixation times over 24 hours) of Davidson's fixative (Hasson *et al.,* 1997; Lightner, 1996a; Lightner & Redman 1998).

4.3.1.2.7.2. Reverse-transcription (RT)-PCR method

Tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9992F and 9195R, amplify a 231 base pair (bp) sequence of the TSV genome (Nunan *et al.,* 1998). The fragment amplified is from a conserved sequence located in the intergenic region and ORF 2 of TSV. Primer 9992F is located near the 3' end of intergenic region and 9195R is located on ORF 2 within VP2 (= CP1) (Mari *et al.,* 2002; Nunan *et al.,* 1998). Recently, 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).

Primer	Product	Sequence	G+C%
9992F	231 bp	5'-AAG-TAG-ACA-GCC-GCG-CTT-3'	55%
9195R		5'-TCA-ATG-AGA-GCT-TGG-TCC-3'	50%
7171F	341 bp	5'-CGA-CAG-TTG-GAC-ATC-TAG-TG-3'	50%
7511R		5'-GAG-CTT-CAG-ACT-GCA-ACT-TC-3'	50%

The RT-PCR method outlined below for TSV generally follows the method used in Nunan *et al.* (1998).

i) *Preparation of RNA template:* RNA can be extracted from fresh, frozen and ethanol-preserved tissues. Extraction of RNA should be performed using commercially available RNA tissue extraction kits, such as the High Pure RNA Tissue Kit (Roche, Penzberg, Germany) and following the manufacturer's procedures for production of quality RNA templates.

ii) The RT-PCR assay is carried out in solution, using 10 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) is used for all amplification reactions described here.

v) The optimised RT-PCR conditions (final concentrations in 50 µl total volume) for detection of TSV in shrimp tissue samples are: primers (0.46 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.

vii) 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.

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.

viii) At 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 product can then be added to the well of a 2.0% 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 PCR (qPCR) method for TSV

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

The real-time RT-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). 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 RT-PCR.

iii) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal

cycler. A positive control should also be included, and this can be an *in-vitro* transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV-infected tissue.

iv) The RT-PCR reaction mixture contains: TaqMan One-step RT-PCR Master Mix (Applied Biosystems, part no. 4309169), 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 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

v) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied osystems; ABI PRISM 7000, 7300, 7500, or newer models and brands can also be used). The cycling consists of reverse transcription at 48°C for 30 minutes and initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The levels of fluorescence are measured at the end of each annealing/extension cycle.

vi) At the end of the reaction, real-time fluorescence measurements are analysed 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. Samples will be defined as negative if there is no Ct (threshold cycle) value is after 40 cycles the Ct (threshold cycle) value is 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive. To confirm the real-time RT-PCR results, an aliquot of RT-PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and exposed to UV light. A 72-bp DNA fragment can be visualised in the samples that are positive for TSV.

vi) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler. A positive control should also be included, and this can be an *in-vitro* transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV infected tissue.

4.3.1.2.7.4. Sequencing

RT-PCR products may be cloned and sequenced when necessary to confirm infection by TSV or to identify false positives or nonspecific amplification (Mari *et al.,* 2002; Nielsen *et al.,* 2005; Srisuvan *et al.,* 2005; Tang & Lightner, 2005; Wertheim *et al.,* 2009).

4.3.1.2.8. Agent purification

Methods for TSV isolation and purification are available (Bonami *et al.,* 1997; Hasson *et al.,* 1995; Mari *et al.,* 2002; Poulos *et al.,* 1999), but these are not recommended for routine diagnosis of TS.

4.3.2.   Serological methods

Not applicable because shrimp are invertebrate animals which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to TSV.

5.   Rating of tests against purpose of use

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

Annex 18 (contd)

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
DNA probes – *in situ*	d	c	b	b	a	a
RT-PCR, qRT-PCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy;
RT-PCR = reverse-transcriptase polymerase chain reaction.

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

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.7.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic TSV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with TSV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is represented by:

• Sudden high mortalities in late PL, juvenile or subadult *P. vannamei* or *P. stylirostris* in regions where TSV is enzootic;

• The sudden presence of numerous sea birds (gulls, cormorants, herons, terns, etc.) 'fishing' in one or more shrimp culture ponds;

• Samples of cultured *P. vannamei* or *P. stylirostris* from ponds with feeding sea birds that present gross signs indicative of acute- or transition-phase 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

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

• Histological demonstration of diagnostic acute-phase TSV lesions in (especially) the cuticular epithelia of the foregut (oesophagus, anterior, or posterior chambers of the stomach) and/or in the gills, appendages, or general cuticle. Such TSV lesions are pathognomonic for TSV only when they occur without accompanying severe acute necrosis (with nuclear pyknosis and karyorrhexis) of the parenchymal cells of the lymphoid organ tubules (which may occur in acute-phase yellowhead virus infections).

• ISH-positive (with a TSV-specific cDNA probe) signal to TSV-type lesions in histological sections (i.e. cuticular acute-phase TS lesions) or to distinctive lymphoid organ spheroids (LOS) in the lymphoid organs of shrimp with chronic phase TS lesions.

• RT-PCR positive results for TSV.

• Sequencing of PCR product encompassing CP2 may be accomplished, as needed, to determine the TSV genotype (Tang & Lightner, 2005; Wertheim *et al.,* 2009).

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CHAPTER 2.2.8.

INFECTION WITH YELLOW HEAD VIRUS

EU comment

The EU supports the proposed changes to this chapter.

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1. Scope

For the purpose of this chapter, yellow head disease (YHD) is considered to be infection with yellow head virus (YHV).

2. Disease information

2.1. Agent factors

2.1.1.   Aetiological agent, agent strains

Yellow head virus (genotype 1) is one of six known genotypes in the yellow head complex of viruses and is the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known genotypes in the complex (genotypes 3–6) occur commonly in healthy *Penaeus monodon* in East Africa, Asia and Australia and are rarely or never associated with disease (Walker *et al.,* 2001, Wijegoonawardane *et al.,* 2008a). YHV and other genotypes in the yellow head complex are classified by the International Committee on Taxonomy of Viruses as a single species (Gill-associated virus) in the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* (Cowley *et al.*, 2012). There is evidence of genetic recombination between genotypes (Wijegoonawardane *et al.*, 2009).

YHV forms enveloped, rod-shaped particles (40–60 nm × 150–200 nm). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids appear as rods (diameter 20–30 nm) and possess a helical symmetry with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p24 and envelope glycoproteins gp64 and gp116) and a ~26 kb positive-sense single-stranded RNA genome.

2.1.2.   Survival outside the host

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

2.1.3.   Stability of the agent (effective inactivation methods)

YHV can be inactivated by heating at 60°C for 15 minutes (Flegel *et al.*, 1995b). Little information is available on other inactivation methods but the virus appears to be susceptible to treatment with chlorine at 30 parts per million (0.03 mg ml^{-1}) (Flegel *et al.,* 1997).

2.1.4.   Life cycle

High multiplicity YHV infections in cell culture have not been reported. Infection at a multiplicity of infection of 0.001 in primary cultures of lymphoid organ cells has indicated that maximum viral titres are obtained 4 days post-infection (Assavalapsakul *et al., 2003*). Clinical signs of YHD occur in *P. monodon* within 7–10 days of exposure. YHV replicates in the cytoplasm of infected cells in which long filamentous pre-nucleocapsids are abundant and virions bud into cytoplasmic vesicles in densely packed paracrystalline arrays for egress at the cytoplasmic membrane (Chantanachookin *et al.,* 1993).

2.2. Host factors

2.2.1.   Susceptible host species

YHD outbreaks have been reported only in the black tiger prawn (*P. monodon*) and the white Pacific shrimp (*P. vannamei*) (Chantanachookin *et al.,* 1993; Senapin *et al.,* 2010). However, natural infections

have also been detected in the kuruma prawn (*P. japonicus*), white banana prawn (*P. merguiensis*), Pacific blue prawn (*P. stylirostris*), white prawn (*P. setiferus*), red endeavour prawn (*Metapenaeus*

ensis), mysid shrimp (*Palaemon styliferus*) and krill (*Acetes* sp.). Other species of penaeid and palemonid shrimp and prawns and krill that have been reported to be susceptible to experimental infection include: brown tiger prawn (*P. esculentus*), brown prawn (*P. aztecus*); pink prawn, hopper and brown-spotted prawn (*P. duorarum*), greentail prawn (*Metapenaeus bennettae*), Sunda river prawn (*Macrobrachium sintangense*), barred estuarine shrimp (*Palaemon serrifer*), the paste prawn (*Ascetes* sp.) and the daggerblade grass shrimp (*Palaemonetes pugio*) (Ma *et al.,* 2009). There are variations in the susceptibility of different species to disease. Laboratory trials have shown that YHV can cause high mortality in *P. monodon, P. vannamei, P. stylirostrus, P. aztecus, P. duorarum, M. sintangense, P. styliferus* and *P. serrifer* (Lightner *et al.,* 1998; Longyant *et al.,* 2005; 2006; Ma *et al.,* 2009). A survey of 16 crab species collected from the vicinity of shrimp farms in Thailand detected no evidence of either natural infection or experimental susceptibility (Longyant *et al.,* 2006). A critical review of susceptibility of crustaceans to yellow head disease and implications of inclusion in European legislation has been conducted (Stentiford *et al.,* 2009). GAV has been detected in *P. monodon* and *P. esculentus* (Walker *et al.,* 2001). To date, infections by other genotypes in the YHV complex have been detected only in *P. monodon* (Wijegoonawardane *et al.,* 2008a).

2.2.2. Susceptible stages of the host

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

2.2.3. Species or subpopulation predilection (probability of detection)

Viruses in yellow head complex genotypes 2–6 are only known to occur commonly (prevalence up to 100%) in healthy *P. monodon*, which appears to be the natural host (Walker *et al.,* 2001; Wijegoonawardane *et al.,* 2008a; 2009). In contrast, YHV (genotype 1) infections are usually detected only when disease is event and whilst they do not occur commonly in healthy *P. monodon*, infections have been detected in healthy wild populations of *P. stylirostris* (Castro-Longoria *et al.,* 2008). During YHD outbreaks in aquaculture ponds, the YHV infection prevalence can be assumed to be high. Natural YHV infections have been detected in *P. japonicus, P. merguiensis, P. setiferus, M. ensis,* and *P. styliferus* (Cowley *et al.,* 2002; Flegel *et al.,* 1995a; 1995b), but there is little information available on the natural prevalence.

2.2.4. Target organs and infected tissue

YHV targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia (Chantanachookin *et al.,* 1993; Lightner, 1996).

2.2.5. Persistent infection with lifelong carriers

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

2.2.6. Vectors

There are no known vectors of YHV.

2.2.7. Known or suspected wild aquatic animal carriers

Infection susceptibility and long-term persistence indicate the potential for a wide range of wild penaeid and palemonid shrimp to act as carriers.

2.3. Disease pattern

2.3.1. Transmission mechanisms

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

2.3.2. Prevalence

The infection prevalence of yellow head complex viruses in healthy *P. monodon* (as detected by nested polymerase chain reaction [PCR]) can be high (50–100%) in farmed and wild populations in Australia, Asia and East Africa as well as in *L. vannamei* farmed in Mexico (Castro-Longoria *et al.,* 2008; Cowley *et al.*, 2004; Sanchez-Barajas *et al.*, 2009; Walker *et al.*, 2001; Wijegoonawardane *et al.*, 2008a). The prevalence of individual genotypes varies according to the geographical origin of the shrimp. In contrast, except in situations of disease outbreaks in aquaculture ponds, the prevalence of YHV (genotype 1) is more commonly low (<1%) in healthy wild or farmed *P. monodon* (pers. Comm.). The use of detection methods less sensitive than nested PCR (e.g. histology, immunoblot, dot-blot, *in-situ* hybridisation), is likely in most cases to result in the real infection prevalence amongst populations of shrimp being underestimated.

2.3.3. Geographical distribution

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

2.3.4. Mortality and morbidity

With *P. monodon* being farmed in ponds, disease caused by YHV (genotype 1) can cause up to 100% mortality within 3–5 days of the first appearance of clinical signs (Chantanachookin *et al.,* 1993). GAV (genotype 2) has also been associated with morbidity and up to 80% mortality in ponds of *P. monodon* farmed in Australia. Whilst mortalities can easily be induced by experimental exposure of *P. monodon* to YHV or GAV, bioassays have identified YHV to be far more virulent (~10^6-fold by lethal dose [LD_{50}] 50% end-point analysis) (Oanh *et al.,* 2011). Genotypes 3, 4, 5 and 6 have not yet been associated with disease (Wijegoonawardane *et al.*, 2008a).

2.3.5. Environmental factors

Elevated virus infection levels accompanied by disease can be precipitated by physiological stress induced by sudden changes in pH or dissolved oxygen levels, or other environmental factors (Flegel *et al.*, 1997). The much higher virulence of YHV compared to GAV and other genotypes appears to ensure that the infection threshold required to cause disease is reached far more easily.

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods have been developed.

2.4.2. Chemotherapy

No effective commercial anti-viral product is yet available.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Resistance breeding

Not reported.

2.4.5. Restocking with resistant species

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

2.4.6. Blocking agents

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

2.4.7. Disinfection of eggs and larvae

Not reported.

2.4.8. General husbandry practices

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

3. Sampling

3.1. Selection of individual specimens

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

3.2. Preservation of samples for submission

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

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

Tissue samples for histology should be preserved in Davidson's fixative. Formalin (10%) in seawater may be a useful alternative.

Tissues for electron microscopy should be sampled from live shrimp.

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

3.3. Pooling of samples

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

3.4. Best organs or tissues

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

3.5. Samples/tissues that are not suitable

Not determined.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Shrimp from late PL stages onwards can be infected experimentally with YHV. In cultured shrimp, infection can result in mass mortality occurring, usually in early to late juvenile stages. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas, which may be exceptionally soft when compared with the brown hepatopancreas of a healthy shrimp. In many cases, the total loss of a pond crop occurs within a few days of the first appearance of shrimp showing gross signs of YHD (Chantanachookin *et al.,* 1993). Cessation of feeding, congregation of moribund shrimp at pond edges and a generally bleached appearance are always seen in YHD outbreaks. However, these disease features are not particularly distinctive for YHD, and in the absence of other more pathognomonic gross signs are not reliable even for preliminary diagnosis of YHD. Gross signs of GAV disease include swimming near the surface and at the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow discoloration of the gills (Spann *et al.*, 1997). However, these signs can occur commonly in response to various stressors and thus are not considered pathognomonic for GAV disease. Shrimp chronically infected with YHV or GAV display normal appearance and behaviour.

4.1.2. Behavioural changes

Exceptionally high feeding activity followed by an abrupt cessation of feeding may occur within 2–4 days of the appearance of gross clinical signs of disease and mortality. Moribund shrimp may congregate at pond edges near the surface (Chantanachookin *et al.,* 1993).

4.2. Clinical methods

4.2.1. Gross pathology

See Section 4.1.

4.2.2. Clinical chemistry

None described.

4.2.3. Microscopic pathology

Fix the cephalothorax tissues of moribund shrimp suspected to be affected by YHD in Davidson's fixative, prepare tissue sections and stain with Meyer's haematoxylin and eosin (H&E) using standard histological procedures (Lightner, 1996). Examine tissues of ectodermal and mesodermal origin by light microscopy for the presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 μm in diameter or smaller(Chantanachookin *et al.,* 1993). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly informative.

4.2.4. Wet mounts

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

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

4.2.5. Smears

For moribund shrimp affected by YHD, haemolymph smears are not useful because haemocytes are usually depleted in the advanced stages of disease. In cases of suspected YHD where moribund shrimp have been sampled from a pond, haemolymph should be collected from grossly normal shrimp from the same pond. Draw the haemolymph into a syringe containing two volumes of either 25% formalin or Davidson's fixative modified by replacing the acetic acid component with either water or formalin. Mix thoroughly, ignore clots in the syringe, place a drop on a microscope slide, smear and then air-dry before staining with H&E or other standard blood smear stains. Dehydrate, add mounting fluid and a cover-slip. Examine under a light microscope using a ×40 objective lens. For YHD-affected shrimp, some smears will show moderate to high numbers of haemocytes with karyorrhectic or pyknotic nuclei. It is important that there is no evidence of concomitant bacterial infection in slides of haemocytes displaying such nuclei,, as bacterial infections may cause similar changes in haemocytes. When making a presumptive diagnosis of YHD, the results from haemolymph smears should be considered in conjunction with the results from rapid-stained whole mounts (see above) or stained tissue sections.

4.2.6. Electron microscopy/cytopathology

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

Stun live shrimp by immersion in iced water until just immobilised or kill by injection of fixative. Quickly dissect and remove small portions of target tissue (no larger than a few mm in diameter) and fix in at least 10 volumes of 6% glutaraldehyde held at 4°C and buffered with sodium cacodylate (Na[CH$_3$]$_2$AsO$_2$$^.$3H$_2$O) solution (8.6 g Na cacodylate, 10 g NaCl, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl) or phosphate solution (0.6 g NaH$_2$PO$_4$$^.H_2$O, 1.5 g Na$_2HPO_4$, 1 g NaCl, 0.5 g sucrose, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl). Fix for at least 24 h prior to processing. For long-term storage in fixative at 4°C, reduce glutaraldehyde to 0.5–1.0%. Processing involves post-fixation with 1% osmium tetroxide, dehydration, embedding, sectioning and staining with uranyl acetate and lead citrate according to standard TEM reagents and methods (Lightner, 1996).

In the cytoplasm of cells infected with YHV, both nucleocapsid precursors and complete enveloped virions are observed. Nucleocapsid precursors appear as long filaments approximately 15 nm in diameter that can vary markedly in length (80–450 nm) and that can sometimes be packed densely in paracrystalline arrays. Virions appear as rod-shaped, enveloped particles (40–60 nm × 150–200 nm) with rounded ends and prominent projections (8–11 nm) extending from the surface. In the cell cytoplasm, virions are commonly seen to be localised or packed densely within intracellular vesicles. Virions may also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV by TEM.

Lymphoid organ spheroids are commonly observed in healthy *P. monodon* chronically infected with YHV or GAV and lymphoid organ necrosis often accompanies disease (Spann *et al.*, 1997). However, spheroid formation and structural degeneration of lymphoid organ tissue also result from infection by other shrimp viruses (Lightner, 1996).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

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

4.3.1.2.2. Antibody–based antigen detection methods

Reagents and protocols for detecting YHV proteins with antibodies have been published (Loh *et al.* 1998; Lu *et al.* 1994). Virions purified from haemolymph of experimentally infected shrimp have been used to produce antiserum in New Zealand white rabbits. From this antiserum, immunoglobulin (IgG) was purified using protein-G-linked columns and cross-reacting normal shrimp antigens were removed by adsorption to acetone-dried, ground shrimp muscle tissue and haemolymph. To detect YHV proteins by Western blotting, dilute 0.1 ml haemolymph collected from a live shrimp in an equal volume of citrate buffer and either run immediately or store at –80°C until used. Clarify 200 µl of the sample at 8000 *g* for 5 minutes and then pellet virions from the clarified supernatant by ultracentrifugation at 140,000 *g* for 5 minutes. Resuspend pellets in 100 µl 2 × loading buffer (2.5 ml 0.5 mM Tris/HCl pH 6.8, 4 ml 10% sodium dodecyl sulphate [SDS], 2 ml glycerol, 1 µl β-mercaptoethanol, 0.5 ml deionised distilled water) and heat at 95°C for 5 minutes. Load 10 µl sample onto a 5% SDS-polyacrylamide gel and electrophorese at 200 V. Blot the gel onto a 0.1 mm pore size nitrocellulose membrane in blotting buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol per litre) at 100 V for 1 hour. Rinse the membrane with phosphate buffered saline (PBS pH 7.4), block in 5% skim milk (in PBS) for 1 hour, and

rinse with PBS for 5 minutes. Soak the membrane in a 1/1000 dilution of the anti-YHV antibody (IgG) for 1 hour, rinse three times with PBS for 5 minutes, and then soak for 1 hour in a 1/2500 dilution of goat anti-rabbit IgG-horseradish-peroxidase (HRP) conjugate. Rinse membrane three times with PBS for 5 minutes and then soak in HRP substrate 3,3',5,5'-tetramethylbenzidine , until blue-purple colour develops. Stop the reaction by soaking the membrane in distilled water. All incubations should be carried out at 25°C ± 2°C. Use a purified viral preparation as a positive control to identify positions of the YHV 116 kDa, 64 kDa and 20 kDa structural proteins. The Western blot YHV detection sensitivity is approximately 0.4 ng YHV protein ($\approx 10^6$ virions).

4.3.1.2.3. Molecular techniques

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

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

Sample preparation: For juvenile or adult shrimp, lymphoid organ, gill tissue or haemolymph may be used to prepare total RNA. Fresh tissue is preferred. Lymphoid organ and gill tissue preserved in 95% analytical-grade ethanol or RNAlater (various manufacturers), or stored frozen at –70°C are also suitable for total RNA preparation. Disrupt 10–20 mg lymphoid organ or gill tissue or 50 µl haemolymph in 500 µl Trizol^{TM4} reagent and extract total RNA according to the product manual. Resuspend RNA in 25 µl water treated with DEPC (diethyl-pyrocarbonate)-, heat at 55°C for 10 minutes, cool on ice and use immediately or store at –70°C until required. Ideally, a 1/200 dilution (i.e. 2.5 µl RNA in 500 µl DEPC-treated water) should be prepared, and UV absorbances at A_{260} nm and A_{280} nm (a UV spectrophotometer is required) should be determined to quantify and check the quality of the RNA (ratio approximately 2:1). RNA yield will vary depending on the type and freshness of tissues, quality of the preservative used, and the length of time tissue has been preserved. However, RNA yields from fresh tissues would be expected to vary from 0.2 to 2.0 µg µl^{-1} and about half these amounts from alcohol-preserved tissues.

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

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

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

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

To synthesise cDNA, mix 2 μl RNA in 20 μl PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV (Moloney murine leukaemia virus) reverse transcriptase, 1.0 U ribonuclease inhibitor, 0.75 μM antisense primer 144R, 1 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM MgCl$_2$, and incubate at 42°C for 15 minutes. Incubate the mixture at 100°C for 5 minutes to inactivate the reverse transcriptase and allow the mixture to cool to 5°C. Add PCR mixture (10 mM Tris/HCl pH 8.3, 50 mM KCl) containing 2.5 U *Taq* DNA polymerase, 2 mM MgCl$_2$ and 0.75 μM of sense primer 10F to give a final volume of 100 μl. Unless the instrument is fitted with a heated lid, overlay the tubes with 100 μl of mineral oil and conduct PCR amplification for 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and finishing at 72°C for 10 minutes. Alongside a suitable DNA ladder, apply a 20 μl aliquot of the PCR to a 2% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gel containing 0.5 μg ml^{-1} ethidium bromide and following electrophoresis, detect the 135 bp DNA band expected for YHV using a UV transilluminator.

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

PCR primer sequences:

10F: 5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'

144R: 5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

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

For cDNA synthesis, 2 μl RNA (ideally 1.0 μg total RNA, if quantified),0.7 μl 50 pmol μl^{-1} primer GY5 and DEPC-treated water are added to 6 μl total, the mixture , incubated at 70°C for 10 minutes and chilled on ice. Add 2 μl Superscript II buffer × 5 (250 mM Tris/HCl pH 8.3, 375 mM KCl, 15 mM MgCl$_2$), 1 μl 100 mM DTT and 0.5 μl 10 mM dNTP stock mixture (i.e. 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP) and mix gently. Preheat to 42°C for 2 minutes, add 0.5 μl 200 U μl^{-1} reverse transcriptase and incubate at 42°C for 1 hour. Heat the reaction at 70°C for 10 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, prepare a 50 μl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl$_2$, 35 pmol of each primer GY1 and GY4, 200 μM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube. Overlay the reaction mixture with 50 μl liquid paraffin, heat at 85°C for 2–3 minutes and then add 1 μl cDNA. Conduct PCR amplification using 35 cycles at 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. For the second PCR step, prepare a 50 μl reaction mixture containing 2 μl of the first step PCR product, 1 × *Taq* buffer (above), 1.5 mM MgCl$_2$, 35 pmol of each primer GY2, Y3 and G6, 200 μM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube and overlay with liquid paraffin. Conduct PCR using amplification conditions as described above. Apply a 10 μl aliquot of the PCR to 2% agarose/TAE gels containing 0.5 μg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

If the viral load is sufficiently high, a 794 bp DNA will be amplified from either GAV or YHV in the first PCR step. In the second PCR step, a 277 bp product indicates detection of YHV and a 406 bp product indicates detection of GAV. The presence of both 406 bp and 277 bp products indicates a dual infection with GAV and YHV. The detection sensitivity of the second-step PCR is ~1000-fold greater than the first-step PCR and GAV or YHV RNA can be detected to a limit of 10 fg lymphoid organ total RNA.

The sequences of RT-PCR primers generic for GAV and YHV (GY) or specific for GAV (G) or YHV (Y) are as follows:

GY1: 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'

GY2: 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'

GY4: 5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'

<u>Annex 19</u> (contd)

GY5: 5'-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3'

Y3: 5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3'

G6: 5'-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3'

NB: Due to reported problems with primer specificity for some emerging strains, all PCR products generated using protocol 2 should be sequenced to confirm the virus genotype.

Protocol 3: Nested *RT*-PCR for detection of all currently known genotypes in the yellow head complex (including YHV and GAV)

For cDNA synthesis, mix 2 µl RNA (ideally 1.0 µg total RNA, if quantified), 50 ng random hexamer primers and 1.0 µl 10 mM dNTP and make up to a total volume of 14 µl in sterile DEPC-treated water, incubate at 65°C for 5 minutes and chill on ice. Add 4.0 µl Superscript III buffer × 5, 1.0 µl 100 mM DTT, 1.0 µl 40 U µl^{-1} RNaseOUT^{TM} (Invitrogen) and 1.0 µl 200 U µl^{-1} reverse transcriptase and mix gently. Incubate at 25°C for 5 minutes and then at 42°C for 55 minutes, stop the reaction by heating at 70°C for 15 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, add 1 µl cDNA to a total 25 µl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 µl 25 mM MgCl$_2$, 0.35 µl primer mix containing 25 pmol µl^{-1} of each primer pool (see below) YC-F1ab and YC-R1ab, 0.5 µl 10 mM dNTP mix and 0.25 µl 5 U µl^{-1} *Taq* DNA polymerase. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final extension at 72°C for 7 minutes. For the second PCR step, use 1 µl of the first PCR product in the reaction mixture as prepared above but substituting primer pools YC-F2ab and YC-R2ab. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Apply an 8 µl aliquot of the PCR to 2% agarose/TAE gels containing 0.5 µg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

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

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

YC-F1ab pool: 5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3'

5'-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3'

YC-R1ab pool: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3'

5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'

YC-F2ab pool: 5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3'

5'-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3'

YC-R2ab pool: 5'-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3'

5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'

Mixed base codes: R(AG), Y(CT), M(AC), K(GT), S(GC), W(AT), H(ACT), B(GCT), V(AGC), D(AGT), N(AGCT).

4.3.1.2.3. *In-situ* hybridisation

The protocol of Tang *et al.* (2002) described is suitable for detecting YHV or GAV (Tang & Lightner, 1999). To preserve viral RNA accessibility, fix tissues sampled from live shrimp in neutral-buffered, modified Davidson's fixative without acetic acid (RF-fixative) (Hasson *et al.*, 1997). To achieve good tissue preservation whilst also preserving RNA accessibility, normal Davidson's fixative can be used as long as the fixation time is limited to 24 hours (maximum of 48 hours). Process the fixed tissue using standard histological methods and prepare 4 μm thick sections on Superfrost Plus slides (Fisher Scientific, Pennsylvania, USA). Prior to hybridisation, incubate sections at 65°C for 45 minutes, remove paraffin with Hemo-De (Fisher Scientific, Pennsylvania, USA), and rehydrate through a reducing ethanol concentration series to water. Digest sections with proteinase K (100 μg ml^{-1}, in 50 mM Tris/HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) for 15 minutes at 37°C, followed by post-fixation in 0.4% formaldehyde for 5 minutes. Rinse in 2 × SSC (standard saline citrate), then pre-hybridise with 500 μl pre-hybridisation solution (4 × SSC, 50% formamide, 1 × Denhardt's, 0.25 mg ml^{-1} yeast RNA, 0.5 mg m^{-1} sheared salmon sperm DNA, 5% dextran sulphate) at 42°C for 30 minutes. For hybridisation, overlay the sections with 250 μl hybridisation solution containing a digoxigenin-labelled DNA probe (20–40 ng ml^{-1}) at 42°C overnight. The next day, wash the sections as follows: 2 × SSC once for 30 minutes at room temperature; 1 × SSC twice for 5 minutes at 37°C; 0.5 × SSC twice for 5 minutes at 37°C. Incubate the sections with sheep anti-digoxigenin-alkaline phosphatase conjugate (Roche) at 37°C for 30 minutes. Wash with 0.1 M Tris/HCl pH 7.5, 0.15 M NaCl twice for 10 minutes at room temperature and rinse with 0.1 M Tris/HCl pH 9.5, 0.1 M NaCl. Incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in the dark for 1–2 h for colour development. Counterstain with Bismarck Brown Y (0.5%), dehydrate through a series of ethanol and Hemo-De, add Permount (Fisher Scientific, Pennsylvania, USA) and cover with a cover-slip. YHV-infected cells give a blue to purple-black colour against the brown counter stain. Include positive controls of YHV-infected tissue and negative controls of uninfected shrimp tissue. The digoxigenin-labelled DNA probe can be prepared by PCR labelling using the following primers:

YHV1051F: 5'-ACA-TCT-GTC-CAG-AAG-GCG-TC-3'

YHV1051R: 5'-GGG-GGT-GTA-GAG-GGA-GAG-AG-3'

4.3.1.2.3 *Agent purification*

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

4.3.1.2.4 *Bioassay*

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

Annex 19 (contd)

Collect moribund shrimp from a YHD-affected ponds or shrimp suspected of being carriers of infection and maintain at 4°C or on ice. Remove and discard the tail and appendages. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at –80°C or in liquid nitrogen until required. Thaw stored samples rapidly in a 37°C water bath within two snap-seal plastic bags and then maintain at 4°C or on ice during all procedures. Remove the carapace and calciferous mouth-parts. Suspend the remaining tissues in six volumes of TN buffer (0.02 M Tris/HCl, pH 7.4, 0.4 M NaCl) and homogenise in a tissue grinder to form a smooth suspension. Clarify the homogenate at 1300 **g** for 20 minutes at 4°C. Remove the supernatant fluid below the lipid layer and pass through a 0.45 µm filter. Maintain the filtrate at 4°C for immediate use or snap-freeze and store in aliquots at –80°C or in liquid nitrogen. Thaw the filtrate rapidly at 37°C and maintain on ice prior to use.

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

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

4.3.2.   Serological methods

Not applicable.

5.   Rating of tests against purpose of use

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

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Bioassay	d	d	d	d	c	b
Direct LM	d	d	d	d	a	d
Histopathology	d	d	c	c	a	d
Transmission EM	d	d	c	c	d	b
Antibody-based assays	d	d	c	c	a	b
DNA probes – *in situ*	d	d	c	c	b	a
PCR	a	a	a	a	a	a
Sequence	a	a	a	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 yellow head disease**

Nested RT-PCR (Section 4.3.1.2.3.1; Protocol 3) followed by confirmatory sequencing of the amplified PCR product is the prescribed method for declaring freedom. Two-step PCR negative results are required. The very rare case when a two-step PCR positive result cannot be confirmed by sequencing is also considered to be a negative result. As genetic recombination between genotypes can occur, the detection of any genotype is considered to be evidence of the presence of YHD.

7. **Corroborative diagnostic criteria**

   7.1. **Definition of suspect case**

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

   7.2. **Definition of confirmed case**

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

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CHAPTER 2.4.7.

INFECTION WITH *PERKINSUS OLSENI*

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

1. Scope

For the purpose of this chapter, infection with *Perkinsus olseni* is considered to be infection with *P. olseni*. *Perkinsus atlanticus* is considered to be a junior synonym.

2. Disease information

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2.2. Host factors

2.2.1. Susceptible host species

Perkinsus olseni has an extremely wide host range. Known hosts include the clams *Anadara trapezia*, *Austrovenus stutchburyi*, *Ruditapes decussatus*, *R. philippinarum, Tridacna maxima, T. crocea, Protothaca jedoensis* and *Pitar rostrata* (Goggin & Lester, 1995; Villalba *et al.,* 2004; Cremonte *et al.,* 2005; Park *et al.,* 2006; Sheppard & Phillips, 2008); oysters ~~*Crassostrea gigas,*~~ <u>*Crassostrea C.*</u> *ariakensis*, and *C. sikamea* (Villalba *et al.,* 2004); pearl oysters *Pinctada margaritifera, P. martensii*, and *P. fucata* (Goggin & Lester, 1995; Sanil *et al.,* 2010); abalone *Haliotis rubra, H. laevigata, H. scalaris,* and *H. cyclobates* (Goggin & Lester, 1995). Other bivalve and gastropod species might be susceptible to this parasite, especially in the known geographical range. Members of the families Arcidae, Malleidae, Isognomonidae, Chamidae and Veneridae are particularly susceptible, and their selective sampling may reveal the presence of *P. olseni* when only light infections occur in other families in the same habitat.

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8. References

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Assessment of acute hepatopancreatic necrosis disease (AHPND), for listing in the *Aquatic Animal Health Code*

The Aquatic Animal Health Standards Commission assessed acute hepatopancreatic necrosis disease (AHPND) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code*, and agreed that AHPND meets the listing criteria 1, 4, 6, 7 and 8 (see Table 1 below).

Table 1. Summary of assessment of AHPND

	Listing criteria								Conclusion
	1	2	3	4	5	6	7	8	
Acute hepatopancreatic necrosis disease	+	NA	NA	+	NA	+	+	+	Listing

NA = not applicable.

A. **CONSEQUENCES**

Criterion No. 1. The disease has been shown to cause significant production losses at a national or multinational (zonal or regional) level.

The host species affected include *Penaeus vannamei*, *P. monodon* and *P. chinensi*s (FAO, 2013), which are economically important for shrimp farming.

Since 2010, increasing losses of farmed shrimp have been reported in China (People's Rep. of), Vietnam and Thailand. These losses were associated with acute hepatopancreatic necrosis syndrome (AHPNS) (Lightner *et al.*, 2012; Flegel 2012; FAO, 2013), now known as acute hepatopancreatic necrosis disease (AHPND). In 2011, production losses of up to 80% were reported from shrimp farms in Hainan, Guangdong, Fujian and Guangxi provinces of China (Leaño and Mohan, 2012).

The disease has also affected shrimp production in Vietnam, Malaysia, Thailand and Mexico (Leaño and Mohan, 2012; FAO 2013; Joshi *et al.*, 2014; Gomez-Gil *et al.*, 2014).

B. **SPREAD**

Criterion No. 4. Infectious etiology of the disease is proven.

AHPND is caused by a pathogenic form of *Vibrio parahaemolyticus* (Tran *et al.*, 2013; Gomez-Gil *et al.*, 2014).

Isolates of *V. parahaemolyticus* from AHPND-affected shrimp have been shown to cause severe mortalities in experimentally challenged shrimp (Zhang *et al.*, 2012; Tran *et al.*, 2013; Gomez-Gil *et al.*, 2014; Joshi *et al.*, 2014). Experimentally infected shrimp develop characteristic AHPND pathology (Joshi *et al.*, 2014; Tran *et al.*, 2013) and the re-isolated bacterium has been shown to induce AHPND in subsequent experimental infections (Tran *et al.*, 2013). AHPND has been transmitted experimentally by immersion and intramuscular injection (Tran *et al.*, 2013; Joshi *et al.*, 2014).

Criterion No. 6. Likelihood of international spread including via live aquatic animals, their products or fomites.

There is significant international trade in the shrimp species that are susceptible to AHPND. The commodities traded include live animals such as shrimp larvae and broodstock.

<u>Annex 21</u> (contd)

Experimental evidence for the transmission of AHPND indicates that infected commodities would provide a pathway for the introduction and spread of AHPND (see Criterion No. 7).

The pattern of reporting of AHPND worldwide is indicative of international spread. AHPND was reported in China (People's Rep. of) and Vietnam in 2010. The distribution of disease in these countries continued to expand throughout 2010. In late 2010, shrimp mortalities associated with AHPND were reported in some coastal provinces of Vietnam. The disease was reported in Malaysia in 2011 and in Thailand in 2012 (FAO, 2013) with distributions expanding over time. AHPND subsequently occurred in Mexico in early 2013 (Gomez-Gil *et al.*, 2014).

Criterion No. 7. Several countries or countries with zones may be declared free from the disease based on the general surveillance principles outlined in Chapter 1.4.

AHPND has been reported in association with mass mortalities during the first 20-30 days of shrimp culture. There are many countries with susceptible species that have not reported mortalities or pathology consistent with AHPND. It is therefore probable that the pathogenic forms of *Vibrio parahaemolyticus* that cause AHPND are absent from these countries. The availability of sensitive and specific molecular diagnostic tests for the AHPND-causing forms of *V. parahaemolyticus* will allow the demonstration of freedom from AHPND.

C.   DIAGNOSIS

Criteria No. 8. A repeatable and robust mean of detection/diagnosis exists.

There is publically available information on diagnostic methods for AHPND including histopathology (Lightner *et al.*, 2012) and molecular methods (Flegel and Lo, 2013). A PCR primer set called AP3 developed by Sirikharin *et al.* (2014) can differentiate AHPND-causing *V. parahaemolyticus* from non-pathogenic forms.

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CHAPTER 11.6.

INFECTION WITH *PERKINSUS OLSENI*

Article 11.6.1.

For the purposes of the *Aquatic Code*, infection with *Perkinsus olseni* means *infection* with *P. olseni*.

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

Article 11.6.2.

Scope

The recommendations in this chapter apply to: primarily venerid clams (*Austrovenus stutchburyi*, *Venerupis pullastra*, *V. aurea*, *Ruditapes decussatus* and *R. philippinarum*), abalone (*Haliotis rubra*, *H. laevigata*, *H. Cyclobates* and *H. scalaris*) and other species (*Anadara trapezia*, *Barbatianovaezelandiae*, *Macomonaliliana*, *Paphies australis*, *Crassostrea ~~gigas~~ and ~~C.~~ ariakensis*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

[…]

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AQUATIC ANIMALS COMMISSION WORK PLAN 2014–2015

Aquatic Code

Task	Sept 2014	March 2015	May GS 2015	Sept 2015
User's guide	Review Member comments and recirculate for comment	Review Member comments	Propose for adoption	
Glossary	AAC revised some definitions and circulated for Member comments	Review Member comments	Propose for adoption	
Article 1.1.5. (Chapter 1.1.)	AAC revised Article 1.1.5. and circulated for Member comments	Review Member comments	Propose for adoption	
Revision of Section 4 to improve guidance on the control of disease		Develop a plan in light of Aquatic Conf. recommendations.		
Chapter 4.3.–Disinfection	AAC reviewed the AHG report and preliminary draft chapter on disinfection and requested AHG to finalise the draft chapter			Review AHG draft chapter and circulate for member comments
Chapter 4.X.–Disinfection of eggs	AAC reviewed the AHG report and draft chapter on disinfection of salmonid eggs and circulated for MCs	Review Member comments	Propose for adoption	
Chapter 4.7.–Control of pathogenic agents in feed	AAC revised chapter 4.7. and circulated for MCs	Review Member comments	Propose for adoption	
Chapter 6.6.–Risk analysis for antimicrobial resistance in aquaculture (new)	Reviewed draft chapter provided by AHG. and circulated for MCs	Review Member comments	Propose for adoption	
Listing of susceptible species in disease-specific chapters	AAC agreed to convene an AHG to apply the criteria for YHD as a pilot	Review AHG report and propose amendments to 9.2.2		Review MCs
AHPND	AAC proposed listing of AHPND and circulated assessment for MC	Review Member comments	Propose for adoption	If adopted. AAC to develop new Chapter

OIE Aquatic Animal Health Standards Commission/September 2014

<u>Annex 23</u> (contd)

Aquatic Manual

Manual tasks	Sept 2014	Feb 2015	May GS 2015	Sept 2015
Crustacean chapters YHD, NHP, TSD, IHHN	Propose some changes and circulated to MC for comment	Review MC comments	Propose for adoption	
Chapter 1.1.3.–Disinfection	Propose for deletion if new *Code* chapter 4.X. is adopted	Propose deletion if new *Code* chapter 4.X. is adopted	Propose for deletion if new *Code* chapter 4.X. is adopted	
Listing of susceptible species in disease-specific chapters		Review AHG report and propose amendments in section 2.2.1. of chaper 2.2.8. YHD		Review MCs
AHPND chapter	AAC agreed to convene AHG to develop a new chapter on AHPND	Review AHG Report and draft chapter and circulate for MC comments		Review MC comments
Test performance	AAC requested that a letter be sent to *Manual* chapter authors regarding test validation	Review progress		
Sections on agent stability (in connection with disinfection)	AAC requested that a letter be sent to *Manual* chapter authors requesting revision of information on agent stability	Review progress		

Other items

Tasks	Sept. 2014	Jan. 2015	Feb-April 2015
OIE Global Aquatic Animal Health Conference (January 2015, TBC)	Review abstracts	Conference (20-22 January 2015, Vietnam)	Review papers
OIE Ref. Lab. Conference (7–9 October 2014)	AAC to provide input into the programme and Scientific Committee.		