The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at OIE Headquarters in Paris from 2 to 6 March 2015.

The list of participants and the adopted agenda are presented at Annex 1 and Annex 2.

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts circulated after the Commission’s September 2014 meeting: Australia, Brazil, Canada, Chile, China (People’s Rep. of), Chinese Taipei, Costa Rica, Cuba, El Salvador, Guatemala, Honduras, Japan, New Zealand, Nicaragua, Norway, Panama, Switzerland, Thailand, the United States of America (USA), the Member States of the European Union (EU), and the African Union–Intercontinental Bureau for Animal Resources (AU-IBAR) on behalf of the OIE Delegates of Africa. The Aquatic Animals Commission acknowledged the large number of comments received and welcomed the receipt of comments from Member Countries that have not submitted comments previously.

The Aquatic Animals Commission reviewed comments that Member Countries had submitted prior to 30 January 2015 and amended texts in the OIE Aquatic Animal Health Code (hereafter referred to as the Aquatic Code) 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 amendments made at the March 2015 meeting are highlighted with a coloured background in order to distinguish them from those made at the September 2014 meeting.

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

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

The table below summarises the texts as presented in the Annexes. Member Countries should note that texts in Annexes 3 to 21 are proposed for adoption at the 83rd General Session in May 2015; Annexes 22 to 25 are presented for Member Countries’ comments; Annexes 26 to 28 are presented for information.

The Commission strongly encourages Member Countries to participate in the development of the OIE’s international standards by submitting comments on this report. Comments should be submitted as specific proposed text changes, supported by a scientific rationale. Proposed deletions should be indicated in ‘strikethrough’ and proposed additions with ‘double underline’. Member Countries should not use the automatic ‘track-changes’ function provided by word processing software as such changes are lost in the process of collating Member Countries’ submissions into the Commission’s working documents.
Comments on Annexes 22 to 25 of this report must reach OIE Headquarters by 21st August 2015 to be considered at the September 2015 meeting of the Aquatic Animals Commission.

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

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1. OIE Aquatic Animal Health Code

1.1. General comments

General comments were received from Chile and Norway. The Aquatic Animals Commission agreed with a Member Country’s comment on the need for more detailed recommendations on disease prevention and control such as biosecurity, including measures for the prevention of emerging diseases. The Aquatic Animals Commission recognised the importance of this need and will continue to work on revisions to chapters on general recommendations for disease prevention and control in Section 4 of the Aquatic Code.

The Aquatic Animals Commission also recognises the need for improved guidance on compartmentalisation as suggested by a Member Country. This issue is discussed further at Item 6.

1.2. User’s guide

Comments on the User’s Guide were received from AU-IBAR, Canada, Chile, EU, Japan, Norway, PR China, Switzerland, Thailand and USA. The Aquatic Animals Commission also considered proposed amendments made by the OIE Terrestrial Animal Health Standards Commission (the Code Commission) at its February 2015 meeting to ensure alignment, where relevant.

The Aquatic Animals Commission considered Member Countries’ comments and revised the text, as appropriate. In response to a Member Country’s comment, the Commission amended text in point 5 in Section A to clarify the application of risk analysis when there are no OIE recommendations on a given pathogenic agent or for specific commodities.

The Aquatic Animals Commission clarified that the reference made to infection with infectious salmon anaemia virus is to illustrate the proposed approach for pathogen differentiation, and provides an example of risk management options based on strain differentiation.

The Aquatic Animals Commission agreed with a Member Country comment to amend text in point 4 (Trade requirements) and point 5 (International health certificates) in Section C to align with the relevant sections of the OIE Terrestrial Animal Health Code (Terrestrial Code) User’s guide.

The revised User’s guide is attached as Annex 3 to be presented for adoption at the 83rd General Session in May 2015.

1.3. Glossary

The Aquatic Animals Commission considered comments provided by Australia, Canada, Chile, Costa Rica, Cuba, EU, PR China, New Zealand and Norway. The Commission also considered proposed amendments to relevant definitions developed by the Code Commission at its February 2015 meeting.

In response to several Member Countries’ comments, the Aquatic Animals Commission amended the definition for “biosecurity”. In response to a Member Country’s comment to include “chemical measures” in the definition, the Commission noted that “physical measures” refers to infrastructure and equipment required to contain pathogenic agents which should not be confused with physical and chemical means of disinfection. The Commission noted the amended definition aligns with that proposed for the Terrestrial Code.
The Aquatic Animals Commission wished to remind Member Countries that the revised definitions for “disinfectant” and “disinfection” were provided by the ad hoc Group on disinfection. This ad hoc Group has been tasked with developing a new draft chapter to provide guidance on disinfection of aquaculture establishments, water and fish eggs for Section 4 of the Aquatic Code. The Commission clarified that the proposed definitions are important to ensure a clear understanding of the use of these terms in the new draft chapter.

The Aquatic Animals Commission considered comments from several Member Countries on the types of processes that should fit within the definition of disinfectant. The Commission considered that physical processes such as drying and heating should be included within the definition because these are applied with a similar purpose to chemical disinfectants, i.e. to destroy or inhibit growth of pathogenic agents.

The Aquatic Animals Commission also considered comments from Member Countries on the types of actions disinfectants have on pathogenic agents. The Commission did not accept a Member Country’s comment to include “removal” of pathogenic agents within the definition. The Commission agreed that exclusion of pathogenic agents and other material, for example through filtration, may be an important step in the disinfection process but should not be considered a “disinfectant”. The Commission requested that the ad hoc Group consider exclusion of pathogenic agents within the context of the new draft chapter on disinfection of aquaculture establishments currently under development.

In response to a Member Country’s comment that many chemicals not normally considered disinfectants, may inhibit the growth of microorganisms, the Commission agreed to amend the definition to include ‘in the course of disinfection’.

The Aquatic Animals Commission considered Member Countries’ comments on the types of things that disinfection may be applied to. The Commission agreed that for the purposes of the definition, it is not necessary to specify what is being disinfected and that “items” means anything that may require disinfection.

The Aquatic Animals Commission noted that no comments were received on its proposal to delete the definition for hazard identification.

The Aquatic Animals Commission did not agree with a Member Country’s comment to retain the definition of ‘Infective period’. Following the proposed amendments to Chapter 1.1, this definition will only appear in Chapter 4.5. and its use in the context of that chapter does not require a specific definition.

The Aquatic Animals Commission noted that only minor comments were provided on the definitions of “risk analysis” and “risk assessment” and agreed that these comments did not justify further amendments to the definitions. The Commission noted that the proposed definitions align with those proposed by the Code Commission.

In response to a Member Country’s comment to explicitly mention veterinary public health in the definition of Veterinary Authority, the Aquatic Animals Commission agreed that as this definition is common to both the Aquatic Code and the Terrestrial Code, that this proposal should be reviewed jointly by the two Commissions.

The revised Glossary is attached as Annex 4 to be presented for adoption at the 83rd General Session in May 2015.

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

The Aquatic Animals Commission reviewed comments provided by Member Countries on proposed changes to Article 1.1.5. and also reviewed amendments proposed by the OIE ad hoc Group on Notification of animal diseases and pathogenic agents to Article 1.1.4.

The Aquatic Animals Commission noted that comments supported the proposed changes to Article 1.1.5. The Commission agreed to the proposed amendments in Article 1.1.4. and revised the text as appropriate.

The report of the OIE ad hoc Group on Notification of animal diseases and pathogenic agents is presented as Annex 26 for Member Countries’ information.
The revised Chapter 1.1. Notification of diseases and provision of epidemiological information is presented at Annex 22 for Member Countries’ comments.

1.5. Criteria for listing diseases (Chapter 1.2.)

The Aquatic Animals Commission reviewed the report of the ad hoc Group on Notification of animal diseases and pathogenic agents. The Commission agreed that it was valuable having representatives of three OIE Specialist Commissions as observers to the meeting of this ad hoc Group. They also recommended further strengthening of coordination between Aquatic Animals Commission the Code Commission to ensure consistency of horizontal chapters in both Codes, where appropriate.

The terms of reference of the OIE ad hoc Group on Notification of animal diseases and pathogenic agents included a review of the listing criteria (i.e. Chapters 1.2.) of the Terrestrial Code and Aquatic Code.

The Aquatic Animals Commission noted that the ad hoc Group proposed a set of simplified criteria for Chapter 1.2. of the Aquatic Code. While the Commission noted that the proposed criteria have been simplified, there is still a need to ensure clarity of those criteria. The Commission noted that the removal of the explanatory notes leads to a loss of guidance on how to apply the criteria and the underlying principles. The Commission agreed that it is critical that the concepts underlying the listing criteria should be easily understood. The explanatory notes currently provide such clarity and their removal could result in changes to the underlying principles reflected by the criteria.

The Aquatic Animals Commission discussed some of the underlying principles of the current listing criteria that are reflected in Chapter 1.2. of the Aquatic Code. These issues are summarised below.

International spread – this criterion is currently satisfied if “under international trading practices, the entry and establishment of the disease is likely”. The wording proposed for this criterion by the ad hoc Group is “International spread of the agent ... has been proven”. This sets a different standard to the current criterion.

Several countries or countries with zones may be declared free – this criterion is currently satisfied if “Several countries or countries with zones may be declared free of the disease...”. The wording proposed for this criterion by the ad hoc Group is “At least one country has demonstrated freedom or impending freedom...”. This sets a different standard to the current criterion in terms of the number of countries and the level of action taken to declare freedom. The current explanatory notes indicate an underlying principle (although not explicitly) that notification should be warranted to support disease control efforts. The proposed new criterion may be problematic because it may be difficult to demonstrate freedom if a disease has not been listed.

Repeatable and robust means of detection – this criterion is currently satisfied if “A repeatable and robust means of detection/diagnosis exists”. The explanatory notes indicate that the diagnostic test should have undergone a formal validation process or a robust case definition should be available. The wording proposed for this criterion by the ad hoc Group reflects the need for a case definition but not a need for test validation.

Consequences – the current criteria for consequences are divided between production impacts, impacts on wild aquatic animal populations, and public health impacts. The wording proposed for this criterion by the ad hoc Group is divided similarly, although the criterion on production impacts less explicitly refers to farmed populations. The Commission noted that the type of impacts can be separated in different ways; for example, by the values affected (commercial or environmental) or the type of resources affected (farmed or wild), noting that wild aquatic animals may simultaneously have environmental and commercial value.

The report of the OIE ad hoc Group on Notification of animal diseases and pathogenic agents is presented at Annex 26 for Member Countries’ information.

The Aquatic Animals Commission encourages Member Countries to comment on the issues raised above rather than specific wording proposed by the ad hoc Group. This will inform further consideration of the listing criteria by the Commission at its September 2015 meeting.

The revised Chapter 1.2. Criteria for listing diseases is presented at Annex 23 for Member Countries’ comments.
1.6. Diseases listed by the OIE (Chapter 1.3.)

a) Acute hepatopancreatic necrosis disease (AHPND)

The Aquatic Animals Commission reviewed comments received from Canada, PR China, Chinese Taipei, EU, Norway and Thailand regarding the listing of acute hepatopancreatic necrosis disease (AHPND) in accordance with Article 1.2.2. of the Aquatic Code. While some Member Countries supported listing, three did not. The Commission noted that comments against listing were based on a recent report that a *Vibrio* species other than *Vibrio parahaemolyticus* was found to be PCR positive for the plasmid that carries the toxin gene necessary to cause AHPND.

While the Aquatic Animals Commission recognised this new development in the knowledge of AHPND, they agreed that this finding must be balanced against a considerable body of scientific information that now exists and which demonstrates that a strain of *V. parahaemolyticus* carrying the pVA-1 plasmid is the cause of AHPND.

In September 2014, the Aquatic Animals Commission recommended that an ad hoc Group be convened to develop a draft chapter on AHPND for inclusion in the Aquatic Manual. The draft Aquatic Manual chapter has been developed (see point 2.6 below) and provides information supporting reliable identification of the aetiological agent of AHPND. Section 1 of the draft Aquatic Manual chapter provides a definition for AHPND and Section 7 provides definitions for suspect and confirmed cases.

The Aquatic Animals Commission noted that, as stated in Chapter 1.2. of the Aquatic Code, the objective of listing is to support Member Countries’ efforts to prevent the transboundary spread of important diseases of aquatic animals through transparent and consistent reporting.

The Aquatic Animals Commission agreed that AHPND should be proposed for listing at the 83rd General Session in May 2015.

See also Item 2.6 regarding development of new draft Aquatic Manual chapter on AHPND.

The Aquatic Animals Commission encourages Member Countries to refer to the Commission’s assessment of AHPND against the listing criteria (presented in Annex 21 of the September 2014 Aquatic Animals Commission meeting report).

b) Infection with HPR-deleted or HPR0 infectious salmon anaemia virus

In response to a Member Country’s comment on the discrepancy between the title of Chapter 10.4. and the name of the disease in Chapter 1.3., the Aquatic Animals Commission clarified that listing in Chapter 1.3. is for the purpose of reporting and notification, and need not necessarily be identical with the title of the disease-specific chapter in the Aquatic Code. For infection with infectious salmon anaemia (ISA) virus, the Commission prefers to keep the name as currently listed in Chapter 1.3. to emphasise the fact that infection with both HPR-deleted and HPR0 forms of the ISA virus are notifiable to the OIE.

c) Crayfish plague (*Aphanomyces astaci*)

In response to a Member Country’s request to change the name of the disease to ‘infection with *Aphanomyces astaci*,’ the Aquatic Animals Commission agreed that the proposal is in line with the OIE approach to naming diseases. However, the Commission decided not to change the name as currently listed until the corresponding disease chapters in the Aquatic Code and Manual have been reviewed. The Commission agreed to review the listing names of diseases and the corresponding Code and Manual Chapter titles when chapters are being amended regarding the lists of susceptible species.

The Aquatic Animals Commission also reminded Member Countries that changes to the name of OIE listed diseases does have implications for the national legislation of Member Countries.
d) Infectious hypodermal and haematopoietic necrosis (IHHN)

The Aquatic Animals Commission did not support a Member Country’s comment to delist IHHN, as no rationale based on the criteria of Chapter 1.2. was provided to substantiate the proposal.

The Aquatic Animals Commission reminds Member Countries that to delist a disease, scientific rationale is required to demonstrate that the disease no longer meets the listing criteria as described in Chapter 1.2. of the Aquatic Code.

e) Infection with ranavirus

The Aquatic Animals Commission considered a Member Country’s comment that Ranavirus is a genus that includes several species of virus. The Commission noted that the objective of listing is to support Member Countries’ efforts to prevent the transboundary spread of important diseases of aquatic animals through transparent and consistent reporting. The Commission agreed that listing at the genus level may not provide a robust basis for decision making and targeted actions.

The Aquatic Animals Commission noted that as infection with ranavirus was first listed in 2008 a review of the case definition of this disease may now be warranted.

The Aquatic Animals Commission agreed to refer the issue to the OIE designated expert for advice and review this issue at their next meeting.

f) Other diseases of amphibians

The Aquatic Animals Commission considered a Member Country’s comment regarding the possible listing of additional diseases of amphibians such as the chytrid fungus Batrachochytrium salamandrivorans, which has caused rapid declines in wild amphibians. The Commission agreed to add this issue to its work plan and noted that there is currently no OIE Reference Laboratory for infection with Batrachochytrium dendrobatidis that could assist with this task.

The revised Chapter 1.3. Diseases listed by the OIE is attached as Annex 5 to be presented for adoption at the 83rd General Session in May 2015.

1.7. Import risk analysis (Chapter 2.1.)

The Aquatic Animals Commission considered comments provided by Australia, Brazil, Chile, PR China, EU and Norway.

In response to a Member Country’s suggestion to add release assessment to the chapter, the Aquatic Animals Commission pointed out that the 2012 edition of the Aquatic Code changed the term ‘release assessment’ to ‘entry assessment’ in Article 2.1.4. and this change is consistent with the second edition of the OIE Handbook on Import Risk Analysis for Animals and Animal Products.

The Aquatic Animals Commission reiterated the importance of ensuring consistency between Chapter 2.1. of the Aquatic Code with the changes in Articles 2.1.5. and 2.1.6. recently adopted in Chapter 2.1 of the Terrestrial Code.

The revised Chapter 2.1. Import risk analysis is attached as Annex 6 to be presented for adoption at the 83rd General Session in May 2015.

1.8. Recommendations for disinfection of salmonid eggs (new Chapter 4.X.)

The Aquatic Animals Commission considered comments provided by Canada, Chile, PR China, EU, New Zealand, Norway and Switzerland and amended the draft chapter as appropriate.

The new Chapter Recommendations for disinfection of salmonid eggs (Chapter 4.X.) is attached as Annex 7 to be presented for adoption at the 83rd General Session in May 2015.

The Aquatic Animals Commission wished to note that if Chapter 4.X. is adopted at the General Session in May 2015, the Aquatic Manual Chapter 1.1.3. ‘Methods for disinfection of aquaculture establishments’ will be deleted as a consequential change. The Commission wished to remind Member Countries that Chapter 1.1.3. is misplaced in the Aquatic Manual and that the ad hoc Group on Disinfection is revising Chapter 4.3. of the Aquatic Code to provide more detailed recommendations on this subject.
1.9. Control of hazards in aquatic animal feed (Chapter 4.7.)

The Aquatic Animals Commission considered comments provided by Australia, Canada, Chile, EU, Norway, New Zealand, PR China, and Switzerland. The Commission reviewed the comments received, and amended the text of draft chapter as appropriate.

The revised Chapter 4.7. Control of hazards in aquatic animal feed is attached as Annex 8 to be presented for adoption at the 83rd General Session in May 2015.

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

The Aquatic Animals Commission considered comments provided by Australia, Chile, EU and Japan. The Commission also considered the text in Chapter 5.1. of the Terrestrial Code as revised by the Code Commission at its February 2015 meeting. The Commission amended the text based on these considerations.

The revised Chapter 5.1. General obligations related to certification is attached as Annex 9 to be presented for adoption at the 83rd General Session in May 2015.

1.11. Certification procedures (Chapter 5.2.)

The Aquatic Animals Commission considered comments provided by Chile and EU. The Aquatic Animals Commission also considered the text in Chapter 5.2. of the Terrestrial Code as revised by the Code Commission at its February 2015 meeting. The Commission amended the text based on these considerations.

The revised Chapter 5.2. Certification procedures is attached as Annex 10 to be presented for adoption at the 83rd General Session in May 2015.

1.12. Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals (draft Chapter 6.5.)

The Aquatic Animals Commission considered comments provided by Australia, EU, PR China, New Zealand, Norway, Switzerland, Thailand and USA and amended the text as appropriate.

The Aquatic Animals Commission moved text defining the use of the terms for hazard, risk and risk to public health, for the purpose of this chapter, into a new point 3 of Article 6.5.1. to improve readability. The lists of factors to be considered in points on release assessment, exposure assessment and consequence assessment in Articles 6.5.3. and 6.5.4. were expanded and text in the points on risk estimation were simplified to improve clarity. The Commission also replaced the term ‘release assessment’ with the term ‘entry assessment’ throughout the chapter to ensure consistency with Chapter 2.1.

The new Chapter 6.5. Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals is attached as Annex 11 to be presented for adoption at the 83rd General Session in May 2015.

1.13. Amphibian disease-specific chapters (Chapters 8.1. and 8.2.)

The Aquatic Animals Commission considered comments provided by Australia, Chinese Taipei, El Salvador, EU and New Zealand and amended the text of these chapters as appropriate.

The Aquatic Animals Commission wished to remind Member Countries that, as noted in their September 2014 meeting report, there is an inconsistency between the Aquatic Code and Aquatic Manual in the recommendations for treatment prior to importation of amphibians intended for the pet trade. Treatment of live animals prior to importation is not considered an adequate risk mitigation measure to prevent spread of infection with Batrachochytrium dendrobatidis. The Commission therefore proposed the removal of the provision for treatment of live aquatic animals from Articles 8.1.8. and 8.1.10.
A Member Country commented that crayfish are now known to be effective carriers of *Batrachochytrium dendrobatidis* and thus that the Aquatic Code and Aquatic Manual chapters may need to be updated. The Commission agreed to review the evidence at its next meeting.

The revised Chapter 8.1. Infection with *Batrachochytrium dendrobatidis* is attached as Annex 12 to be presented for adoption at the 83rd General Session in May 2015.

The revised Chapter 8.2. Infection with ranavirus is attached as Annex 13 to be presented for adoption at the 83rd General Session in May 2015.

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

The Aquatic Animals Commission considered a Member Country’s comment to add ‘genotype 1’ after the name of infection with yellow head virus (YHV) given that genotype 1 is the only known pathogenic agent of yellow head disease. (See also Item 1.18.).

The revised Chapter 9.2. Infection with yellow head virus is attached as Annex 24 for Member Countries’ comments.

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

In its September 2014 meeting report, the Aquatic Animals Commission recognised that the text in Articles X.X.7. and X.X.11. of disease-specific chapters is almost identical and therefore proposed to merge these two articles in all disease-specific chapters of the Aquatic Code. The Commission noted that the model articles apply to Article X.X.7. (importation of live aquatic animals) and Article X.X.11. (importation of aquatic animal products) from a country, zone or compartment declared free for all disease-specific chapters except in Chapter 10.4. where this issue applies to Articles 10.4.10., 10.4.11., 10.4.15. and 10.4.16.

The Commission noted that only comments supportive of this proposal were received.

The revised model Articles X.X.7. and X.X.11. is attached as Annex 14 to be presented for adoption at the 83rd General Session in May 2015.

[Note: for Chapter 10.4., this issue applies to Articles 10.4.10., 10.4.11., 10.4.15. and 10.4.16.]

1.16. Corrections in Articles 10.4.4. and 10.4.6.

In its September 2014 meeting report, the Aquatic Animals Commission noted that the text in point 2 of Articles 10.4.4. and 10.4.6. in Chapter 10.4. was incorrect. Only comments supportive of the proposed changes were received.

The revised Articles 10.4.4. and 10.4.6. are attached as Annex 15 to be presented for adoption at the 83rd General Session in May 2015.

1.17. Infection with *Perkinsus olseni* (Article 11.6.2.)

In its September 2014 meeting report, the Aquatic Animals Commission had proposed the deletion of *Crassostrea gigas* as a species susceptible to infection with *Perkinsus olseni* as there is no information to substantiate that this species is susceptible. No objections to this proposal were received.

The revised Article 11.6.2. is attached as Annex 16 to be presented for adoption at the 83rd General Session in May 2015.

1.18. List of susceptible species for OIE listed crustacean diseases

The Aquatic Animals Commission considered the report of the *ad hoc* Group on Susceptibility of crustacean species to infection with OIE listed diseases and commended the members of this *ad hoc* Group for their excellent work.
The Aquatic Animals Commission reviewed the ad hoc Group’s proposed changes to the list of susceptible species for Chapter 9.2. Infection with yellow head virus. The Commission noted that the application of the new criteria for listing species as susceptible to infection with a specific pathogen (described in Chapter 1.5.) to infection with yellow head virus, by the ad hoc Group, resulted in an amended list of susceptible species (see details in the ad hoc Group report at Annex 27). The Commission agreed to circulate the proposed changes to the list of susceptible species in Article 9.2.2. for Member Countries’ comments.

The Aquatic Animals Commission also requested that authors of the Aquatic Manual Chapter 2.2.8. Yellow head disease to amend the list of susceptible species in this chapter to align with the recommendations of the ad hoc Group report. The Commission will review the amended Manual chapter at its next meeting in September 2015.

The Commission also recommended that the ad hoc Group continue its work to review the list of susceptible species for other OIE listed crustacean pathogenic agents.

The report of the OIE ad hoc Group on Susceptibility of crustacean species to infection with OIE listed diseases is attached as Annex 27 for Member Countries’ information.

The revised Article 9.2.2. Infection with yellow head virus is attached as Annex 24 for Member Countries’ comments.

2. OIE Manual of Diagnostic Tests for Aquatic Animals

2.1. Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis

The Aquatic Animals Commission, in consultation with the chapter’s authors, reviewed Member Countries’ comments and amended the text, as appropriate.

The revised Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis is attached as Annex 17 to be presented for adoption at the 83rd General Session in May 2015.

2.2. Chapter 2.2.4. Necrotising hepatopancreatitis

The Aquatic Animals Commission, in consultation with the chapter’s authors, reviewed Member Countries’ comments and amended the text, as appropriate.

The revised Chapter 2.2.4. Necrotising hepatopancreatitis is attached as Annex 18 to be presented for adoption at the 83rd General Session in May 2015.

2.3. Chapter 2.2.5. Taura syndrome

The Aquatic Animals Commission, in consultation with the chapter’s authors, reviewed Member Countries’ comments and amended the text, as appropriate.

The revised Chapter 2.2.5. Taura syndrome is attached as Annex 19 to be presented for adoption at the 83rd General Session in May 2015.

2.4. Chapter 2.2.8. Infection with yellow head virus

The Aquatic Animals Commission, in consultation with the chapter’s authors, reviewed Member Countries’ comments and amended the text, as appropriate.

The revised Chapter 2.2.8. Infection with yellow head virus is attached as Annex 20 to be presented for adoption at the 83rd General Session in May 2015.
2.5. Chapter 2.4.6. Infection with *Perkinsus olseni*

No Member Countries’ comments had been received on the revised section 2.2.1. of Chapter 2.4.6.

The revised Chapter 2.4.6. Infection with *Perkinsus olseni* is attached as Annex 21 to be presented for adoption at the 83rd General Session in May 2015.

2.6. New draft chapter on acute hepatopancreatic necrosis disease

At their September 2014 meeting, the Aquatic Animals Commission recommended that an ad hoc Group be convened to develop a draft chapter on acute hepatopancreatic necrosis disease (AHPND) for inclusion in the *Aquatic Manual*, in light of the proposal to list the disease.

The Aquatic Animals Commission acknowledged the ad hoc Group’s work and revised the draft chapter. The Commission clarified the definition of AHPND to address the disease caused by unique strains of *Vibrio parahaemolyticus*, carrying a plasmid, designated pVA1, containing genes coding for a deadly binary toxin (Pir). The Commission emphasised that the main purpose of listing a disease is to share important information on its diagnosis, distribution and notification. For this purpose, Section 7 of disease-specific chapters in the *Aquatic Manual* provides definitions of a suspect and of a confirmed case of the disease in question. For AHPND, a case is suspicious based on histopathology findings or detection of *V. parahaemolyticus* carrying the plasmid or mortality associated with clinical signs; a case can be confirmed by detection of *V. parahaemolyticus* carrying the plasmid and histopathology findings or mortality associated with clinical signs or bioassay.

The draft new Chapter Acute hepatopancreatic necrosis disease (Chapter X.X.X.) is presented at Annex 25 for Member Countries’ comment.

3. OIE Reference Centers

3.1. Applications for OIE Reference Centre status or changes of experts

The Aquatic Animals Commission noted that there are currently no OIE Reference Laboratories for necrotising hepatopancreatitis or for infection with *Batrachochytrium dendrobatidis* and invites applications from Member Countries where expertise exists for these crustacean and amphibian diseases.

3.2. Annual reports of Reference Centre activities in 2014

The Aquatic Animals Commission was presented with an analysis of the activities of the Reference Laboratories and Collaborating Centres for aquatic animal diseases in 2014. Reports had been received from 35 of the 42 OIE Reference Laboratories and one of the two Collaborating Centres for aquatic animals.

The Aquatic Animals Commission was impressed with the quality of the work carried out by the laboratories and expressed its gratitude to the experts for their valuable work.

The Aquatic Animals Commission was pleased to note that the number of OIE Reference Laboratories that do not have an internationally recognised quality management system in place had decreased. The Commission encourages non-accredited OIE Reference Laboratories to take the necessary steps to achieve this goal.

The Aquatic Animals Commission also noted that a large number of OIE Reference Laboratories are producing diagnostic reagents but none are included in the current list of OIE-approved standard reference reagents (see: [http://www.oie.int/en/our-scientific-expertise/veterinary-products/reference-reagents/](http://www.oie.int/en/our-scientific-expertise/veterinary-products/reference-reagents/)). The Commission encourages the OIE Reference Laboratories for aquatic animal diseases to consider submitting their reference reagents for approval by the OIE.

4. Twinning projects

The Aquatic Animals Commission was updated on the status of aquatic animal disease twinning projects. As of September 2014, the project on infectious salmon anaemia between Canada and Chile has been completed. The following projects are ongoing: infectious haematopoietic necrosis between USA and PR China, Infectious salmon anaemia between Norway and Brazil, Koi Herpesvirus between Japan and Indonesia and Shrimp diseases between USA and Indonesia.

The Aquatic Animals Commission reviewed a twinning project proposal and provided technical comments.
5. **Follow-up on Recommendations from the Third Global Conference of OIE Reference Centers, Seoul (Rep. of Korea), 14–16 October 2014**

The Aquatic Animals Commission took note of the Recommendations from the Third Global Conference of OIE Reference Centres.

The Aquatic Animals Commission appreciated the inclusion of a session on aquatic animal diseases, which included valuable presentations focused on diagnostic test validation and quality assurance. The Commission noted that the main recommendations of this session were to improve standards and guidelines for the validation of diagnostic tests for aquatic animal diseases, to improve communication among the Aquatic Reference Laboratories and to continue to encourage OIE Reference Laboratories for aquatic animal diseases to become accredited to ISO 17025 or an equivalent quality management system.


6. **Follow-up actions from the 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 discussed outcomes and recommendation of the OIE Global Conference on Aquatic Animal Health held in Ho Chi Minh City (Vietnam), 20-22 January 2015.

The Aquatic Animals Commission noted strong interest from participants during the surveillance session. The presentations and panel discussions raised several points regarding strengthening recommendations on surveillance in the *Aquatic Code*. In particular, the session highlighted the need to provide guidance on more flexible approaches to surveillance while also addressing the challenges of conducting surveillance for aquatic animal diseases. The Commission recognises the need for guidance on cost effective approaches to surveillance (outcome and risk based surveillance). The Commission also recognises the need to consider underlying epidemiological principles when specifying required surveillance periods in the disease-specific chapters.

The Aquatic Animals Commission also noted strong interest from participants during the compartmentalisation session. The Commission noted that the panel discussion highlighted the need to clarify the concept of compartmentalisation and reminds Member Countries that the purpose of compartmentalisation in the *Aquatic Code* is to facilitate international trade. This may be distinct from biosecurity measures not primarily designed to facilitate international trade.

The Aquatic Animals Commission noted the need to provide updated and separate guidance for zoning and compartmentalisation in the *Aquatic Code*. This revised guidance should support practical application of these concepts by the private sector and Competent Authorities. These revised chapters would require supporting guidance in Section 4 of the *Aquatic Code* on principles of biosecurity and disease control.

The conference emphasised the importance of the PVS pathway to support Member Countries to build capacity of their Veterinary and Aquatic Animal Health Services. The Commission recommended that Member Countries consider requesting an OIE PVS Tool: Aquatic evaluation of their Veterinary Service or Aquatic Animal Health Service with the objective of improving competencies and general compliance with OIE standards for aquatic animals. The Commission noted that many countries expressed an interest in an OIE PVS Tool: Aquatic evaluation and considered this to a positive development.

In line with the outcomes of the Third Global Conference of OIE Reference Centers the conference highlighted ways in which the *Aquatic Manual* can be improved to provide clear guidance on test validation. The Aquatic Animals Commission sees the opportunity to draw on the knowledge and expertise of the OIE Reference Centres network to pursue this work.

The Aquatic Animals Commission expressed its appreciation to donors for financing this event, the Government of Vietnam for hosting the conference, to the speakers for their excellent presentations and to participants for their contribution to the discussions.
An aim of the conference was to set priorities for future work of the Aquatic Animals Commission. The Commission agreed this goal was achieved and the issues noted above should be considered in the formulation of the new Commission’s Work Plan at its next meeting in September 2015.

The conference recommendations, presentations and abstracts are available on the OIE website at http://www.oie.int/eng/A_AAHWF2015/recommandations.htm


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

The Aquatic Animals Commission’s Work Plan for 2015/16 is presented in Annex 28 for Member Countries’ information.

8. Other business

8.1. Notification of new susceptible species to infection with Perkinsus olseni

Following the notification by a Member Country of a new species Haliotis iris susceptible to infection with P. olseni, the Aquatic Animals Commission agreed to request the OIE designated expert to review the scientific evidence supporting listing this new susceptible host species according to the criteria in Chapter 1.5. of the Aquatic Code. The Commission noted that the current Aquatic Manual indicates a wide range of susceptible species, and explicitly suggests that more susceptible species are yet to be identified. The Aquatic Animals Commission agreed to include this item on its Work Plan.

8.2. Fish-borne Zoonotic Trematodes

The Aquatic Animals Commission held a teleconference with Dr Rohana Subasinghe (FAO) to discuss the issue of Fish-borne Zoonotic Trematodes (FZT) raised during the OIE Global Conference on Aquatic Animal Health in Vietnam.

The Aquatic Animals Commission recognises the importance of FZT which are estimated to infect over 18 million people worldwide, and a number of other mammals (e.g. cats, dogs, pigs, fish-eating birds). The Commission also noted that FZT have recently been added to the WHO list of neglected tropical emerging diseases. In aquatic systems one of the main risk factors for FZT infection and transmission is the contamination of pond environments with FZT eggs from infected hosts (i.e. humans, domestic animals and fish-eating birds). Factors that promote the growth of snail intermediate host populations also increase the risk of FZT. In aquaculture systems, it should be possible to reduce environmental contamination with FZT by mechanically removing the intermediate hosts (snails). This is particularly important as future fish availability will significantly and increasingly depend on aquaculture.

The Aquatic Animals Commission agreed to include this important subject in its Work Plan and consider developing a chapter to provide guidance on managing the risks associated with FZT.

9. Next meeting

September 2015 (specific date to be confirmed).
MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION
Paris, 2–6 March 2015

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MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 2–6 March 2015

Adopted agenda

1. OIE Aquatic Animal Health Code
   1.1. General comments
   1.2. User’s guide
   1.3. Glossary
   1.4. Notification of diseases and provision of epidemiological information (Chapter 1.1.)
   1.5. Criteria for listing (Chapter 1.2.)
   1.6. Diseases listed by the OIE (Chapter 1.3.)
   1.7. Import risk analysis (Chapter 2.1.)
   1.8. Recommendations for disinfection of salmonid eggs (new Chapter 4.X.)
   1.9. Control of hazards in aquatic animal feed (Chapter 4.7.)
   1.10. General obligations related to certification (Chapter 5.1.)
   1.11. Certification procedures (Chapter 5.2.)
   1.12. Risk analysis for antimicrobial resistance arising from the use of antimicrobial agents in aquatic animals (draft Chapter 6.5.)
   1.13. Amphibian disease-specific chapters (8.1. and 8.2.)
   1.14. Infection with yellow head virus (Chapter 9.2)
   1.15. Articles X.X.7. and X.X.11. of disease-specific chapters
   1.16. Corrections in Articles 10.4.4. and 10.4.6.
   1.17. Infection with Perkinsus olseni (Article 11.6.2.)
   1.18. List of susceptible species for OIE listed crustacean diseases

2. OIE Manual of Diagnostic Tests for Aquatic Animals
   2.1. Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis
   2.2. Chapter 2.2.4. Necrotising hepatopancreatitis
   2.3. Chapter 2.2.5. Taura syndrome
   2.4. Chapter 2.2.8. Yellow head disease
Annex 2 (contd)

2.5. Chapter 2.4.7. Infection with *Perkinsus olseni*

2.6. New draft chapter on acute hepatopancreatic necrosis disease

3. OIE Reference Centres

   3.1. Applications for OIE Reference Centre status or changes of experts

   3.2. Annual reports of Reference Centre activities in 2014

4. Twinning projects

5. Follow-up on Recommendations from the Third Global Conference of OIE Reference Centres, Seoul (Rep. of Korea), 14–16 October 2014

6. Follow-up actions from the OIE Global Conference on Aquatic Animal Health: ‘Riding the wave to the future’, Ho Chi Minh City (Vietnam), 20–22 January 2015


8. Other business

   8.1. Notification new susceptible species to infection with *Perkinsus olseni*

   8.2. Fish-borne Zoonotic Trematodes

9. Next meeting
GUIDE TO THE USER’S GUIDE OF THE
AQUATIC ANIMAL HEALTH CODE

A. Introduction

1) The OIE Aquatic Animal Health Code (hereafter referred to as the Aquatic Code) sets out provides standards for the improvement of aquatic animal health worldwide. More recently, the Aquatic Code has also included standards for the welfare of farmed fish and use of antimicrobial agents use in aquatic animals. 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 develop 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.

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

45) The absence of chapters, articles or When the Aquatic Code does not include recommendations on particular pathogenic agents or commodities, does not mean that Veterinary Authorities and other appropriate sanitary measures can still be applied by Competent Authorities may not apply appropriate aquatic animal health and welfare measures provided they are based on a risk analysis conducted in accordance with the Aquatic Code. However, such measures should be based on sound scientific justification according to the principles of the WTO SPS Agreement.

56) The complete text of the Aquatic Code is available on the OIE website and individual chapters 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 online 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 section standards includes the criteria for listing aquatic animal diseases, the diseases which are listed by the OIE, procedures for notification to the OIE, and criteria for listing species as susceptible to infection with a specific pathogen.

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 exceed more trade restrictive stricter than existing OIE trade standards.
Annex 3 (contd)

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 Competent Authorities 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 address certification and the measures applicable by the exporting, transit and importing countries. Section 5 also includes a range of model international aquatic animal health certificates to be used as a harmonised basis of 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 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 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. An example, Infection with infectious salmon anaemia virus is a first listed disease for which offering risk management options based on strain differentiation are provided for infection with infectious salmon anaemia virus 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 importance in the context of aquaculture, given the large number of existing species and the number of new aquaculture species existing in being introduced to aquaculture.

4) Trade requirements

International aquatic animal health measures related to international 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 different from or less restrictive than those recommended by the Aquatic Code. To scientifically justify However, if measures exceed are more trade restrictive than OIE standards, measures the importing country should provide scientific justification by conducting 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 responsibility of importing and exporting countries in international trade. Veterinary Authorities and other Competent Authorities and all veterinarians or and certifying officials directly involved in international trade should be familiar with Chapters. These Chapters 5.1. also provide guidance the OIE for informal dispute mediation by the OIE.

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 exporting country or zone for the pathogenic agent in question. Where such a list is present, importing countries should not require any conditions related to the agent in question apply measures trade restrictions to the listed commodities with respect to the listed commodities.

5) Trade in aquatic animal commodities

Chapter 5.4. describes the criteria used to assess the safety of aquatic animal commodities that are listed in Articles X.X.3. and X.X.11. (crustacean and mollusc) or X.X.12. (amphibian and fish) disease-specific chapters.

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 commodities 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 commodities in point 1 of Article X.X.3. are based on the absence of the pathogenic agent in the traded aquatic animals commodity 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. (for Chapter 10.4 the relevant Article is 10.4.17.) (amphibian and fish disease chapters) and Article X.X.11. (crustacean and mollusc disease chapters) lists aquatic animals or aquatic animal products commodities for retail trade for human consumption from a country, zone or compartment not declared free from the disease in question. The criteria for inclusion of aquatic animals commodities 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.
Annex 3 (contd)

56) International health certificates

An international aquatic animal health certificate is an official document that is drawn up by the Veterinary Authority or other Competent Authority of the exporting country in accordance with Chapter 5.1. and Chapter 5.2. It certifies the aquatic animal health requirements for the exported commodity. The quality of the exporting country’s Veterinary Services or Aquatic Animal Health Services is essential in providing assurances to trading partners regarding the safety of exported aquatic animals commodities and aquatic animal products. This includes the Veterinary Services or Aquatic Animal Health Services’ ethical approach to the provision of international health certificates and their history in meeting their notification obligations.

International health certificates underpin international trade and provide assurances to the importing country regarding the health status of the aquatic animals commodities and aquatic animal products imported. The measures prescribed should take into account the health status of both exporting and importing countries and be based upon the standards in the Aquatic Code.

The following steps should be taken when drafting international aquatic animal health certificates:

a) identify list the diseases, for from which the importing country is justified in seeking protection in regards to because of its own aquatic animal health status. Importing countries should not impose measures in regards to diseases that occur in their own territory but are not subject to official control or eradication programmes;

b) for commodities capable of transmitting these diseases through international trade, the importing country should apply the relevant articles addressing the commodity in question in the relevant disease-specific chapters. The application of the articles should be adapted to the disease status of the exporting country, zone or compartment. Such status should be established according to Article 1.4.6. except when articles of the relevant disease chapter specify otherwise;

c) when preparing international aquatic animal health certificates, the importing country should endeavour to use terms and expressions in accordance with the definitions given in the Glossary. As stated in Article 5.2.3., international aquatic animal health certificates should be kept as simple as possible and should be clearly worded, to avoid misunderstanding of the importing country’s requirements;

76) Guidance notes for importers and exporters

It is recommended that Veterinary Authorities and other Competent Authorities are recommended to prepare ‘guidance notes’ to assist importers and exporters to understand trade requirements. These notes should identify and explain the trade conditions, including the measures to be applied before and after export, and during transport and unloading, and the relevant legal obligations and operational procedures. The guidance notes should advise on all details to be included in the health certification accompanying the consignment to its destination. Exporters should also be reminded of the International Air Transport Association rules governing air transport of aquatic animals and aquatic animal products.

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GLOSSARY

**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.

**DISINFECTANTS**

means chemical compounds or physical processes capable of destroying pathogenic agents microorganisms or inhibiting their growth or survival ability in the course of disinfection.

**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.

means the process of cleaning and applying disinfectants to inactivate pathogenic agents on potentially contaminated items.

**HAZARD IDENTIFICATION**

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

**INFECTION 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, 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.
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 *Martelia refringens*
- Infection with *Perkinsus marinus*
- Infection with *Perkinsus olseni*
- Infection with *Xenohaliotis californiensis*. 
The following diseases of crustaceans are listed by the OIE:

- Acute hepatopancreatic necrosis disease
- 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.

The following diseases of amphibians are listed by the OIE:

- Infection with *Batrachochytrium dendrobatidis*
- Infection with ranavirus.
CHAPTER 2.1.

IMPORT RISK ANALYSIS

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).

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.
Article 2.1.2.

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.

Article 2.1.3.

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.
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).

   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:
Annex 6 (contd)

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. **Risk estimation**

*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.

Article 2.1.5.

**Principles of risk management**

1) *Risk management* is the process of deciding upon and implementing measures to address the *risks* identified in the *risk assessment* in order to 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.

Article 2.1.6.

**Risk management components**

1) *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* in order to bring in line with the Member Country’s appropriate level of protection.

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.
Annex 6 (contd)

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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CHAPTER 4.X.

RECOMMENDATIONS FOR SURFACE DISINFECTION OF SALMONID EGGS

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 between incubators with eggs and ultimately between facilities and forms a part of normal routine 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, povidine-iodine. Different protocols should be applied depending on the stage of egg development.

Iodophores, commonly povidone-iodine solutions, 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. It is recommended to follow manufacturer’s instructions/directions to identify circumstances where pH may be a concern. Povidine-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.

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

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

2) immersed in a iodophore 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 iodophore solution should be a minimum of 1:4; then

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

4) held in pathogen free water.
Annex 7 (contd)

All rinsing and disinfection solutions should be prepared using pathogen free water. Iodophore solutions may be buffered using 100 mg sodium bicarbonate (\(\text{NaHCO}_3\)) per litre of diluted iodophore solution if the pH is low.

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CHAPTER 4.7.

CONTROL OF PATHOGENIC AGENTS IN AQUATIC ANIMAL FEED

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 raw and live feed continues to be a common practice, the risk of disease transmission via feed needs to should 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 about 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 is performed in a manner to prevent the spread of infectious diseases of aquatic animals. 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 biosecurity plans health programmes on their farms in order to manage risks related to the use of unprocessed or semi-processed raw and live feed. This can be done through identification of disease free sources and 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).
Article 4.7.4.

Hazard 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.

Article 4.7.5.

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.

Article 4.7.6.

Risk management

1. Use of safe 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 likelihood 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 or 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 10.4.174.) of all disease-specific chapters in Sections 8 to 11; or

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 cleaning 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 in a manner that avoids 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

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.

Certificates 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 certifying official authorised 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 align restrict their requirements with those recommended in the relevant standards of the OIE necessary to achieve the national appropriate level of protection. If there are no such standards recommendations or if the country chooses a level of protection requiring measures these are stricter than the standards of the OIE, these should be based on an import risk analysis conducted in accordance with Chapter 2.1.

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 exceed stricter require a higher level of protection than those that provide protection.
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.

Article 5.1.3.

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.

Article 5.1.4.

Responsibilities in case of an incident related to importation

1) International trade involves a continuing ethical responsibility. Therefore, if within a reasonable period subsequent to an export taking place, the Competent Authority becomes aware of the appearance or reappearance of a disease that has been specifically included in the international aquatic animal health certificate or other disease of potential epidemiological importance to the importing country there is an obligation for the Competent Authority to notify the importing country, so that the imported commodities may be inspected or tested and appropriate action be taken to limit the spread of the disease should it have been inadvertently introduced.
2) If a disease condition appears in imported aquatic animals within a reasonable period after importation, the Competent Authority of the exporting country should be informed so as to enable an investigation to be made, because this may be the first available information on the occurrence of the disease in a previously free aquatic animal population. The Competent Authority of the importing country should be informed of the result of the investigation because the source of infection may not be in the exporting country.

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.

If a disease appears in aquatic animals in an importing country and is associated with importation of commodities, the Competent Authority of the exporting country should be informed. This will enable the exporting country to investigate as this may be the first available information on the occurrence of the disease in a previously free aquatic animal population.

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.
CHAPTER 5.2.

CERTIFICATION PROCEDURES

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.
Annex 10 (contd)

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 exchange of data 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 (W3C) 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).

c) 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.
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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CHAPTER 6.5.

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

Article 6.5.1.

Recommendations for analysing the risks to aquatic animal health and public 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 environment, including human, animal and other usages (under study); problems related to antimicrobial resistance are inherently related to antimicrobial agent use in any environment, including human and non-human uses.

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

2. Objective

For the purposes 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 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. Definitions

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.

For the purposes of this chapter, risk to aquatic animal health relates to the infection of aquatic animals with microorganisms in which resistance has emerged due to antimicrobial agent usage in aquaculture, and resulting in the loss of benefit of antimicrobial therapy used to manage aquatic animal diseases.

For the purpose of this chapter, risk to public health relates to the infection of humans with microorganisms in which resistance has emerged due to antimicrobial agent usage in aquatic animals, and resulting in the loss of benefit of antimicrobial therapy used to manage the human infection.
Annex 11 (contd)

34. The risk analysis process

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

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 contribution to the likelihood of humans and aquatic animals could be being 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 Article Chapter 2.1.3 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 information on use of 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. Data needs Definition of the risk

The definitions of risk used in this chapter are those associated with the use of antimicrobial agents within aquaculture.
Special care is required in the design of data collection programmes for risk assessment to take account of possible confounding factors.

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 standardised methods for antimicrobial susceptibility testing (AST)

Currently, situation in aquaculture is that standardised methods for antimicrobial susceptibility testing for many relevant aquaculture 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.

5. Lack of approved drugs

The small number of approved antimicrobial agents for use in 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 in aquaculture, have the potential to increase the size of the reservoir.
Annex 11 (contd)

There is a risk that the use of antimicrobial agents in aquaculture will result in a rise in the frequency of resistance determinants in the 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.

Article 6.5.3.

Analysis of risks to human health

1. Definition of the risk

The infection of humans with microorganisms in which that have acquired resistance has emerged 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 Entry assessment

A release entry assessment describes the biological pathways necessary for by which the use of a specific antimicrobial agent in aquatic animals leads to the release of resistant microorganisms or resistance determinants into a particular environment. This assessment includes and estimating either qualitatively or quantitatively the probability of that complete process occurring. The release entry assessment describes the probability of the entry 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.

The following factors should be considered in the release entry assessment:
- species of aquatic animals treated with the antimicrobial agent(s) in question;
- aquaculture production system (intensive or 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;
– site and type of infection;
– development of resistant microorganisms;
– prevalence of pathogenic agents that are likely to develop resistance in an aquatic animal species;
– 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.

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;
– transfer of resistant microorganisms and their resistance determinants between humans, aquatic animals, and the environment;
– 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;
Annex 11 (contd)

- 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;
- level of direct contact of workers in the aquaculture or processing industries to the antimicrobial resistant organisms.

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 (e.g. illness and hospitalisation);
- 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 in animal health and public health (see OIE list of antimicrobial agents of veterinary importance and WHO list of Critically Important Antimicrobials);
- prevalence of resistance in human bacterial pathogens under consideration.

6. Risk estimation

A risk estimation integrates the results from the release entry 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. Risk management

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 aquaculture 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.
Annex 11 (contd)

Analysis of risks to aquatic animal health

1. Definition of the risk

The *infection* of aquatic animals with microorganisms in which that have acquired resistance *has emerged* 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. ReleaseEntry assessment

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

- aquatic animal species treated with the antimicrobial agent in question;
- aquaculture production system (intensive or 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 or sales 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);
- development of resistant microorganisms;
- prevalence of pathogenic agents that are likely to develop resistance in an aquatic animal species;
– mechanisms and pathways of direct or indirect transfer 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.

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;
Annex 11 (contd)

- potential linkage of virulence attributes and resistance;
- importance of the antimicrobial agent in aquatic animal health and public health (see OIE list of antimicrobial agents of veterinary importance and WHO list of Critically Important Antimicrobials);
- 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.

6. Risk estimation

A risk estimation integrates the results from the entry 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:

- 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 BATRACHOCYTRIUM DENDROBATIDIS

[...]

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

   b) 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 and equipment 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 and 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;
Annex 12 (contd)

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.

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

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

[...]
Article 8.1.13.

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

When importing, for use in laboratories and zoos, 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:

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 and equipment used in transport and of all effluent and waste materials in a manner that inactivates *B. dendrobatidis*; and

3) the disposal of carcasses in accordance with Chapter 4.6.

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Text deleted.
CHAPTER 8.2.

INFECTION WITH RANAVIRUS

[...]

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 be delivered directly to and held in quarantine facilities for slaughter and processing to products authorised by the Competent Authority; and

2) water and equipment used in transport and all effluent and waste materials from the processing facility 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 and equipment used in transport and of all effluent and waste materials in a manner that inactivates ranavirus; and

3) the disposal of carcasses in accordance with Chapter 4.6.

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— Text deleted.
Articles X.X.7. and X.X.11.

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

Article X.X.7.

Importation of live aquatic animals and aquatic animal products 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.X.X. 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.
CHAPTER 10.4.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

[...]

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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Text deleted.
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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CHAPTER 2.2.2.

INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS

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 Taxonomy of Viruses has assigned IHHNV (a parvovirus) as a tentative species in the genus Brevidensovirus, family Parvoviridae with the species name of PstDNV (for Penaeus stylirostris densovirus) (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 two 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 is from South-East Asia. These genotypes are infectious to P. vannamei and P. monodon. Two putative related sequences are found embedded in the genome of penaeids Type 3A) from East Africa, India and Australia, and Type 3B) from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang et al., 2007). The first two genotypes are infectious to the representative penaeids, P. vannamei and P. monodon. There is evidence that these sequences are not infectious to P. vannamei and P. monodon. 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). Primer sets 309F/309R can distinguish the infectious forms of IHHNV from non-infectious forms. Primer sets MG831F/MG831R will distinguish the non-infectious forms of IHHNV.

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective inactivation methods)

IHHNV is believed to be the most 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; Kalaagayan 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 & Quintio, 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 (Lightner, 1996a; Lightner et al., 1983a; 1983b; 1985), and vertical transmission via infected eggs (Motte et al., 2003) have been demonstrated.

2.3.2. Prevalence

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

2.3.3. Geographical distribution

IHHNV appears to have a world-wide distribution in both wild and cultured penaeid shrimp (Brock & Lightner, 1990; Lightner, 1996a; 1996b; Owens et al., 1992). In the Western Hemisphere, IHHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although 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).

IHHNV was detected for the first time in farmed prawns in Australia in 2008. Additionally, an IHHN-like virus sequence has been reported from Australia (Krabsetsve et al., 2004; Owens et al., 1992), and the presence of IHHN in farmed prawns in Australia was reported to the OIE in 2008. As discussed in Section 2.1.1, IHHNV-related sequences have been found inserted into the genome of P. monodon from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang et al., 2007).

2.3.4. Mortality and morbidity

Depending on the host 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 in P. monodon, this variant of inserted IHHNV sequence is infectious to other penaeids (Tang & Lightner, 2002; 2006).

IHHNV interferes with normal egg and larval development: poor hatching success of eggs, and poor survival and culture performance of the larval and PL stages is observed when broodstock are used from wild or farmed stocks where IHHNV is enzootic (Motte et al., 2003).

Farmed stocks of P. stylirostris, juveniles, subadults and adults may show persistently high mortality rates. In P. vannamei, P. stylirostris, and possibly P. monodon, IHHNV-infected stocks 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.

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

2.4.5. Restocking with resistant species

There has been some limited application and success with IHHNV-resistant *P. stylirostris* (Clifford, 1998; Lightner, 1996a; Weppe, 1992; Zarin-Herzberg & Ascencio 2001). The relative resistance of *P. vannamei* to 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 is transmitted vertically by the transovarian route (Motte et al., 2003). Hence, while disinfection of eggs and larvae is good management practice (Chen et al., 1992) and is recommended for its potential to reduce IHHNV contamination of spawned eggs and larvae produced from them (and contamination by other disease agents), the method is not effective for preventing 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) pre-screening of wild or pond-reared broodstock and/or their spawned eggs/nauplii, and discarding those that test positive for the virus (Fegan & Clifford, 2001; Motte et al., 2003), as well as the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Lotz et al., 1995; Pruder et al., 1995; Wyban 1992). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (Jaenike et al., 1992; Lightner, 2005; Pruder et al., 1995). Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status (Lightner et al., 2009). The development of SPF *P. vannamei* that were free not only of IHHNV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Lightner et al., 2009; Rosenberry, 2004).

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by 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 haloe (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 DNA probe specific to IHHNV provides a definitive diagnosis of IHHNV infection (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4.2.4. Wet mounts
No reliable methods have been developed for direct microscopic pathology.

4.2.5. Smears
Not applicable.

4.2.6. Fixed sections
Histopathology: histology may be used to provide a definitive diagnosis of 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.
Annex 17 (contd)

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™ System User's Guide for Membrane Hybridization and from Boehringer Mannheim’s Nonradioactive In Situ Hybridization Application Manual (Roche Applied Science, 2006a; 2006b). The protocols given below use a DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (Lightner, 1996a; Mari et al., 1993). An alternative method for producing DIG-labelled probes uses specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit™.

**Dot-blot hybridisation procedure:** 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 equivalent¹): 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 30 minutes 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.

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)
  Buffer I 3 × 15 minutes at 68°C
  Buffer II 1 × 5 minutes at room temperature
  Buffer I 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
Annex 17 (contd)

xi) 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 x 10 dips each
- Absolute alcohol: 3 x 10 dips each
- Xylene (or suitable substitute): 4 x 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

\[
\begin{align*}
\text{NaCl} & : 160 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 4 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 23 \text{ g} \\
\text{KCl} & : 4 \text{ g} \\
\text{DD H}_2\text{O} & : 1950 \text{ ml (qs to 2 litres)}
\end{align*}
\]

pH to 8.2 with NaOH; autoclave to sterilise; store at room temperature. To make 1 × PBS, dilute 100 ml 10 × PBS in 900 ml DD H₂O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

ii) 10 × Tris/NaCl/EDTA (TNE) buffer

\[
\begin{align*}
\text{Tris base} & : 60.57 \text{ g} \\
\text{NaCl} & : 5.84 \text{ g} \\
\text{EDTA} & : 3.72 \text{ g} \\
\text{DD H}_2\text{O} & : 900 \text{ ml (qs to 1 litre)}
\end{align*}
\]

pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 × TNE in 900 ml DD H₂O; Filter 1 × solution through a 0.45 µm filter; store at 4°C.

iii) Proteinase K, 100 µg ml⁻¹ (prepare just prior to use)

\[
\begin{align*}
\text{PBS} & : 10 \text{ ml 1 × PBS} \\
\text{Proteinase K} & : 1 \text{ mg}
\end{align*}
\]

iv) 0.4% formaldehyde

\[
\begin{align*}
37\% \text{ formaldehyde} & : 5.4 \text{ ml} \\
\text{DD H}_2\text{O} & : 500 \text{ ml}
\end{align*}
\]

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 Na₂C₆H₅O₇·2H₂O  88.23 g Na citrate.2H₂O
DD H₂O  1000 ml (qs)

pH to 7.0; autoclave; store at 4°C.

To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H₂O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H₂O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H₂O. Filter solutions through a 0.45 µm filter; store at 4°C.

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 polyvinylpyrollidone
DD H₂O  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 H₂O  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 H₂O  25 ml

To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18-gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.

x) 10 × Buffer I

1 M Tris/HCl  121.1 g Tris base
1.5 M NaCl  87.7 g NaCl
DD H₂O  1000 ml (qs)

pH to 7.5 with HCl. Autoclave; store at 4°C.

To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H₂O. Filter through a 0.45 µm filter; store at 4°C.

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.
Annex 17 (contd)

xii) Buffer III

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris/HCl</td>
<td>1.21 g Tris base</td>
<td>pH to 9.5 with HCl Then add:</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.58 g NaCl</td>
<td>50 mM MgCl(_2) 1.02 g MgCl(_2).6H(_2)O</td>
</tr>
<tr>
<td>DD H(_2)O</td>
<td>100 ml (qs)</td>
<td>Filter through a 0.45 µm filter; store at 4°C.</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 µl NBT</td>
<td>75 mg NBT ml(^{-1}) in 70% dimethylformamide</td>
<td></td>
</tr>
<tr>
<td>3.5 µl X-phosphate</td>
<td>5-bromo-4-chloro-3-indoyl phosphate, toluidine salt</td>
<td></td>
</tr>
</tbody>
</table>

xv) Buffer IV

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris/HCl</td>
<td>1.21 g Tris base</td>
<td>pH to 8.0 with HCl Filter through a 0.45 µm filter; store at 4°C.</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.37 g EDTA.2H(_2)O (disodium salt)</td>
<td></td>
</tr>
<tr>
<td>DD H(_2)O</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

xvi) 0.5% Bismarck Brown Y

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bismarck Brown Y</td>
<td>2.5 g</td>
<td>Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.</td>
</tr>
<tr>
<td>DD H(_2)O</td>
<td>500 ml</td>
<td></td>
</tr>
</tbody>
</table>

Polymerase chain reaction for IHHNV: several single-step PCR methods (Krabsetsve et al., 2004; Nunan et 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). However these tests also detect IHHNV-related sequences called 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 392F/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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product</th>
<th>Sequence</th>
<th>G+C%/Temp.</th>
<th>GenBank &amp; References</th>
</tr>
</thead>
<tbody>
<tr>
<td>389F</td>
<td>389 bp</td>
<td>5’-CGG-AAC-ACA-ACC-CGA-CTT-TA-3’</td>
<td>50%/72°C</td>
<td>AF218266</td>
</tr>
<tr>
<td>389R</td>
<td></td>
<td>5’-GGC-CAA-GAC-CAA-AAT-ACG-AA-3’</td>
<td>45%/71°C</td>
<td>(Tang et al., 2000)</td>
</tr>
<tr>
<td>77012F</td>
<td>356 bp</td>
<td>5’-ATC-GGT-GCA-CTA-CTC-GGA-3’</td>
<td>50%/68°C</td>
<td>AF218266</td>
</tr>
<tr>
<td>77353R</td>
<td></td>
<td>5’-TCG-TAC-TGG-CTG-ATC-3’</td>
<td>55%/63°C</td>
<td>(Nunan et al., 2000; 2001)</td>
</tr>
<tr>
<td>392F</td>
<td>392 bp</td>
<td>5’-GGG-CAA-ACC-AGA-ATC-CTA-3’</td>
<td>50%/68°C</td>
<td>AF218266</td>
</tr>
<tr>
<td>392R</td>
<td></td>
<td>5’-ATC-CGG-AGG-AAT-CTG-ATG-TG-3’</td>
<td>50%/71°C</td>
<td>(Tang et al., 2000; 2007)</td>
</tr>
<tr>
<td>309F</td>
<td>309 bp</td>
<td>5’-TCC-AAC-ACT-TAG-TCA-AAA-CCA-A-3’</td>
<td>36%/68°C</td>
<td>AF218266</td>
</tr>
<tr>
<td>309R</td>
<td></td>
<td>5’-TGT-CTG-CTA-CCA-TCA-TCC-A-3’</td>
<td>40%/69°C</td>
<td>(Tang et al., 2007)</td>
</tr>
<tr>
<td>MG831F</td>
<td>831 bp</td>
<td>5’-TTG-GGG-ATG-CAG-CAA-TAT-CT-3’</td>
<td>45%/58°C</td>
<td>DQ228358</td>
</tr>
<tr>
<td>MG831R</td>
<td></td>
<td>5’-GTC-CAT-CCA-CTG-ATC-GGA-CT-3’</td>
<td>55%/62°C</td>
<td>(Tang et al., 2007)</td>
</tr>
</tbody>
</table>

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

**General PCR method for IHHNV:** The PCR method described below for IHHNV generally follows the methods outlined in 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™ Nucleic 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:
Annex 17 (contd)

<table>
<thead>
<tr>
<th>Hot start</th>
<th>Programme 1</th>
<th>5 minutes 95°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linked to</td>
<td>Programme 2</td>
<td>30 seconds 95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 seconds 55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 minute 72°C</td>
</tr>
<tr>
<td>Linked to</td>
<td>Programme 3</td>
<td>7 minutes 72°C</td>
</tr>
<tr>
<td>Linked to</td>
<td>Programme 4</td>
<td>4°C until off</td>
</tr>
</tbody>
</table>

v) Prepare a ‘master mix’ consisting of water, 10 × PCR buffer, the four dNTPs, the two primers, MgCl₂, 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 H₂O: 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
- 25 mM MgCl₂: 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). Using primers 309F/309R, it is possible to distinguish infectious forms of IHHNV from non-infectious forms. Using MG831F/MG831R it is possible to distinguish the non-infectious forms.

The qPCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (2001).
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-carboxyfluorescein (FAM) on the 5' end and N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (Applied Biosystems, part no. 450025).

Preparation of DNA template: the extraction and purification of DNA template is the same as that described in the section of traditional PCR.

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.

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.

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 after 40 cycles, the Ct (threshold cycle) values exceed 40 cycles, or if the Ct value is 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.

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 IHHNV viral sequence and do not react with IHHNV-related sequences present in the P. monodon stocks from Africa or Australia (Tang et al., 2007), or Thailand (e.g. Saksmerprome et al., 2011) have been developed.

PCR commercial kits are available for 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>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Bioassay</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Direct LM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Histopathology</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Antibody-based assays</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>DNA probes – in situ</td>
<td>d</td>
<td>d</td>
<td>b</td>
</tr>
<tr>
<td>PCR, qPCR</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Sequence</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

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

IHHN shall be suspected if at least one of the following criteria is met:

i) Clinical signs indicative of IHHN and a positive result by in-situ hybridisation

or

ii) Histopathology indicative of IHHN and a positive result by in-situ hybridisation.
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 IHHNV is enzootic.

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
IHHN is considered to be confirmed if two of the following criteria are met:

i) Positive result by *in-situ* hybridisation

ii) Positive result by PCR (always genotype specific)

iii) Sequence analysis to confirm IHHNV nucleic acid sequence.

The two methods must target different areas of the genome.

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.

8. References


Annex 17 (contd)


Annex 17 (contd)


Annex 17 (contd)


ROCHE APPLIED SCIENCE (2006b). DIG Nonradioactive Labeling and Detection Product Selection Guide. Catalog Number 03 908 089 001. Roche Diagnostics, Indianapolis, USA.


* * *

**NB:** There are OIE Reference Laboratories for Infectious hypodermal and haematopoietic necrosis (see Table at the end of this Aquatic Manual or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on Infectious hypodermal and haematopoietic necrosis.
1. Scope

Necrotising hepatopancreatitis (NHP) disease is caused by infection with a Gram-negative, pleomorphic intracellular alpha-proteobacterium (Frelier et al., 1992; Lightner & Redman, 1994; Lightner et al., 1992; Loy et al., 1996a; 1996b) preliminarily called Candidatus Hepatobacter penaei. The principal host species in which necrotising hepatobacterium (NHPB) can cause significant disease outbreaks and mortalities are Penaeus vannamei and P. stylirostris (Del Río-Rodríguez et al., 2006; Frelier et al., 1993; Ibarra-Gámez et al., 2007; Lightner & Redman, 1994; Morales-Covarrubias et al., 2011).

NHP has four distinct phases: initial, acute, transition and chronic. In acute and transition-phase disease, pathognomonic lesions are typically present in histological sections of the hepatopancreas, while in the initial and chronic phases of the disease, there are no pathognomonic lesions, and molecular and antibody-based methods for NHPB detection are necessary for diagnosis (Morales-Covarrubias, 2010; Morales-Covarrubias et al., 2010; 2012; Vincent & Lotz, 2005).

Synonyms: necrotising hepatobacterium (NHPB) or NHP bacterium (NHPB); rickettsial-like organism (RLO).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

NHPB is a pleomorphic, Gram-negative, intracytoplasmic bacterium preliminarily called Candidatus Hepatobacter penaei. 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 form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier et al., 1992; Lightner & Redman, 1994; Loy et al., 1996a; 1996b). Genetic analysis of the NHPB 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).
Annex 18 (contd)

NHPB causes chronic disease in *P. vannamei*, the main effects of which are slow growth, a soft cuticle and a flaccid body (Morales-Covarrubias, 2010; Morales-Covarrubias et al., 2012).

Outbreaks of NHP disease have been reported in *P. aztecus* (Johnson, 1990; Jory, 1997; Lightner, 1996; Morales-Covarrubias, 2010). NHP has also been seen in *P. californiensis* and *P. setiferus* (Frelier et al., 1995; Lightner, 1996). *Penaeus setiferus* is reportedly less susceptible to disease than *P. vannamei* (Frelier et al., 1995).

In an NHP survey of the Gulf of Mexico, *P. setiferus* and *P. duorarum* in the vicinity of coastal prawn farms along the Yucatan and Campeche coast revealed no histological evidence of NHP (Del Rio-Rodriguez 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; 2011; 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., 2012; 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 between 0.6% and 1.3% in *P. vannamei* collected from shrimp farms in Belize, Brazil, Guatemala, Honduras, Mexico, Nicaragua and Venezuela (Morales-Covarrubias *et al*., 2011).

### 2.3.3. Geographical distribution

NHPB appears to have a Western hemisphere distribution in both wild and cultured penaeid shrimp (Aguirre-Guzman *et al*., 2010; Del Rio-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*., 2011).

### 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 a low prevalence (<7%) in shrimp farms in the months of April, May, July and August. However, in the months of September and October when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

### 2.4. Control and prevention

#### Control

The use of the antibiotics, oxytetracycline and florfenicol 50%, in medicated feeds every 8 hours for 10 days is probably the best NHP treatment currently available, particularly if disease is detected in the initial phase (Frelier *et al*., 1995; Morales-Covarrubias *et al*., 2012).

#### 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 quick 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’Byren, 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 pre-screening 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-Reantaso et al., 2001; Bradley-Dunlop et al., 2004; Briñez et al., 2003; Frelier et al., 1993; Lightner, 1996; Morales-Covarrubias et al., 2012).

3.5. Samples/tissues those are not suitable
NHPB 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. This results in increased prevalence and severity of infections that can be more easily detected using the available diagnostic and detection methods for NHPB.
4.1.1. Clinical signs

A wide range of gross signs can be used to indicate the possible presence of NHP. These include: lethargy, reduced food intake, atrophied hepatopancreas, anorexia and empty guts, noticeably reduced growth and poor length weight ratios (‘thin tails’); soft shells and flaccid bodies; black or darkened gills; heavy surface fouling by epicommensal organisms; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic.

4.1.2. Behavioural changes

In acute NHP disease, *P. vannamei* may present behavioural changes including lethargy and reduced feeding activity.

4.2. Clinical methods

4.2.1. Gross pathology

NHPB often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adult and broodstock, the incubation period and severity of the disease are somewhat size or age dependent. Infected adults seldom show signs of the disease or mortalities (Aranguren *et al.*, 2006; 2010; Bastos Gomes *et al.*, 2010, Brock & Main, 1994; Morales-Covarrubias *et al.*, 2012). Gross signs are not NHP specific, but acute NHP shows a marked reduction in food consumption, followed by changes in behaviour and appearance (see Section 4.1.1).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute and chronic NHP in *P. vannamei* can be readily diagnosed using routine haematoxylin and eosin (H&E) stain histological methods (see Section 4.2.6).

4.2.3.1. Initial phase of necrotising hepatopancreatitis

Initial NHPB infection is more difficult to diagnose using routine H&E histological methods. For diagnosis of initial infections, 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 foam cells and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations). Hypertrophic cells, individual epithelial cells appeared to be separated from adjacent cells, undergo necrosis and desquamation in to the tubular lumen. The tubular epithelial cell lipid content is variable.

4.2.3.3. Transition phase of 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 centre 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 numbers of haemocyte nodules.
Annex 18 (contd)

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 centre; pale with black stripes (melanised tubules); pale centre 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 uses is based on the tubular deformation or tubular atrophy, mainly of the apical region to indicate early stages of NHP.

NHP disease has four phases (a semiquantitative scale):

- **Initial phase:** low presence of tubular deformation (1–5 field–1 organism–1) and cell detachment.
- **Acute phase:** infiltration of haemocytes, increased numbers of deformed tubules (6–10 field–1 organism–1), encapsulation present in different regions of the sample, which is atrophied tubules surrounded by multiple layers of haemocytes.
- **Transition phase:** infiltration of haemocytes, increased numbers of deformed tubules (11–15 field–1 organism–1), melanised tubules, necrotic tubules and a high level of encapsulation present in different regions of the sample. At this stage haemocyte nodules were observed with masses of bacteria in the centre of the nodule.
- **Chronic phase:** areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells with masses of bacteria in the cytoplasm.

4.2.5. Smears

Not applicable.

4.2.6. Electron microscopy/cytology

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 *Penaeus vannamei* serving as the indicator of the intracellular bacteria (Cock *et al.*, 2009; Johnson, 1990; Lee & O’Brien, 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 monoclonal antibodies (MAbs) 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 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., 2012). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled cDNA probe to NHPB, 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) fragment against 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 98°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 (Roche, Germany) and following the manufacturer’s procedures for production of quality DNA 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.

iii) The following controls should be included in every when performing the RT-PCR assay for NHPB: a) known NHPB negative tissue sample; b) a known NHPB-positive sample (hepatopancreas); and c) a ‘no template’ control.

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

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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.
iv) The optimised RT-PCR conditions (5–50 ng DNA) (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, 60–0.1 U μl⁻¹), magnesium 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.

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,N-tetramethyl-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 DNA, and water in a reaction volume of 25 μl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the master cycler Realplex 2.0 (Eppendorf). The cycling consists of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. After each cycle, the levels of fluorescence are measured.

v) At the end of the reaction, real time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase in PCR product. 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.
4.3.1.2.3.4. Sequencing

RT-PCR products may be cloned and sequenced or sequenced directly 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 et al., 2004; Vincent & Lotz, 2005), but these are not recommended for routine diagnosis of NHP. The NHP bacterium is unculturable using traditional bacteriological methods, thus NHPB infection must be maintained through continual exposure of uninfected L. vannamei stock to a population undergoing an NHPB epidemic.

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

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
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</thead>
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<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
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<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Bioassay</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Direct LM</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Histopathology</td>
<td>d</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>In-situ DNA probes</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>d</td>
<td>d</td>
<td>d</td>
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<tr>
<td>Antibody-based assays</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>qPCR</td>
<td>a</td>
<td>a</td>
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<tr>
<td>PCR</td>
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<tr>
<td>Sequencing</td>
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</tbody>
</table>

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

The presence of NHPB shall be suspected if at least one of the following criteria is met. 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 f- 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.

8. References


Annex 18 (contd)


Annex 18 (contd)


* * *

NB: At the time of publication (2015) there was not yet an OIE Reference Laboratory for Necrotising hepatopancreatitis (see Table at the end of this Aquatic Manual or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
CHAPTER 2.2.5.

TAURA SYNDROME

1. Scope

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is TSV, as described by Bonami et al. (1997) and Mari et al. (1998; 2002). At least four genotypes (strains) have been documented based on the gene sequence encoding VP1 (= 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 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. 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 or epizootics may carry the virus for life (Hasson *et al.*, 1999a; 1999b) and, although not documented, are assumed to pass the virus to their progeny by vertical transmission.

2.2.6. 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 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; Zarín-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 it is possible TSV might be transmitted vertically (transovarian transmission), despite there being no published report documenting this route of transmission. Disinfection of eggs and larvae (Chen *et al.*, 1992) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry and disease control and management practices have been used successfully to reduce the risks TSV infections and disease occurring during farm grow-out. These include the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Lotz *et al.*, 1995; Moss *et al.*, 2001; Pruder *et al.*, 1995; Wyban 1992; Wyban *et al.*, 2004). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of TS. Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF *P. vannamei* that were free not only of TSV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its surpassing *P. monodon* in 2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (FAO, 2006; Lightner, 2005; Rosenberry, 2004).
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.
Annex 19 (contd)

**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).

**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.
Annex 19 (contd)

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.

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).

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 MgCl₂, pH 9.5.

vi) Reactions are stopped with distilled water.

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3 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual.*
Annex 19 (contd)

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

4.3.1.2.6. Other antibody-based methods

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

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

4.3.1.2.7. Molecular techniques

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

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

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

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

4.3.1.2.7.2. Reverse-transcription (RT)-PCR method

Tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9992F and 9195R, amplify a 231 base pair (bp) sequence of the TSV genome (Nunan et al., 1998). The fragment amplified is from a conserved sequence located in the intergenic region and ORF 2 of TSV. Primer 9992F is located near the 3' end of intergenic region and 9195R is located on ORF 2 within VP2 (= CP1) (Mari et al., 2002; Nunan et al., 1998). A new pair of TSV primers (7171F and 7511R) has been developed and shown to have an improved sensitivity for TSV detection (Navarro et al., 2009). These replacement primers are 9992F/9195R and they are located within ORF 2.
### Primer Product Sequence Temperature G+C%

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product</th>
<th>Sequence</th>
<th>Temperature</th>
<th>G+C%</th>
</tr>
</thead>
<tbody>
<tr>
<td>9992F</td>
<td>231 bp</td>
<td>5'-AAG-TAG-ACA-GCC-GCG-CTT-3'</td>
<td>69°C</td>
<td>55%</td>
</tr>
<tr>
<td>9195R</td>
<td>5'-TCA-ATG-AGA-GCT-TGG-TCC-3'</td>
<td>55%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7171F</td>
<td>341 bp</td>
<td>5'-CGA-CAG-TTG-GAC-ATC-TAG-TG-3'</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>7511R</td>
<td>5'-GAG-CTT-CAG-ACT-GCA-ACT-TC-3'</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

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) was is used for all amplification reactions described here. Alternative kits can be used and adjusted for use for this assay.

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.

xii) **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-ACA-ATG-GCC-ACA-ATA-TTC-GAG-CAT-C-3’), which corresponds to the region from nucleotide 1024 to 1051, is synthesised and labelled with fluorescent dyes 5-carboxyfluorescein (FAM) on the 5’ end and N,N,N’,N’-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3’ end (Applied Biosystems, catalog no. 450025).

ii) Preparation of RNA template: the extraction and purification of RNA template from haemolymph, or shrimp tissue, is the same as that described in the section for traditional 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 can be performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, 7500, or newer models or equivalent thermocycler 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 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.

Table 5.1. TSV surveillance, detection and diagnostic methods in penaeids

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Bioassay</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Direct LM</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>Histopathology</td>
<td>d</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Antibody-based assays</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>DNA probes – in situ</td>
<td>d</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>RT-PCR, qRT-PCR</td>
<td>a</td>
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<td>a</td>
</tr>
<tr>
<td>Sequence</td>
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<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

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;
Annex 19 (contd)

• 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).

8. References


Annex 19 (contd)


Annex 19 (contd)


OIE Aquatic Animal Health Standards Commission/March 2015
Annex 19 (contd)


ROSENBERRY B. (2004). WORLD SHRIMP FARMING 2004. NUMBER 17, PUBLISHED BY SHRIMP NEWS INTERNATIONAL, SAN DIEGO, CALIFORNIA, USA, 276 PP.


Annex 19 (contd)


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NB: There is an OIE Reference Laboratory for Taura syndrome (see Table at the end of this Aquatic Manual or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on Taura syndrome.
CHAPTER 2.2.8.
INFECTION WITH YELLOW HEAD VIRUS

1. Scope

For the purpose of this chapter, yellow head disease (YHD) is considered to be infection with yellow head virus genotype 1 (YHV1).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Yellow head virus genotype 1 (YHV1) 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 (40–50 nm × 150–180 nm) (Chantanachookin et al., 1993; Wongteerasupaya et al., 1995). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids appear as rods (diameter 20–30 nm) and possess a helical symmetry with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p20–24 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).
Annex 20 (contd)

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). The Pacific blue shrimp (P. stylirostris), the daggerblade grass shrimp (Palaemonetes pugio), and the Jinga shrimp (Metapenaeus affinis) also fulfill the criteria required for listing a species susceptible to infection with YHV1 according to Article 1.5 of Aquatic Animal Health Code. Natural infections have also been detected in the kuruma prawn (P. japonicus), white banana prawn (P. merguiensis), Pacific blue shrimp (P. stylirostris), white prawn (P. setiferus), red endeavour prawn (Metapenaeus ensis), mysid shrimp (Palaemon styliferus) and krill (Aécetes sp.). Other species of penaeid and palamomid shrimp and prawns and krill that have been reported to be susceptible to experimental infection include: brown tiger prawn (P. esculentus), brown prawn (P. aztecas); pink prawn, hopper and brown-spotted prawn (P. duorarum), green tail prawn (Metapenaeus bennettiae), Suna 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. aztecas, 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 (Wijegooneawardane et al., 2008a). Metapenaeus brevicomis and P. aztecas also fulfill some of the criteria required for listing as susceptible but evidence was lacking to either confirm the identity of the pathogen under study as YHV1, to demonstrate a natural route of infection, or to definitively confirm an ‘infected’ status.

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; Wijegooneawardane et al., 2008a; 2009). In contrast, YHV (genotype 1) infections are usually detected only when disease is evident and whilst they do not occur commonly in healthy P. monodon, infections have been detected in healthy wild populations of P. stylirostris (Castro-Longoria et al., 2008). During YHD outbreaks in aquaculture ponds, the 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. 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; Wijegooneawardane et al., 2008a; 2009).

2.2.4. Target organs and infected tissue

YHV targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematoopoietic 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 persist for life (Walker et al., 2001; Wijegooneawardane 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 palaemonid 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 (Acestes 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 [LD50] 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. Gills or haemolymph can be used for non-sacrificial sampling.

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.

4.2.5. **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[CH3]2AsO2·3H2O) 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 NaH2PO4·H2O, 1.5 g Na2HPO4, 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 40–50 nm × 150–180 nm with rounded ends and prominent projections (8–11 nm) extending from the surface. In the cell cytoplasm, virions are commonly seen to be localised or packed densely within intracellular vesicles. Virions may also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from 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-mercaptopethanol, 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 (≈ 10⁸ 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™ 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 A260nm and A280 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™ reagent and extract RNA according to the product manual. Based on the standard Trizol™ 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.

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/Cl 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 mM antisense primer 144R, 1.0 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM MgCl₂, 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/Cl pH 8.3, 50 mM KCl) containing 2.5 U Taq DNA polymerase, 2 mM MgCl₂ and 0.75 µM of sense primer 10F to give a final volume of 100 µl. Unless the instrument is fitted with a heated lid, overlay the tubes with 100 µl of mineral oil and conduct 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.

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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.
The sensitivity of the PCR is approximately 0.01 pg of purified YHV RNA (~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 MgCl₂), 1 µl 100 mM DTT and 0.5 µl 10 mM dNTP stock mixture (i.e. 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP) and mix gently. Preheat to 42°C for 2 minutes, add 0.5 µl 200 U µl⁻¹ reverse transcriptase and incubate at 42°C for 1 hour. Heat the reaction at 70°C for 10 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, prepare a 50 µl reaction mixture containing 1 × Taq buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 35 pmol of each primer GY1 and GY4, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U Taq polymerase in a 0.5 ml thin-walled tube. Overlay the reaction mixture with 50 µl liquid paraffin, heat at 85°C for 2–3 minutes and then add 1 µl cDNA. 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 MgCl₂, 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’
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™ (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 MgCl₂, 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, 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 shrimp. At 2 days post-infection, harvest moribund shrimp showing typical signs of YHD. Use a syringe to draw haemolymph from the sinuses at the base of the walking legs and mix carefully on ice with the same volume of lobster haemolymph medium (LHM) (486 mM NaCl, 15 mM CaCl₂, 10 mM KCl, 5 mM MgCl₂, 0.5 mM Na₂HPO₄ 8.1 mM MgSO₄, 36 mM NaHCO₃, 0.05% dextrose in Minimal Eagle’s Medium, adjusted pH 7.6 with 1 N NaOH).

Centrifuge the mixture at 480 g for 30 minutes at 4°C to remove cellular debris. Ultracentrifuge the supernatant at 100,000 g for 1 hour at 4°C. Discard the supernatant and gently resuspend the pellet overnight at 4°C in 1 ml LHM. Layer this suspension over a continuous gradient of 20-40% Urografin and ultracentrifuge at 100,000 g for 1 hour at 4°C. After centrifugation, collect the viral band by using a Pasteur pipette and dilute with NTE buffer (0.02 M EDTA, 0.2 M NaCl, 0.2 M Tris/HCl [pH 7.4]) to a final volume of 12 ml. Ultracentrifuge the suspension at 100,000 g for 1 hour at 4°C and resuspend the pellet (purified virus) in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA [pH 7.4]) and store in 20 µl aliquots at –80°C until required.

4.3.1.2.4 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.

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

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
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<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
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<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>c</td>
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<tr>
<td>Bioassay</td>
<td>d</td>
<td>d</td>
<td>d</td>
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<tr>
<td>Direct LM</td>
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<td>d</td>
<td>d</td>
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<td>Histopathology</td>
<td>d</td>
<td>d</td>
<td>c</td>
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<td>Transmission EM</td>
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<td>c</td>
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<tr>
<td>Antibody-based assays</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>DNA probes – in situ</td>
<td>d</td>
<td>d</td>
<td>c</td>
</tr>
<tr>
<td>PCR</td>
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<td>a</td>
<td>a</td>
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<tr>
<td>Sequence</td>
<td>a</td>
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</table>

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

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

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

7.2. Definition of confirmed case

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.

8. References


Annex 20 (contd)


**Annex 20 (contd)**


* * *

**NB:** There is an OIE Reference Laboratory for Yellow head disease (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on Yellow head disease
CHAPTER 2.4.7.

INFECTION WITH *PERKINSUS OLSENI*

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 synonym.

2. Disease information

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*, *C. ariakensis*, and *C. sikamea* (Villalba et al., 2004); pearl oysters *Pinctada margaritifera*, *P. martensis*, 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.

8. References


NB: There is an OIE Reference Laboratory for Infection with *Perkinsus olseni* (see Table at the end of this Aquatic Manual or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the OIE Reference Laboratories for any further information on infection with *Perkinsus olseni*.
CHAPTER 1.1.
NOTIFICATION OF DISEASES, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

Article 1.1.1.

For the purposes of the Aquatic Code and in terms of Articles 5, 9 and 10 of the OIE Organic Statutes, every Member Country of the OIE shall recognise the right of the Headquarters to communicate directly with the Competent Authority of its territory or territories.

All notifications and all information sent by the OIE to the Competent Authority shall be regarded as having been sent to the country concerned and all notifications and all information sent to the OIE by the Competent Authority shall be regarded as having been sent by the country concerned.

Article 1.1.2.

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

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

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

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

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

Article 1.1.3.

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

1) in accordance with relevant provisions in the disease-specific chapters, notification, through the World Animal Health Information System (WAHIS) or by fax or e-mail within 24 hours of any of the following events:
   a) first occurrence of a listed disease in a country, a zone or a compartment;
   b) re-occurrence of a listed disease in a country, a zone or a compartment following the final report that declared the outbreak ended;
Annex 22 (contd)

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

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

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

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

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

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

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

Article 1.1.4.

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

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

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

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

      b) the disease has been eradicated; or

      c) the situation has become sufficiently stable; or

   b) until sufficient scientific information is available to determine whether it meets the criteria for listing.

   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 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, 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.

Article 1.1.6.

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

2) The Headquarters shall communicate by e-mail or World Animal Health Information Database (WAHID) to Competent Authorities all notifications received as provided in Articles 1.1.2. to 1.1.5. and other relevant information.

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CHAPTER 1.2.

CRITERIA FOR LISTING AQUATIC ANIMALS: THE INCLUSION OF DISEASES IN THE OIE LIST

Article 1.2.1.

Introduction

This chapter describes the criteria for listing aquatic animal diseases in the OIE list. The objective of listing is to support Member Countries by providing information needed to take appropriate action efforts to prevent the transboundary spread of important diseases of aquatic animals. This is achieved through transparent, timely and consistent reporting notification.

For the diseases listed in accordance with Article 1.2.2., the corresponding disease-specific chapters in the Aquatic Code assist Member Countries in the harmonisation of disease detection, prevention and control and provide standards for safe international trade in aquatic animals and their products.

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

Principles for selection of diagnostic tests are described in Chapter 1.1.2 of the Aquatic Manual.

Article 1.2.2.

The criteria for listing an aquatic animal disease in the OIE list are as follows:

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

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<thead>
<tr>
<th>No.</th>
<th>Criteria for listing</th>
<th>Explanatory notes</th>
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<tbody>
<tr>
<td>A.</td>
<td></td>
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<tr>
<td>OR</td>
<td>The disease has been shown to cause a significant production losses at a national or multinational (zonal or regional) level impact on the health of aquatic animals at the level of country or a zone taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality.</td>
<td>There is a general pattern that the disease will lead to losses in susceptible species, and that morbidity or mortality are related primarily to the infectious agent and not management or environmental factors. (Morbidity includes, for example, loss of production due to spawning failure.) The direct economic impact of the disease is linked to its morbidity, mortality and effect on product quality.</td>
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<thead>
<tr>
<th>No.</th>
<th>Criteria for listing</th>
<th>Explanatory notes</th>
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<tr>
<td>2-OR</td>
<td><strong>c.</strong></td>
<td>The disease has been shown to, or scientific evidence indicates that it is likely to, would cause a significant impact on the health of morbidity or mortality in wild aquatic animal populations, taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats.</td>
</tr>
<tr>
<td>AND</td>
<td></td>
<td>Wild aquatic animal populations can be populations that are commercially harvested (wild fisheries) and hence are an economic asset. However, the asset could be ecological or environmental in nature, for example, if the population consists of an endangered species of aquatic animal or an aquatic animal potentially endangered by the disease.</td>
</tr>
<tr>
<td>3-OR</td>
<td><strong>c.</strong></td>
<td>The disease has been shown to, or scientific evidence indicates that it is likely to, would cause a significant impact on the health of morbidity or mortality in wild aquatic animal populations, taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats.</td>
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<tr>
<td>AND</td>
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<td>Wild aquatic animal populations can be populations that are commercially harvested (wild fisheries) and hence are an economic asset. However, the asset could be ecological or environmental in nature, for example, if the population consists of an endangered species of aquatic animal or an aquatic animal potentially endangered by the disease.</td>
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<td>3.4</td>
<td><strong>c.</strong></td>
<td>The agent is of public health concern. Natural transmission to humans has been proven, and human infection is associated with severe consequences.</td>
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<td>AND</td>
<td></td>
<td>Infectious aetiology of the disease is proven.</td>
</tr>
<tr>
<td>4.</td>
<td>-</td>
<td>Infectious aetiology of the disease is proven.</td>
</tr>
<tr>
<td>5.</td>
<td><strong>c.</strong></td>
<td>An infectious agent is strongly associated with the disease, but the aetiology is not yet known.</td>
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<tr>
<td>AND</td>
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<td>Infectious diseases of unknown aetiology can have equally high-risk implications as those diseases where the infectious aetiology is proven. Whilst disease occurrence data are gathered, research should be conducted to elucidate the aetiology of the disease and the results be made available within a reasonable period of time.</td>
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<tr>
<td>6-1</td>
<td><strong>And</strong></td>
<td>Likelihood of international spread, of the agent including (via live aquatic animals or their products, or fomites) has been proven.</td>
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<td>AND</td>
<td></td>
<td>International trade in aquatic animal species susceptible to the disease exists or is likely to develop, and, under international trading practices, the entry and establishment of the disease is likely.</td>
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<td>7-2</td>
<td><strong>And</strong></td>
<td>At least one country or countries with zones has demonstrated freedom or impending freedom from the disease in populations of susceptible aquatic animals, may be declared free of the disease based on the general surveillance provisions principles outlined in chapters 1.4. and 1.5.</td>
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<tr>
<td>AND</td>
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<td>Free countries/zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a disease can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread disease, or the protection of the last remaining free zones from a widespread disease.</td>
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### C. Diagnosis

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8-3 A repeatable and robust Reliable means of detection and diagnosis exists and a precise case definition is available to clearly identify cases and allow them to be distinguished from other diseases.

A diagnostic test should be widely available and preferably has undergone a formal standardisation and validation process using routine field samples (See Aquatic Manual) or a robust case definition is available to clearly identify cases and allow them to be distinguished from other pathologies.

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CHAPTER 9.2.

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

Article 9.2.1.

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

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

Article 9.2.2.

Scope

The recommendations in this chapter apply to: *Penaeus monodon*, *Penaeus vannamei*, *Penaeus stylirostris*, *Palaemonetes pugio* and *Metapenaeus affinis* giant tiger prawn (*Penaeus monodon*), brown tiger prawn (*P. esculentus*) and Kuruma prawn (*P. japonicus*). These recommendations also apply to any other susceptible species referred to in the Aquatic Manual when traded internationally.

[...]
CHAPTER 2.2.X.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

1. Scope

For the purpose of this chapter, acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), is considered to be infection with unique strains of *Vibrio parahaemolyticus*, namely AHPND-causing *V. parahaemolyticus* (VP_{AHPND}).

The disease has two distinct phases:

i) An acute phase characterised by acute progressive, massive degeneration of the hepatopancreas (HP) tubules from medial to dorsal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (FAO, 2013; NACA, 2012; 2014; Nunan *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Tran *et al.*, 2013a; 2013b; 2014a; 2014b).

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent isolates

AHPND has a bacterial aetiology (Tran, 2013a; 2013b; Zhang *et al.*, 2012). It is caused by specific virulent strains of *Vibrio parahaemolyticus*, namely VP_{AHPND}, which contains one or more extrachromosomal plasmids, including a unique, previously unreported, large, plasmid with a size of ~70 kbp (Gomez-Gil *et al.*, 2014; Gomez-Jimenez *et al.*, 2014; Kondo *et al.*, 2014; Yang *et al.*, 2014). This plasmid has been designated pVPA3-1, and its size may vary slightly. Removal (or “curing”) of pVA1 abolishes the AHPND-causing ability of the virulent strain of *V. parahaemolyticus*. A pVA1-cured strain fails to induce the massive sloughing of cells in the hepatopancreatic tubules that is a primary histopathological characteristic of disease (Lee *et al.*, 2015).

VP_{AHPND} expresses a deadly plasmid-encoded toxin (Pir^{vp}), which is homologous to the Pir (*Photorhabdus* insect-related) binary toxin. The toxin is formed from two subunits, PirA^{vp} and PirB^{vp}, but unlike other Pir binary toxins, *V. parahaemolyticus* PirB (PirB^{vp}, a 50.1 kDa protein) alone is capable of inducing AHPND histopathology in the hepatopancreatic tubules, while PirA^{vp} (a 12.7 kDa protein) causes only minor histological changes (Han *et al.*, 2015; Lee *et al.*, 2015; Sirikharin *et al.*, 2015).

Within a population of AHPND-causing bacteria, natural deletion of the Pir^{vp} region may occur in a few individuals (Tinwongger *et al.*, 2014). This deletion is due to the instability caused by the repeat sequences/transposase that flank the pir toxin operon, and although different strains exhibit different levels of stability, when the deletion occurs, it means that a virulent strain of *V. parahaemolyticus* will lose its ability to induce AHPND. However, if the pir toxin sequence is used as a target for detection, then a colony that has this deletion will produce a negative result even though the colony was derived from an isolate of virulent bacteria.

The plasmid pVA1 also carries a cluster of genes related to conjugative transfer, which means that this plasmid is potentially able to transfer to other bacteria. So far, however, there have been no published reports that any bacteria other than *V. parahaemolyticus* carry pVA1. The pVA1 plasmid also carries the *pndA* gene, which is associated with a post-segregational killing (psk) system. For a bacterium that
harbours a plasmid with the psk system (PSK*), only progeny that inherit the PSK* plasmid will be viable. Progeny that do not inherit the PSK* plasmid will die because the stable pndA mRNA will be translated to PndA toxin that will kill the bacterium. The presence of a psk system on a plasmid thus ensures that the plasmid is inherited during bacterial replication. The pVA1 plasmid will therefore be passed on to subsequent generations of VP$_{ANPND}$ producing PirA$^\text{VP}$ and PirB$^\text{VP}$. Hence, when PirA$^\text{VP}$ is present there is little or no histopathology. When PirB$^\text{VP}$ is present, its larger size of 50.1 kDa is adequate to produce an enzyme that denudes the hepatopancreatic tubules (Lee et al., 2015).

2.1.2. Survival outside the host (i.e. in the natural environment)
Not known.

2.1.3. Stability of the agent
Not known.

2.1.4. Life cycle
Not applicable.

2.2. Host factors

2.2.1. Susceptible host species (common and Latin names)
*Penaeus vannamei* (white leg or Pacific white shrimp); *P. monodon* (black tiger prawn) and *P. chinensis* (fleshy prawn).

2.2.2. Susceptible stages of the host
In the acute phase, this disease is characterised by a massive acute progressive degeneration of the HP tubules from medial to dorsal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (FAO, 2013; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; 2013b; 2014a; 2014b).

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

2.2.3. Species or sub-population predilection (probability of detection)
Not applicable.

2.2.4. Target organs and infected tissue
Gut-associated tissues and organs.

2.2.5. Persistent infection with lifelong carriers
See Section 2.1.4 Life cycle.

2.2.6. Vectors
None are known.

2.2.7. Known or suspected wild aquatic animal carriers
None are known (except in South-East Asia, some molluscs and certain polychaetes).

2.3. Disease pattern

2.3.1. Transmission mechanisms
Mortalities are expected within 30 days of stocking shrimp ponds with postlarvae (PL) or juveniles (from 15 mg to ~1 g in weight) (Nunan et al., 2014; Leaño & Mohan, 2013; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

With laboratory infections, mortality can be induced within 12 hours of exposure to strains of \( V_{AHPND} \) by the per os route if the coated feed contains \( 10^8 \) CFU (colony-forming units) per gram of inoculum (Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

Alternatively mortalities can be induced is with a bath challenge, provided that the challenge bath begins with \( 10^8 \) CFU per gram of inoculum (Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

### 2.3.2. Prevalence

Nunan et al., 2014, Soto-Rodriguez et al., 2015, and Tran et al., 2013b found a near 100% prevalence in pond-reared stocks in South-East Asia and in Mexico after 2013.

### 2.3.3. Geographical distribution

The disease has been introduced into south-east China (People's Rep. of), Vietnam, Malaysia, Thailand and Mexico. In other countries in the East and South-East Asia regions, and neighbouring Mexico, farms may have been exposed to the toxin-producing strains of \( V. \text{parahaemolyticus} \) (Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b; Zhang et al., 2012).

### 2.3.4. Mortality and morbidity

Joshi et al., 2014a; 2014b; Nunan et al., 2014; Soto-Rodriguez et al., 2015; and Tran et al., 2013b found a near 100% mortality and morbidity prevalence in pond-reared stocks in South-East Asia and in Mexico.

### 2.3.5. Environmental factors (e.g. temperature, salinity, season, etc.)

Water sources with low salinity (below ~5 to ~10 ppt) seem to reduce the prevalence of the disease. Although AHPND can be found year round in South-East Asia, the hot and dry season from April to July seems to be the peak. Bad feed management, algal bloom or crash are also factors that may lead to AHPND in endemic areas.

### 2.4. Control and prevention

#### 2.4.1. Vaccination

Not applicable.

#### 2.4.2. Chemotherapy

Not useful.

#### 2.4.3. Immunostimulation

Not useful.

#### 2.4.4. Resistance breeding

An AHPND line with some resistance to the disease has been developed in Mexico and in Ecuador. This was accomplished through mass selection over 10 years for growth and survival, rather than for SPF (specific-pathogen free) stock development (Lightner, unpublished data).

#### 2.4.5. Restocking with resistant species

None available.

#### 2.4.6. Blocking agents

None available.
2.4.7. Disinfection of eggs and larvae
None known.

2.4.8. General husbandry practices
None known.

3. Sampling

3.1. Selection of individual specimens
Samples of moribund shrimp or shrimp that show clinical signs (see Section 4.1.1) should be selected for AHPND detection. It is assumed that adults (broodstock) can carry toxin-bearing strains (especially PirB<sup>vP</sup>) of <i>V. parahaemolyticus</i> (Han et al., 2015; Lee et al., 2015; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b). Therefore, broodstock without clinical signs may also be selected for testing, but only for testing for the presence of PirB<sup>vP</sup> toxin.

3.2. Preservation of samples for submission
Carefully selected shrimp samples can be submitted to a variety of laboratories for diagnosis of AHPND. The samples can be submitted in 90% ethanol for polymerase chain reaction (PCR) detection, or preserved in Davison’s AFA fixative for routine histopathology (Joshi et al., 2014a; 2014b; Leaño & Mohan, 2013; Lee et al., 2015; Nunan et al., 2104; Sirikharin et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

3.3. Pooling of samples
For molecular testing, samples of shrimp cephalothoracies can be selected (pooled when less than 0.5 g).

Samples, especially PL or specimens up to 0.5 g can be pooled. Larger shrimp should not be pooled and should be processed individually (Lightner, unpublished data).

3.4. Best organs or tissues
Gut-associated tissues and organs, such as hepatopancreas, stomach, the midgut, the hindgut, and faeces of selected shrimp for samples. Valuable broodstock may be worth saving, and from these only faeces should be collected.

3.5. Samples/tissues that are not appropriate (i.e. when it is never possible to detect)
Samples other than gut-associated tissues and organs are not appropriate (FAO, 2013; NACA, 2012; 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

4. Diagnostic methods

4.1. Field diagnostic methods
4.1.1. Gross signs
The clinical signs could be used for presumptive diagnosis, which can be further confirmed by histopathology observed at the animal level include a pale to white HP due to pigment loss in the connective tissue capsule, significant atrophy of the HP, soft shells and guts with discontinuous contents or no contents, black spots or streaks sometimes visible within the HP, soft HP which does not squash easily between the thumb and forefinger, and the onset of clinical signs and mortality starting as early as 10 days post-stocking (NACA, 2012; 2014).

4.1.2. Behavioural changes
Not applicable.

4.2. Clinical methods
4.2.1. Clinical chemistry
None are known.
4.2.2. Microscopic pathology
An acute phase characterised by an acute, massive progressive degeneration of the HP tubules from medial to dorsal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (FAO, 2013; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013a; 2013b; 2014a; 2014b).

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

4.2.3. Wet mounts
Not applicable.

4.2.4. Smears
Not applicable.

4.2.5. Fixed sections (for ISH):
*In-situ* hybridisation is a useful technique provided it is done properly. The result of an ISH test will be apparent as a Bismarck Brown stained material will remain. This will be used to distinguish ANHPD tissues from those tissues which are not affected.

4.2.6. Electron microscopy/cytopathology
None reported to date (February 2015).

4.3. Agent detection and identification methods
4.3.1. Direct detection methods
4.3.1.1. Microscopic methods
See Section 2.2.2.

4.3.1.1.1. Wet mounts
Not applicable.

4.3.1.1.2. Smears
Not applicable.

4.3.1.1.3. Fixed sections
Not applicable.

4.3.1.2. Agent isolation and identification
On marine or blood agar, the strains of VP AHPND are capable of swarming (Han et al., 2015). Hence, it is possible to isolate PirB\textsuperscript{vp} toxin-producing forms of *V. parahaemolyticus* on standard media used for isolation of bacteria from diseased shrimp or other samples, especially because PirB\textsuperscript{vp} produces a more potent toxin than PirA\textsuperscript{vp} (Lee et al., 2015; Soto-Rodriguez et al., 2015). The identity of the *V. parahaemolyticus* may be confirmed by use of a PCR method to detect lecithin dependent haemolysin gene (Taniguchi et al., 1985) and their probable ability to cause AHPND by PCR methods described in section 4.3.1.2.3. This must be followed by bioassay to confirm ability to cause AHPND.
4.3.1.2.1. Cell culture/artificial media

No methods are available.

4.3.1.2.2. Antibody-based antigen detection methods

None is available to date (February 2015).

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. PCR protocols for detection of AHPND causing bacteria from cultures or infected shrimp

Although the AP1 and AP2 methods are not recommended for detection of VP AHPND, they are included here because they may find utility in studying the environmental prevalence and distribution of bacterial isolates carrying pVA1 plasmid variants that lack the PirA\(_{VP}\) and PirB\(_{VP}\) genes, would not cause AHPND and would give negative results with the methods recommended here for VP AHPND detection.

Methods for detection of isolates of VP AHPND by PCR have been developed. The most successful methods target the unique genes for AHPND toxin Pir\(_{AVP}\) (12.7 kDa) and/or PirB\(_{VP}\) (50.1 kDa) that together cause sloughing of shrimp hepatopancreatic cells. These toxin genes have been found as episomal elements in all AHPND isolates so far sequenced (Gomez-Gil et al., 2014; Kondo et al., 2014; Yang et al., 2014) and GenBank Accession number KM067908.

Two earlier, preliminary PCR detection methods (AP1 and AP2) that targeted the plasmid carrying the toxin genes (Yang et al., 2014) were announced on the internet at the website of the Network of Aquaculture Centres in Asia-Pacific (NACA) in December 2013 (Flegel & Lo, 2014) but were later abandoned because of 3% false positive results from non-AHPND bacteria that carried the plasmid without the toxin genes.

To overcome the problem of false positive results, methods have been developed that target the AHPND toxin genes. The first such method (AP3) was announced in June 2014 and targeted the 12.7 kDa \(\text{pir}_{AVP}\) gene (Sirikharin et al., 2014). It was validated for 100% positive and negative predictive value by testing with 104 AHPND-causing and non-pathogenic bacteria (including other \textit{Vibrio} and non-\textit{Vibrio} species) that had previously been confirmed by bioassay (Kwai et al., 2014; Sirikharin et al., 2015). A subsequent publication using 9 AHPND-causing and 11 non-pathogenic isolates of \textit{V. parahaemolyticus} from Mexico (Soto-Rodriguez et al., 2015) reported that the AP3 method gave the highest positive (90%) and negative (100%) predictive values of five PCR methods tested, including one commercial method.

The AP3 method and four other more recently published methods that target the AHPND \(\text{pir}_{AVP}\) gene (the \(\text{Pir}_{AVP}\)A method and the VpPirA-284) and \(\text{pirB}_{VP}\) (\(\text{Pir}_{BVP}\)B method and the VpPirB-392) are one-step PCR methods of relatively low sensitivity when used for detection of AHPND-causing bacteria at carrier levels or in environmental samples such as sediments and biofilms. For such samples, a preliminary enrichment step is recommended since experience has shown that these PCR methods are not sensitive enough to detect low numbers of bacterial cells at carrier levels and that adaptation to a nested PCR protocol was not successful due to the occurrence of non-specific amplicons.

An additional two-tube nested PCR method called AP4 has been devised and found to give 100% positive predictive value for AHPND-causing bacteria using the same 104 bacterial isolates used to validate AP3 above (announcement at www.enaca.org and manuscript in preparation). This method does not give rise to non-specific amplicons and has a minimum sensitivity for 1 fg of DNA extracted from AHPND-causing bacteria, allowing it to be used directly with tissue and environmental samples without an enrichment step.

4.3.1.2.3.1.1 Enrichment of samples prior to DNA extraction

Preliminary enrichment cultures for detection of AHPND-causing bacteria at carrier levels or in environmental samples may be carried out in any suitable medium (e.g. tryptic-soy broth) or alkaline peptone water containing 2.5% NaCl supplement) incubated for 4 hours at around 30°C with shaking. After this, let any debris settle, remove the cloudy supernatant for centrifugation to pellet the bacteria it contains and discard the supernatant solution. Extract DNA from the bacterial pellet.
4.3.1.2.3.1.2 Agent purification

The causative agent of AHPND may be isolated in pure culture from diseased shrimp, carrier shrimp or environmental samples using standard microbiological media used for isolation of *Vibrio* species from such sources (Lightner, 1996; Tran et al., 2013a; 2013b). Isolation of pure cultures must be followed by PCR analysis and/or bioassays to confirm the ability to cause AHPND.

AP1 (AHPND Primer set 1)

**AP1F:** 5’-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3’

**AP1R:** 5’-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3’

AP1 Amplicon sequence 700 bp (Lee et al., 2015).

AP2 (AHPND Primer set 2)

**AP2F:** 5’-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3’

**AP2R:** 5’-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3’

AP2 Amplicon sequence 700 bp (Lee et al., 2015).

4.3.1.2.3.1.3 DNA extraction

A general DNA extraction method may be used to extract DNA from the stomach or hepatopancreatic tissue of a putatively infected shrimp, from cultures of purified bacterial isolates or from bacterial pellets from enrichment cultures (see above). The amount of template DNA in a 25 µl PCR reaction volume should be in the range of 0.01–1 ng of DNA when extracted from bacterial isolates (i.e. directly from a purified culture) and in the range of 10–100 ng of total DNA when extracted from shrimp tissues or from a bacterial pellet from an enrichment culture.

4.3.1.2.3.1.4 PCR primers for one-step PCR detection of AHPND bacteria

Five one-step PCR methods called AP3, Pir*vpA*, Pir*vpB*, VpPirA and VpPirB have been developed (see above) for detection of VP*AHPND*. The AP3, Pir*vpA* and VpPirA methods target the *pirA* gene while the Pir*vpB* and VpPirB methods target the *pirB* gene. These primers are listed in Table 4.1 together with the size of their expected amplicons.

<table>
<thead>
<tr>
<th>Method name</th>
<th>Primers</th>
<th>Target gene</th>
<th>Expected amplicon size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| **AP3**     | AP3-F: 5’-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3’  
AP3-R: 5’-GTG-GTA-GTA-ATA-GAT-TGT-ACA-GAA-3’ | *pirA* | 333 | Sirikharin et al., 2014  
Sirikharin et al., 2015 |
| **Pir*vpA** | Pir*vpA* F: 5’-ATG-AGT-AAC-AAT-ATA-AAA-CAT-G-3’  
Pir*vpA* R: 5’-TTA-GTG-GTA-ATA-GAT-TGT-ACA-G-3’ | *pirA* | 336 | Lee et al., 2015 |
| **Pir*vpB** | Pir*vpB* F: 5’-GAG-CCA-GAT-ATT-GAA-AAC-ATT-TGG-3’  
Pir*vpB* R: 5’-CCA-GCG-AGG-TAG-TGT-ATA-GTA-3’ | *pirB* | 438 | Lee et al., 2015, |
Han et al., 2015 |
| **VpPirB-392** | VpPirB-392F: 5’-TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG-3’  
VpPirB-392R: 5’-TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG-3’ | *pirB* | 392 | KM067908  
Han et al., 2015 |

*Note that the primer sequences and amplicons for the two methods that target the *pirA* gene differ slightly.*

4.3.1.2.3.1.5 PCR primers for nested PCR for detection of AHPND bacteria

A two-tube, nested PCR method called AP4 has been devised and found to give 100% positive predictive value for AHPND-causing bacteria using the same 104 bacterial isolates used to validate AP3 above (www.enaca.org) (Sritunyalucksana et al., 2015). Nonspecific amplicons do not arise from this method and it has a minimum sensitivity for 1 fg of DNA.
Annex 25 (contd)

extracted from AHPND-causing bacteria, allowing it to be used directly with tissue and environmental samples that may have low levels of AHPND bacteria. The target sequence consists of a chimeric DNA fragment comprising the full PirA\textsuperscript{vp} gene sequence plus the 12 bp linker plus the full succeeding PirB\textsuperscript{vp} gene sequence for a total of 1269 bp. The first-step and second-step PCR primers are listed in Table 4.2 below. The primers were designed from the China (People’s Rep. of) isolate of AHPND bacteria (Yang et al., 2014). The expected amplicons are 1269 bp for the outer primers AP4-F1 and AP4-R1 and 230 bp for the inner primers AP4-F2 and AP4-R2. At high concentrations of target DNA, additional bands for amplicons may occur as the product of residual primer AP4-F1 working with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

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

<table>
<thead>
<tr>
<th>Method name</th>
<th>Primers</th>
<th>Expected amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP4 Step 1</td>
<td>AP4-F1: 5’-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3’ AP4-R1: 5’-ACG-ATT-TCG-ACG-TTC-CCC-AA-3’</td>
<td>1269</td>
<td>Sirikharin et al., 2015</td>
</tr>
<tr>
<td>AP4 Step 2</td>
<td>AP4-F2: 5’-TTG-AGA-ATA-CGG-GAC-GTG-GG-3’ AP4-R2: 5’-GTT-AGT-CAT-GTG-AGC-ACC-TTC-3’</td>
<td>230</td>
<td></td>
</tr>
</tbody>
</table>

Note that the AP4-F1 primer sequence is equal to that of AP3-F.

4.3.1.2.3.1.6 Protocol for the AP3, 1-step PCR method

This protocol follows the method described by Sirikharin et al. 2014. The PCR reaction mixture consists of 10x PCR mix (Invitrogen\textsuperscript{5}) 2.5 µl, 50 mM MgCl\textsubscript{2}, 0.7 µl, 10 mM dNTPs, 0.4 µl, 10 µM AP3-F1, 0.5 µl, 10 µM AP3-R1, 0.5 µl, 0.3 µl of Taq DNA pol (5 units µl\textsuperscript{-1}, Invitrogen) and 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds with a final extension step at 72°C for 5 minutes and hold at 4°C.

4.3.1.2.3.1.7 Protocol for the VpPirA-284 1-step PCR method

Perform PCR with PuReTaq ready-to-go PCR beads (GE Healthcare). This consists of a 3-minute step at 94°C to denature DNA prior to the primers binding and activation of the Taq DNA polymerase, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

Prepare a 25 µl PCR reaction with a PuReTag ready-to-go PCR bead. Each reaction contains 0.2 µM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 2.5 U of Taq DNA polymerase, and 1 µl of extracted DNA.

4.3.1.2.3.1.8 Protocol for the PirB\textsuperscript{vp} 1-step PCR method

PCR consists of an initial preheating stage of 5 minutes at 94°C, followed by 25–30 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 60 seconds extension at 72°C, and a final 10 minutes extension at 72°C. The amplified PCR products are analysed in 2% agarose gels, stained with ethidium bromide, and visualised under ultraviolet transillumination. AHPND positive samples give a positive band at 336 bp and 400 bp with the PirA\textsuperscript{vp} and PirB\textsuperscript{vp} primer sets, respectively. No band is produced by non-AHPND samples (Lee et al., 2015).

4.3.1.2.3.1.9 Protocol for the VpPirB-392 1-step PCR method

The protocol for this method is the same as that for the VpPirA-284 method above

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\textsuperscript{5} Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in thisAquatic Manual.
4.3.1.2.3.1.10 Protocol for the AP4 nested PCR method

This protocol follows the method described by Sritnyalucksana et al., 2015. The first PCR reaction mixture consists of 10× PCR mix (Invitrogen) 2.5 µl, 50 mM MgCl₂, 1.5 µl, 10 mM dNTPs, 0.5 µl, 10 µM AP4-F1, 0.5 µl, 10 µM AP4-R1, 0.5 µl, 0.3 µl of Taq DNA pol (5 units µl⁻¹, Invitrogen) and 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension step at 72°C for 2 minutes and hold at 4°C.

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

4.3.1.2.3.1.11 Controls for all PCR methods

The following controls should be included in all AHPND PCR assays: a) DNA template extracted from a known negative sample, such as specific-pathogen-free shrimp tissues; b) DNA template from a known positive sample, including AHPND-affected shrimp tissue, or DNA from a VP AHPND bacteria culture, or plasmid DNA that contains the target region of the specific set of primers; c) a none-template control, i.e. adding nuclease-free water as the template.

4.3.1.2.3.1.12 Analysis of PCR products by agarose gel electrophoresis

After PCR, load 5–10 µl of the PCR reaction mix onto a 1.5% agarose gel (containing 0.5 µg ml⁻¹ ethidium bromide). Look for the expected amplicons appropriate for the PCR method used (Tables 4.1 and 4.2).

4.3.2. Serological methods

Not applicable.

5. Rating of tests against purpose of use

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

Table 5.1. Methods for targeted surveillance and diagnosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PL</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>d</td>
<td>d</td>
<td>b</td>
</tr>
<tr>
<td>Bioassay</td>
<td>d</td>
<td>d</td>
<td>b</td>
</tr>
<tr>
<td>Direct LM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>Histopathology</td>
<td>d</td>
<td>c</td>
<td>a</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>PCR</td>
<td>d</td>
<td>b</td>
<td>a</td>
</tr>
<tr>
<td>Sequence</td>
<td>d</td>
<td>d</td>
<td>a</td>
</tr>
</tbody>
</table>

PL = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.
Annex 25 (contd)

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

Two years of freedom from AHPND is adequate to declare freedom from the acute hepatopancreatic necrosis disease (VP\textsubscript{AHPND}) (Leaño & Mohan, 2013; NACA, 2012; 2014; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013b).

7. **Corroborative diagnostic criteria**

7.1. **Definition of suspect case**
AHPND shall be suspected if at least one of the following criteria is met:

- Histopathology indicative of AHPND
- Detection of VP\textsubscript{AHPND}
- Mortality associated with clinical signs of AHPND.

7.2. **Definition of confirmed case**
AHPND is considered to be confirmed if the following criteria are met:

- Detection of VP\textsubscript{AHPND}
- Histopathology indicative of AHPND
- Mortality associated with clinical signs of AHPND
- Positive results by bioassay.

8. **References**


Annex 25 (contd)


* *

* *
The OIE ad hoc Group on Notification of Animal Diseases and Pathogenic Agents met at the OIE Headquarters from 6 to 8 January 2015.

The members of the Group and other participants are listed in Appendix I. The meeting was chaired by Dr Toni Tana and Dr Allan Sheridan acted as rapporteur.

Dr Alex Thiermann, Advisor of the Director General and President of the Terrestrial Animal Health Code Commission, welcomed the participants on behalf of the Director General, Dr Bernard Vallat, and thanked them for having accepted the OIE’s invitation. He reminded the participants of the importance of this Group in the OIE’s work, gathering together many experts coming from different regions. He reiterated that this Group has a unique and critical mandate. The aim is to review disease listing criteria bearing in mind all diseases, not just specific ones considered important at particular times. He also reminded the Group that a listed disease was not more important than other diseases, but the criteria define it as a disease, based on its epidemiologic characteristics, that requires rapid dissemination of information to facilitate control efforts by the veterinary services. The Group was also asked to consider providing more clarity and discipline on how to report on emerging diseases and when reporting ceases to be necessary. The conclusions of this Group should also help the OIE to find a way to encourage Member Countries to improve the level and quality of disease notification and events reporting.

Dr Paula Cáceres, Head of World Animal Health Information and Analysis Department, presented the objectives of the meeting: to examine and evaluate the disease listing criteria in both the Terrestrial and Aquatic Codes for inclusion of diseases, infections and infestations in the OIE Lists. The Group was requested to assess the need for further definition of disease notification obligations of Member Countries for emerging diseases. The Group was also asked to assist the OIE considering deleting the reporting of non OIE-listed diseases in the annual report and, based on the amended Chapter 1.1 adopted by the OIE World Assembly of May 2014, replacing it by the information on emerging diseases declared endemic. Dr Cáceres also spoke with the Group on day two and clarified the description of the role and responsibilities of expert members that was presented by Dr Thiermann. The aim of an expert member of an ad hoc Group appointed by the OIE is to work to further the OIE’s mission, bringing their unique regional experiences and awareness to the task at hand without advocating a position at odds with that of the OIE.

The Group reviewed and agreed to its terms of reference; they are listed in Appendix II.
Annex 26 (contd)

The Group endorsed the proposed agenda presented in Appendix III.

Dr Vallat, the Director General, joined the meeting on day two to support the activities of the Group. He reminded the Group that the OIE organises such Groups periodically to review existing standards and improve them on the basis on new scientific information. He highlighted that one of the main missions of the OIE is to promote the transparency of the animal health situation worldwide using appropriate standards and reporting mechanisms. He explained that the system for disease notification was built and modernised over years, with increasing requirements for data collection and reporting. He also stated that the OIE’s capacity building work with Member Countries through the network of focal points aims to facilitate disease reporting and improve the quality of information. He emphasised the importance of recent changes in the definition and obligations of emerging diseases due to increasing numbers and importance of these diseases.

He reminded the Group that one of the main objectives should be to simplify the criteria for the listing of diseases for the benefit of Members. Dr Vallat emphasised that the OIE continued to work on modernising the WAHIS reporting system aiming to improve the mapping system, official status displaying, data mining and analysis of the information collected, as well as to include the information on genotype collected from the network of reference laboratories and the information related to antimicrobial resistance. He added also that three departments, namely the Scientific and Technical Department, the International Trade Department and the World Animal Health Information and Analysis Department, worked on the harmonisation of definitions between the Terrestrial and Aquatic Codes and WAHIS guidelines.

The desire is to have this report discussed by three Specialist Commissions concerned during their February 2015 meetings. Observers from the three Commissions whose work is directly linked to this ad hoc Group are listed in Appendix I as other participants who attended this meeting.

The Group appointed a Chair and rapporteur and briefly discussed their role and responsibilities. The Group agreed that it was critically important to remember that the criteria for listing of diseases could not be looked at in isolation from each other or other elements of Chapters 1.1 and 1.2 in each of the OIE Terrestrial Animal Health Code (Terrestrial Code) and Aquatic Animal Health Code (Aquatic Code). In addition the Group noted that the purpose of listing and reporting on diseases was subordinate to the OIE’s overall mandate for animal health.

Following the meeting the proposed changes to the chapters were compiled and are presented in this report as the following Appendices:

- **Terrestrial Code, Chapter 1.1** Appendix IV
- **Aquatic Code, Chapter 1.1** Appendix V
- **Terrestrial Code, Chapter 1.2** Appendix VI
- **Aquatic Code, Chapter 1.2** Appendix VII (showing proposed changes)
- **Aquatic Code, Chapter 1.2** Appendix VIII (changes proposed)

Chapter 1.3 of the Aquatic Code is equivalent to Article 1.2.3. of the Terrestrial Code. It consists of the Aquatic Code’s listed diseases.

1. **Examine and evaluate the disease listing criteria in the Terrestrial and Aquatic Codes**

   **Chapter 1.2**

   The Group discussed the need to clearly define for Members the purposes for listing diseases contained in Chapter 1.2. of the Terrestrial Code and Chapter 1.3 of the Aquatic Code.
The Group reviewed each article in the chapters related to the listing criteria for including terrestrial and aquatic animal diseases. The Group noted that there were some differences between the Chapters in the two Codes; however, and as per earlier requests by some Member Countries, the Group looked for ways to better harmonise the criteria for listing of terrestrial and aquatic diseases where it was feasible.

The Group noted that the titles of the two chapters and the first line of the introduction were different but conveyed the same information. For that reason the Group requested the Code Commissions to assess whether these aspects of the Chapters could be harmonised.

The Group also discussed the need for specificity when an ad hoc Group is appointed to consider whether a disease, infection or infestation is proposed for listing under these criteria. The Group advised the Code Commission representative that the criteria can be applied to either a disease or to a specified strain of a disease. The Group agreed it was essential that ad hoc groups are provided with clarity as to whether they are to consider a disease or a specified strain of a disease in their terms of reference.

Terrestrial Code: Article 1.2.1

The Group agreed that the aim of listing was to facilitate notification by and to the Member Countries to allow them to take appropriate and (where possible) co-ordinated action to prevent the spread of diseases as far as possible through control exercised by the veterinary authorities over animals and animal products. The Group agreed that this should be clear in the first paragraph of this article and recommended also insertion of the term ‘timely’ to highlight the importance of providing information soon enough so other Members can take effective action.

The Group agreed that the role of the Terrestrial Code in providing standards for disease control and safe trade in animals and animal products was important and should be mentioned. This had already been done in the Aquatic Code and insertion of that form of wording in the second paragraph of the introduction was recommended by the Group.

The Group agreed that details on the mechanisms for notification are provided in Chapter 1.1 and there was no benefit gained by restating them.

The Group further agreed that it would assist Members to have reference in this section to the principles for selection of an appropriate diagnostic test. This was discussed when considering point C. 8 of Article 1.2.2. of the Aquatic Code.

Aquatic Code: Article 1.2.1

The same rationale for amending the article in the Terrestrial Code was seen as appropriate in this instance.

Terrestrial Code: Article 1.2.2

Point 1. The Group was advised that two aspects of this article elicited Member comments – ‘international spread’ and ‘proven’. After discussion the Group agreed that the term ‘international spread’ was clear and in need of no further elaboration. The Group also agreed that the term ‘proven’ had a clear scientific meaning in the context of the Terrestrial Code and that it was not to be considered as a legal term.

Point 2. The Group was advised by the Code Commission and agreed that the term ‘demonstrated freedom’ would incorporate historical disease freedom as per the provisions of Article 1.4.6. of the Terrestrial Code. The Code Commission further advised and the Group agreed that ‘impending freedom’ would be applicable to countries with control programmes with eradication as the end point in an advanced stage. In addition the Group was advised by the Code Commission and agreed that the ‘negligible risk’ categorisation of certain countries in respect of BSE would be equivalent to ‘freedom’ for this Article. The Group recommended to simplify the wording related to surveillance provisions in the Terrestrial Code, as has been done in the Aquatic Code.
Annex 26 (contd)

Point 3. a. The Group agreed to maintain this criterion as written.

Point 3.b. The Group was advised of comments by Members indicating a lack of consistent understanding of the terms ‘significant morbidity and mortality’. Some members wished for quantification of incidence and some requested a specific definition for the term ‘morbidity’ as it applies to the Terrestrial Code. The Group discussed whether a definition of the term or further elaboration of its meaning within the article was the best way to improve clarity. After extensive discussion and review of a draft definition the Group agreed that simplification of the higher level statement and specification of the criteria that should be used within the article would be more useful. The use of the term ‘significant impact on health’ is now proposed to be accompanied by a mechanism by which it can be evaluated.

The Group also discussed the significance of positive serological results in relation to the listing criteria. A positive serological result, or titre, in an animal is evidence of prior exposure to an agent (micro-organism, protein, etc.) leading to an immunological response. However, exposure to an infectious agent may, or may not, result in illness in an individual. The Group agreed that positive serological results in the absence of clinical signs are not considered to be signs of disease or morbidity and are not to be considered as evidence of ‘a significant impact on health’.

During these discussions the Group also considered whether additional criteria were necessary. One suggestion was to allow for relisting of a disease that has been delisted but for which control measures remain in place in a number of countries. The Group discussed this proposal and did not support the suggestion. Countries are allowed to implement animal health-based measures under WTO rules for non-listed diseases if they provide a risk assessment and the measures are the least-trade-restrictive that are necessary to protect that country’s status. Delisting decisions are agreed at General Session when a disease does not meet the listing criteria. However, delisted diseases could be proposed for relisting if their behaviour changed in such a way that they subsequently met the listing criteria, so the Group did not see any benefit to include this proposal.

The Code Commission advised that use of the term ‘zone’ in this article would include containment zones established to control disease.

Point 3.c. The Group aligned the wording of this point with that of point 3.b. In addition the Group agreed to change the term ‘wild animal populations’ to ‘wildlife’. ‘Wildlife’ is defined in the Terrestrial Code and includes other wildlife categories of economic value previously excluded, in particular captive wild animals. The Group further considered this aspect in relation to the equivalent requirement in the Aquatic Code (point A. 2.of Article 1.2.2) and agreed that addition of the term ‘ecological threats’ was of significant value here as well.

Point 4. The Group agreed to maintain this criterion as written.

Re-ordering of the Article 1.2.2: The Group considered it easier for Members to apply the criteria if the only ‘or’ options were at the end of the section. Reordering to suit the suggestion was performed by moving the previous point 4 of Article 1.2.2. to point 3 of Article 1.2.2.

Aquatic Code: Article 1.2.2

The Group reviewed the reason for having explanatory notes in light of recent proposed changes to the Aquatic Code, and agreed to incorporate relevant information into the assessment criteria. In addition it was noted that removal of the explanatory notes means that the table format is no longer required and the Group recommends to the Code Commission alignment of the format of this Article with the corresponding Article 1.2.2. of the Terrestrial Code.

The Group further agreed that the second paragraph of Article 1.2.2. that describes in detail how to apply the criteria was unnecessary and that alignment of the first sentence of the article with that in the equivalent section of the Terrestrial Code was appropriate.
Annex 26 (contd)

No. A. 1. This article corresponds to point 3.b of Article 1.2.2. of the Terrestrial Code. The Group agreed that the new article in the Terrestrial Code was broader and should be proposed for use in this article of the Aquatic Code. The Group also agreed that the explanatory note referring to morbidity was liable to create confusion and was no longer required.

No. A. 2. This article corresponds to point 3. c. of Article 1.2.2. of the Terrestrial Code. The Group aligned the wording of this clause with that in point A.1 of Article 1.2.2. The Group decided following review of the explanatory notes that consideration of ecological aspects of the disease impact was of significant value given the broad mandate of the OIE. As the term ‘ecological’ also incorporates consideration of environmental factors the Group did not feel it was appropriate to add ‘environmental’ as was previously in the explanatory note. The explanatory note was no longer seen as necessary given these changes to the article.

No. A. 3. This article corresponds to point 3. a of Article 1.2.2. of the Terrestrial Code. The Group noted that this article did not incorporate the concept of severity of consequences, which the Group agreed was important. The Group reviewed use of the corresponding article in the Terrestrial Code and agreed to recommend that the same wording be used in the Aquatic Code.

No. B. 4. This article corresponds to no article in the Terrestrial Code. The Group agreed this article was no longer necessary as the glossary definition of ‘disease’ in the Aquatic Code specifies an infectious aetiology.

No. B. 5. This article corresponds to no article in the Terrestrial Code. The Group agreed this Article was not appropriate as a disease with a suspected infectious aetiology would be reported as an emerging disease (as defined in the glossary to the Aquatic Code).

No. B. 6. This article corresponds to point 1 of Article 1.2.2. of the Terrestrial Code. The Group noted when discussing this point that, in light of removal by the Code Commission of the article on emerging diseases from this chapter, the current wording was no longer appropriate. The Group agreed that use of the same wording as in the Terrestrial Code would be appropriate and that the information in the guidance note was not needed.

No. B. 7. This article corresponds to point 2 of Article 1.2.2. of the Terrestrial Code. The use of the term ‘zone’ in this context was explained as covering bodies of water within a country as well as those that may be shared by a number of countries. The Group agreed that the competent authority of at least one country would need to propose ‘freedom’ as the term ‘several countries’ is undefined and if a single country is free then this status is worth protecting. The Group also discussed that the minimum requirement in the Terrestrial Code was the important feature. If one country could be free, others could take action to gain that same status and may be encouraged to do so. For these reasons the Group agreed to harmonise the text with that used in point 2 of Article 1.2.2. of the Terrestrial Code and remove the explanatory text.

No. C. 8. This article corresponds to point 4 of Article 1.2.2. of the Terrestrial Code. The Group discussed the terms ‘repeatable and robust’ in relation to diagnostic testing. The single term ‘reliable’ is often used in relation to test performance and the Group agreed that this term could be used here together with some information on the criteria that can be applied when selecting a test for use. The Group reviewed the explanatory notes for this article and agreed that the appropriate chapter of the Aquatic Manual, Chapter 1.1.2, should provide that information for application by Members. It was further agreed that this would be best placed in the Introduction, under Article 1.2.1. Following these changes it was seen that the wording of the equivalent article in the Terrestrial Code, that includes specification of the need for case definition in the explanatory note, would be appropriate for the Aquatic Code as well. The explanatory note was then removed.

Re-ordering of points of the Article 1.2.2 of the Aquatic Code: For the same reason that reordering of these points was performed in the Terrestrial Code, and for harmonisation, reordering of the revised Article 1.2.2 was performed, as shown in Appendix VII (in tracked changes) and Appendix VIII (as a clean version with all suggested changes to that chapter accepted).
Annex 26 (contd)

**Terrestrial Code: Article 1.2.3**

While no changes to the text were made, the Group agreed that it was worthwhile asking the Code Commission to consider splitting Article 1.2.3. of the Terrestrial Code, which includes all diseases currently listed in the Terrestrial Code, into a separate chapter. This has been done in the Aquatic Code, where Chapter 1.3 is the disease list. The Group considered there could be advantages in that approach as a change suggested to the Terrestrial Code’s disease list would then purely affect the list and not open the criteria for review without reason.

2. **Assessment of the need for further definition of disease notification obligations of Member Countries for emerging diseases**

The Group sought clarification of what the OIE was seeking by raising this agenda item. Dr Caceres presented the current situation related to the notification of emerging diseases and a flow chart describing the World Animal Health Information System (WAHIS). The Group was informed that, once an emerging disease has been declared as endemic or stable, a country is no longer required to provide the OIE with further information concerning the disease. Is there a need to change point 1.1.4 in the Terrestrial Code to facilitate on-going reporting of information on these diseases?

The Group discussed the provisions of Article 1.1.4 of the Terrestrial Code regarding notification of emerging diseases. The Group agreed that the phrase in point 2 of Article 1.1.4. ‘as described under point 1’ was unnecessary and poorly referenced. The Group proposed it be deleted.

A suggestion was made that point 2 of Article 1.1.4. might benefit from having a time period specified during which countries would be required to continue submitting reports. That would be cut short if listing was proposed or the disease became sufficiently stable. The Group discussed this point in relation to whether a net benefit would be gained from the additional reporting that may occur. The Group agreed that the existing criteria for reporting ensure that the situation on a country is well described for other Members. In addition, the existing criteria allow for a return to reporting of the disease under appropriate circumstances so there is no net benefit by mandating a time period. The discussion did include however that there needs to be reliability of reporting up to the time a disease was sufficiently stable, or eradicated. The Group agreed to incorporate the phrase ‘sufficient time to have reasonable certainty that’ in the first sentence of point 2 of Article 1.1.4. for that reason and amend the punctuation of sub points a., b. and c. to clarify that reporting should continue until either the disease has been eradicated or becomes sufficiently stable within the country, or until it has been assessed for listing. The Group agreed that determining whether a disease was emerging and whether it had met either of the first two of those criteria for ceasing reporting was the responsibility of the Member country’s Delegate.

The Group considered whether the WAHIS/WAHID system facilitates reporting in line with the Terrestrial Code requirements of 1.1.3 and 1.1.6. After discussion it was agreed the existing system does not support countries to supply the data mandated in point 4 of Article 1.1.3. In addition, the level of detail requested in WAHIS is not reflected in the mandatory reporting requirements of Article 1.1.3.

The Group recommends that the OIE consider appointing an ad hoc Group to refine WAHIS and Article 1.1.3. of the Terrestrial Code in order to define clearly for Members the level of compulsory data that is expected in reports. The Group agreed that this ad hoc Group should also consider changes to the WAHIS system to facilitate the agreed reporting in an appropriate manner. This should include facilitation of reporting of non-listed diseases (including those previously delisted) under Article 1.1.6 that Members consider would be of use to other Members and should also be consistent with Member’s obligations under Article 1.1.4.
3. **In case of significant proposed changes in criteria, analyse and comment on the results of recent ad hoc Groups on some emerging diseases (e.g. PED, MERS, Schmallenberg)**

The Group analysed the newly proposed criteria for the listing of diseases and agreed that the changes it recommended were to clarify already existing criteria. As there were no major modifications proposed the Group was not requested, under its Terms of Reference, to review the reports on emerging diseases such as PED, MERS and Schmallenberg.

4. **Analyse new emerging diseases such as Ebola and the consequences for disease information reporting**

Dr Marija Popovic, chargée de mission at the World Animal Health Information and Analysis Department, gave a brief background and on the evolution on the voluntary reporting of non OIE listed wildlife diseases using the spreadsheet questionnaire and lately by the WAHIS –Wild platform. She stated that Ebola virus disease is classified under the disease “infection with filovirus” and that this include Marburg virus. The reporting was developed to provide Member Countries with an early warning system as the diseases involved had an impact on livestock health, human health, wildlife conservation, biodiversity and environmental integrity.

Dr Popovic advised that no quantitative data has been received so far from any Member Country on Ebola. The OIE followed up unofficial information in the last quarter of 2014 regarding domestic pigs affected by Ebola disease but the respective Member Country advised the information was incorrect.

The Group critically assessed the consequences of reporting on new emerging diseases by Members. Regarding Ebola, as specified by the Terms of Reference, the Group agreed it was not competent to assess Ebola disease (infection with filovirus). The Group then considered whether listing would be of value to Members if Ebola could be seen to meet the amended listing criteria. The Group acknowledged that Ebola is an important disease due to its potential impact on human health. However, it needs to be considered in the framework of Article 1.2.1 with the purpose of listing in mind. If listing and consequent mandatory reporting of information on a given disease facilitates the taking of appropriate action by Members to prevent transboundary spread of that disease then listing is consistent with the mission of the OIE. The Group agreed that, for Ebola, it was difficult to see how listing would achieve that outcome. The Group discussed that this situation illustrated how important it was, not to apply the listing criteria to a disease without considering the broader context of the OIE’s mission in terms of official notification, particularly as captured in the revised Article 1.2.1. Members with Ebola can nevertheless provide reports as it could be considered an emerging disease or voluntary reporting may be considered under Article 1.1.6 as an important animal health event. The Group agreed in relation to Ebola that Members should be encouraged to report.

5. **Consider the deletion of reporting non-OIE-listed diseases in the annual report (Article 1.1.3 point 4) and consider replacing it by the information on emerging diseases declared endemic (Article 1.1.4)**

The Group reviewed point 4 of Article 1.1.3. of the Terrestrial Code regarding the notification of diseases, infections and infestations, and provision of epidemiological information particularly related to the annual report.

Dr Cáceres briefly informed the Group about the current content of the annual report.

The Group discussed the content of the annual report on the non OIE-Listed diseases. The Group agreed that the information requested in the annual report on non-listed diseases is not supported by point 4 of Article 1.1.3. The discussion included consideration of how useful this information is for Members on the basis of advice from the OIE advisers present that the reports are rarely if ever interrogated by Members. The Group agreed that it was no longer appropriate to include these diseases in WAHIS and suggested that the WAHIS form be changed to reflect this.
Annex 26 (contd)

The Group agreed that the WAHIS system for gathering disease information should be flexible, facilitating the input of information on the reporting form that Members consider may be of use for other Members, and reiterated that this was not the case at present. The Group agreed that allowing provision of free text in an ‘other comments’ box could also be considered to encourage the Member Countries to provide information as per Article 1.1.6. The Group considered a suggestion that OIE should encourage Members to provide data on emerging diseases and that this be done by providing a specific area on the OIE website where emerging diseases are listed. While not covered by the Terms of Reference, the Group discussed this point and considered that its usefulness could be assessed by the suggested ad hoc Group for the WAHIS system if appointed by OIE.

The Group agreed that the official data collection should be focused on the OIE-Listed and emerging diseases. Previous recommendations by the group on appointing an ad hoc Group to review the WAHIS and WAHID systems were reiterated.

6. Other business

The Group discussed the definitions of emerging disease in the Terrestrial and Aquatic Codes. The Group agreed that, while it would be better for Members if the definitions were consistent, it did not have sufficient background information to consider whether there were necessary reasons for the differences in the definitions. The Group suggested both Code Commissions to work together to assess whether and how it would be possible to more closely harmonise the definitions of ‘emerging disease’ in both Codes.

7. Finalisation and adoption of the draft report

The Group finalised and adopted the draft report

…/Appendices
MEETING OF THE

AD HOC GROUP ON NOTIFICATION OF ANIMAL DISEASES AND PATHOGENIC AGENTS

Paris, 6–8 January 2015

List of participants

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Annex 26 (contd)

Appendix I (contd)

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<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
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TERMS OF REFERENCE
OF
THE AD HOC GROUP ON NOTIFICATION OF ANIMAL DISEASES AND PATHOGENIC AGENTS

Paris, 6–8 January 2015

The ad hoc Group is kindly requested to:

1) On the basis of Chapter 1.2 of the OIE Terrestrial Animal Health Code and Aquatic Animal Health Code, assist the OIE in addressing the following points:

   a) Examine and evaluate the disease listing criteria for inclusion of diseases, infections and infestations in the OIE List.

   b) Assess the need for further definition of disease notification obligations of Member Countries for emerging diseases and modification of the reporting obligations when an emerging disease becomes endemic.

   c) In case of significant proposed changes in criteria, analyse and comment on the results of recent ad hoc Groups on some emerging diseases (e.g. PED, MERS, Schmallenberg).

   d) Analyse new emerging diseases such as Ebola and the consequences for disease information reporting.

2) On the basis of the adopted amended Chapter 1.1 during the OIE World Assembly of May 2014, assist the OIE in addressing the following point:

   a) Consider the deletion of reporting non-OIE-listed diseases in the annual report (Article 1.1.3 point 4) and consider replacing it by the information on emerging diseases declared endemic (Article 1.1.4).

3) Any other business
MEETING OF THE
AD HOC GROUP ON NOTIFICATION OF ANIMAL DISEASES AND PATHOGENIC AGENTS
Paris, 6–8 January 2015

Agenda

1. Opening

2. Appointment of chairperson and rapporteur

3. Terms of reference for the ad hoc Group meeting

  3.1. Examine and evaluate the disease listing criteria for inclusion of diseases, infections and infestations in the OIE List.

  3.2. Assess the need for further definition of disease notification obligations of Member Countries for emerging diseases and modification of the reporting obligations when an emerging disease becomes endemic.

  3.3. In case of significant proposed changes in criteria, analyse and comment on the results of recent ad hoc Groups on some emerging diseases (e.g. PED, MERS, Schmallenberg).

  3.4. Analyse new emerging diseases such as Ebola and the consequences for disease information reporting.

  3.5. Consider the deletion of reporting non-OIE-listed diseases in the annual report (Article 1.1.3 point 4) and consider replacing it by the information on emerging diseases declared endemic (Article 1.1.4).

4. Any other business

5. Finalisation and adoption of the draft report
CHAPTER 1.1.

NOTIFICATION OF DISEASES, INFECTIONS AND INFESTATIONS, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

Article 1.1.1.

For the purposes of the Terrestrial Code and in terms of Articles 5, 9 and 10 of the OIE Organic Statutes, Member Countries shall recognise the right of the Headquarters to communicate directly with the Veterinary Authority of its territory or territories.

All notifications and all information sent by the OIE to the Veterinary Authority shall be regarded as having been sent to the country concerned and all notifications and all information sent to the OIE by the Veterinary Authority shall be regarded as having been sent by the country concerned.

Article 1.1.2.

1) Member Countries shall make available to other Member Countries, through the OIE, whatever information is necessary to minimise the spread of important animal diseases, and their aetiological agents, and to assist in achieving better worldwide control of these diseases.

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

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

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

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

Article 1.1.3.

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

1) in accordance with relevant provisions in the disease-specific chapters, notification through the World Animal Health Information System (WAHIS) or by fax or e-mail, within 24 hours, of any of the following events:
   a) first occurrence of a listed disease, infection or infestation in a country, a zone or a compartment;
   b) re-occurrence of a listed disease, infection or infestation in a country, a zone or a compartment following the final report that declared the outbreak ended;
   c) first occurrence of a new strain of a pathogen of a listed disease, infection or infestation in a country, a zone or a compartment;
Annex 26 (contd)

Appendix IV (contd)

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

e) occurrence of a listed disease, infection or infestation in an unusual host species;

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

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

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

Article 1.1.4.

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

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

2) periodic reports subsequent to a notification of an emerging disease, as described under point 1. These should continue until for sufficient time to have reasonable certainty that:

   a) the disease, infection or infestation has been eradicated; or

   b) the situation becomes sufficiently stable;

   or until

   c) sufficient scientific information is available to determine whether it meets the criteria for listing.

Article 1.1.5.

1) The Veterinary Authority of a country in which an infected zone was located shall inform the Headquarters when this zone is free from the disease, infection or infestation.

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

3) A Member Country may be considered to regain freedom from a specific disease, infection or infestation when all relevant conditions given in the Terrestrial Code have been fulfilled.

4) The Veterinary Authority of a Member Country which sets up one or several free zones 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 on a map of the territory of the Member Country.
Article 1.1.6.

1) Although Member Countries are only required to notify listed diseases, infections and infestation and emerging diseases, they are encouraged to inform the OIE of other important animal health events.

2) The Headquarters shall communicate by e-mail or World Animal Health Information Database (WAHID) to Veterinary Authorities all notifications received as provided in Articles 1.1.2. to 1.1.5. and other relevant information.

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CHAPTER 1.1.

NOTIFICATION OF DISEASES, AND PROVISION OF EPIDEMIOLOGICAL INFORMATION

Article 1.1.1.

For the purposes of the Aquatic Code and in terms of Articles 5, 9 and 10 of the OIE Organic Statutes, every Member Country of the OIE shall recognise the right of the Headquarters to communicate directly with the Competent Authority of its territory or territories.

All notifications and all information sent by the OIE to the Competent Authority shall be regarded as having been sent to the country concerned and all notifications and all information sent to the OIE by the Competent Authority shall be regarded as having been sent by the country concerned.

Article 1.1.2.

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

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

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

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

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

Article 1.1.3.

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

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

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

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

c) first occurrence of a new strain of a pathogenic agent of a listed disease in a country, a zone or a compartment;
Annex 26 (contd)

Appendix V (contd)

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

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

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

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

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

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

Article 1.1.4.

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

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

2) periodic reports subsequent to a notification of an emerging disease, as described under point 1. These should continue until for sufficient time to have reasonable certainty that:

   a) the disease has been eradicated; or

   b) the situation becomes sufficiently stable;

   or until

   c) sufficient scientific information is available to determine whether it meets the criteria for listing.

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

2) The Headquarters shall communicate by e-mail or World Animal Health Information Database (WAHID) to Competent Authorities all notifications received as provided in Articles 1.1.2. to 1.1.5. and other relevant information.

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CHAPTER 1.2.

CRITERIA FOR THE INCLUSION OF DISEASES, INFECTIONS AND INFESTATIONS IN THE OIE LIST

Article 1.2.1.

Introduction

The aim of this chapter is to describe the criteria for the inclusion of diseases, infections and infestations on the OIE list.

The objective of listing is to support Member Countries by providing information needed to take appropriate actions to prevent the transboundary spread of important animal diseases, including zoonoses. This is achieved by transparent, timely and consistent notification reporting.

Each listed disease normally has a corresponding chapter that assists Member Countries in harmonising disease detection, prevention and control, and provides standards for safe international trade in animals and their products.

Requirements for notification are detailed in Chapter 1.1, and notifications are to be made through WAHIS or, if not possible, by fax or e-mail as described in Article 1.1.3.

Principles for selection of diagnostic tests are described in Chapter 1.1.5 of the Terrestrial Manual.

Article 1.2.2.

The criteria for the inclusion of a disease, infection or infestation in the OIE list are as follows:

1) International spread of the agent (via live animals or their products, vectors or fomites) has been proven.

AND

2) At least one country has demonstrated freedom or impending freedom from the disease, infection or infestation in populations of susceptible animals, based on the animal health surveillance provisions of the Terrestrial Code, in particular those contained in Chapter 1.4.

AND

3) A reliable means of detection and diagnosis exists and a precise case definition is available to clearly identify cases and allow them to be distinguished from other diseases, infections and infestations.

AND

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

OR

b) The disease has been shown to cause a significant impact on the health of morbidity or mortality of domestic animals at the level of a country or a zone, taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality.
Annex 26 (contd)

Appendix VI (contd)

OR

c) The disease has been shown to, or scientific evidence indicates that it would, cause a significant impact on the health of morbidity or mortality in wild wildlife animal populations taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats.

AND

4. A reliable means of detection and diagnosis exists and a precise case definition is available to clearly identify cases and allow them to be distinguished from other diseases, infections and infestations.

Article 1.2.3.

The following diseases, infections and infestations are included in the OIE list.

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

1) The following are included within the category of multiple species diseases, infections and infestations:
   - Anthrax
   - Bluetongue
   - Brucellosis (*Brucella abortus*)
   - Brucellosis (*Brucella melitensis*)
   - Brucellosis (*Brucella suis*)
   - Crimean Congo haemorrhagic fever
   - Epizootic haemorrhagic disease
   - Equine encephalomyelitis (Eastern)
   - Foot and mouth disease
   - Heartwater
   - Infection with Aujeszky's disease virus
   - Infection with *Echinococcus granulosus*
   - Infection with *Echinococcus multilocularis*
   - Infection with rabies virus
   - Infection with Rift Valley fever virus
   - Infection with rinderpest virus
   - Infection with *Trichinella* spp.
   - Japanese encephalitis
   - New World screwworm (*Cochliomyia hominivorax*)
   - Old World screwworm (*Chrysomya bezziana*)
   - Paratuberculosis
   - Q fever
   - Surra (*Trypanosoma evansi*)
   - Tularemia
   - West Nile fever.
2) The following are included within the category of cattle diseases and infections:
   - Bovine anaplasmosis
   - Bovine babesiosis
   - Bovine genital campylobacteriosis
   - Bovine spongiform encephalopathy
   - Bovine tuberculosis
   - Bovine viral diarrhoea
   - Enzootic bovine leukosis
   - Haemorrhagic septicaemia
   - Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis
   - Infection with *Mycoplasma mycoides* subsp. *mycoides* SC (Contagious bovine pleuropneumonia)
   - Lumpy skin disease
   - Theileriosis
   - Trichomonosis
   - Trypanosomosis (tsetse-transmitted).

3) The following are included within the category of sheep and goat diseases and infections:
   - Caprine arthritis/encephalitis
   - Contagious agalactia
   - Contagious caprine pleuropneumonia
   - Infection with *Chlamydophila abortus* (Enzootic abortion of ewes, ovine chlamydiosis)
   - Infection with peste des petits ruminants virus
   - Maedi–visna
   - Nairobi sheep disease
   - Ovine epididymitis (*Brucella ovis*)
   - Salmonellosis (*S. abortus ovis*)
   - Scrapie
   - Sheep pox and goat pox.

4) The following are included within the category of equine diseases and infections:
   - Contagious equine metritis
   - Dourine
   - Equine encephalomyelitis (Western)
   - Equine infectious anaemia
   - Equine influenza
   - Equine piroplasmosis
   - Glanders
   - Infection with African horse sickness virus
   - Infection with equid herpesvirus-1 (EHV-1)
   - Infection with equine arteritis virus
   - Venezuelan equine encephalomyelitis.
Annex 26 (contd)

Appendix VI (contd)

5) The following are included within the category of swine diseases and infections:
   - African swine fever
   - Infection with classical swine fever virus
   - Nipah virus encephalitis
   - Porcine cysticercosis
   - Porcine reproductive and respiratory syndrome
   - Transmissible gastroenteritis.

6) The following are included within the category of avian diseases and infections:
   - Avian chlamydiosis
   - Avian infectious bronchitis
   - Avian infectious laryngotracheitis
   - Avian mycoplasmosis (*Mycoplasma gallisepticum*)
   - Avian mycoplasmosis (*Mycoplasma synoviae*)
   - Duck virus hepatitis
   - Fowl typhoid
   - Infection with avian influenza viruses
   - Infection with influenza A viruses of high pathogenicity in birds other than poultry including wild birds
   - Infection with Newcastle disease virus
   - Infectious bursal disease (Gumboro disease)
   - Pullorum disease
   - Turkey rhinotracheitis.

7) The following are included within the category of lagomorph diseases and infections:
   - Myxomatosis
   - Rabbit haemorrhagic disease.

8) The following are included within the category of bee diseases, infections and infestations:
   - Infection of honey bees with *Melissococcus plutonius* (European foulbrood)
   - Infection of honey bees with *Paenibacillus larvae* (American foulbrood)
   - Infestation of honey bees with *Acarapis woodi*
   - Infestation of honey bees with *Tropilaelaps* spp.
   - Infestation of honey bees with *Varroa* spp. (Varroosis)
   - Infestation with *Aethina tumida* (Small hive beetle).

9) The following are included within the category of other diseases and infections:
   - Camelpox
   - Leishmaniosis.

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AQUATIC CODE

CHAPTER 1.2.

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

Introduction

This chapter describes the criteria for listing diseases in Chapter 1.3.

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

For the diseases listed in accordance with Article 1.2.2., the corresponding disease-specific chapters in the Aquatic Code assist Member Countries in the harmonisation of disease detection, prevention and control and provide standards for safe international trade in aquatic animals and their products.

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

Principles for selection of diagnostic tests are described in Chapter 1.1.2 of the Aquatic Manual.

Article 1.2.2.

The criteria for the inclusion of a listing an aquatic animal disease in the OIE list are as follows:

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

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<td>4 OR 5</td>
<td>The disease has been shown to cause a significant production losses at a national or multinational (zonal or regional) level impact on the health of aquatic animals at the level of country or a zone taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality.</td>
<td>There is a general pattern that the disease will lead to losses in susceptible species, and that morbidity or mortality are related primarily to the infectious agent and not management or environmental factors. (Morbidity includes, for example, loss of production due to spawning failure.) The direct economic impact of the disease is linked to its morbidity, mortality and effect on product quality.</td>
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2 OR 5 | The disease has been shown to, or scientific evidence indicates that it is likely to, would cause a significant impact on the health of morbidity or mortality in wild aquatic animal populations taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats. | Wild aquatic animal populations can be populations that are commercially harvested (wild fisheries) and hence are an economic asset. However, the asset could be ecological or environmental in nature, for example, if the population consists of an endangered species of aquatic animal or an aquatic animal potentially endangered by the disease. |
3. The agent is of public health concern. Natural transmission to humans has been proven, and human infection is associated with severe consequences.

4. Infectious aetiology of the disease is proven.

5. An infectious agent is strongly associated with the disease, but the aetiology is not yet known.

6. 1. Likelihood of international spread of the agent including (via live aquatic animals, their products or fomites) has been proven. International trade in aquatic animal species susceptible to the disease exists or is likely to develop and, under international trading practices, the entry and establishment of the disease is likely.

7. 2. At least one country in the world has demonstrated freedom or impending freedom from the disease in populations of susceptible aquatic animals, may be declared free of the disease based on the general surveillance provisions principles outlined in of Chapters 1.4. and 1.5. Free countries/zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a disease can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread diseases, or the protection of the last remaining free zones from a widespread disease.

8. A repeatable and robust Reliable means of detection and diagnosis exists and a precise case definition is available to clearly identify cases and allow them to be distinguished from other diseases. A diagnostic test should be widely available and preferably has undergone a formal standardisation and validation process using routine field samples. (See Aquatic Manual) or a robust case definition is available to clearly identify cases and allow them to be distinguished from other pathologies.

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AQUATIC CODE

CHAPTER 1.2.

CRITERIA FOR THE INCLUSION OF DISEASES IN THE OIE LIST

Article 1.2.1.

Introduction

This chapter describes the criteria for listing diseases in Chapter 1.3.

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

For the diseases listed in accordance with Article 1.2.2., the corresponding disease-specific chapters assist Member Countries in the harmonisation of disease detection, prevention and control and provide standards for safe international trade in aquatic animals and their products.

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

Principles for selection of diagnostic tests are described in Chapter 1.1.2 of the Aquatic Manual.

Article 1.2.2.

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

1) International spread, of the agent (via live aquatic animals, their products or fomites) has been proven.

AND

2) At least one country has demonstrated freedom or impending freedom from the disease in populations of susceptible aquatic animals, based on the general surveillance provisions of Chapters 1.4. and 1.5.

AND

3) Reliable means of detection and diagnosis exist and a precise case definition is available to clearly identify cases and allow them to be distinguished from other diseases.

AND

4)

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

OR

b) The disease has been shown to cause a significant impact on the health of aquatic animals at the level of country or a zone taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality.
Annex 26 (contd)

Appendix VIII (contd)

OR

c) The disease has been shown to, or scientific evidence indicates that it would, cause a significant impact on the health of wild aquatic animal populations taking into account the occurrence and severity of the clinical signs, including direct production losses and mortality, and ecological threats.

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REPORT OF THE FIRST MEETING OF OIE AD HOC GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 10–12 February 2015

The OIE ad hoc Group on Susceptibility of crustacean species to infection with OIE listed diseases (the ad hoc Group) met at OIE Headquarters on 10–12 February 2015. Dr Grant Stentiford chaired the meeting.

1. Welcome and introduction

The members of the ad hoc Group and other participants at the meeting are listed at Annex I. The adopted agenda is provided as Annex II.

On behalf of Dr Bernard Vallat, Director General of the OIE, the Head of the International Trade Department, Dr Derek Belton, welcomed all members and thanked them for their agreement to work with the OIE on this important topic.

2. Objectives of the meeting

Chapter 1.5. ‘Criteria for listing species as susceptible to infection with a specific pathogen’ was introduced into the 2014 edition of the OIE Aquatic Animal Health Code (Aquatic Code). The purpose of this chapter is to provide criteria for determining which host species should be listed as susceptible in each disease-specific chapter of the Aquatic Code. These criteria are to be applied progressively to each disease-specific chapter in the Aquatic Code. Furthermore, for species where there is some evidence of susceptibility but insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3., information will be included in the relevant disease-specific chapter of the Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual).

Assessment of the scientific evidence will be undertaken by the ad hoc Group and the assessments will be provided to Member Countries for comment prior to any change in the list of susceptible species in Article X.X.2. of the crustacean disease-specific chapters of the Aquatic Code and Manual.

The objectives of the meeting were therefore as follows:

1) To consider the general elements of the three-stage approach for determining susceptibility of given host taxa to infection with the listed crustacean pathogens

2) To determine the specific evidence required to fulfil stages 2 (identification of the pathogen) and 3 (determination of infection) for yellow head virus (YHV) genotype 1 (hereafter YHV1)

3) To apply specific criteria defined in 2 to the available peer-reviewed and grey literature pertaining to YHV1 for determining susceptibility according to Aquatic Code chapter 1.5.

4) To classify evidence pertaining to susceptibility of specific host taxa to YHV1 to either Group 1 (Aquatic Code-listed), Group 2 (Aquatic Manual-listed) or Group 3 (other, e.g. mechanical vector)

5) To report on the outcome of the analysis prior to the meeting of the Aquatic Animal Health Standards Commission in March 2015.

3. Terms of Reference

The Terms of Reference were adopted and the final version is shown in Annex III.
4. Discussion of working documents and other relevant documents

Prior to the meeting, the ad hoc Group was provided with the European Food Safety Authority (EFSA) opinion relating to susceptibility of aquatic animals to pathogens listed in EC Directive 2006/88 (EFSA, 2008). Furthermore, a peer-reviewed paper arising from this work and focussed on application of the criteria outlined by EFSA to susceptibility of hosts to the crustacean diseases white spot disease (WSD), Taura syndrome (TS) and yellowhead disease (YHD), was also provided (Stentiford et al., 2009).

The ad hoc Group agreed that the three-stage approach as outlined in Article 1.5.3. would be adequate (given appropriate supporting data) to define a host as ‘susceptible’ to ‘infection’ with YHV1.

The Group wished to raise the following specific points related to Articles within Chapter 1.5.: 

Article 1.5.4. In reference to crustacean diseases, the ad hoc Group agreed that while injection of an infectious agent can be considered as an experimental invasive route, it does not mimic a natural route of infection. This contrasts the previous review of susceptibility of crustacean hosts to pathogens by EFSA (EFSA, 2008) in which injection was considered to potentially mimic a natural route of infection in crustaceans.

Article 1.5.4. In reference to consideration of ‘environmental factors’ associated with route of transmission/natural pathways, the Group considered this a relevant inclusion when considering the evidence reported in specific studies (e.g. did the temperature at which a given challenge study was conducted mimic expected natural conditions for the host/pathogen?).

Article 1.5.5. In reference to use of section 7 of the appropriate chapter of the Aquatic Manual to provide a means to ‘identify’ the pathogen under consideration, the Group considered it vital that section 7 be modified to include appropriate molecular diagnostic (PCR) and phylogenetic elements. In most cases this should take the form of PCR followed by sequencing of the amplicon, whilst in some cases pathogen taxon-specific PCR assays may be sufficient. If this information is not consistently provided in section 7, it is difficult to confirm the taxonomy of the pathogen under consideration against the reference listed pathogen.

Article 1.5.6. In reference to criteria A-D, the Group discussed and agreed a set of descriptive elements for each criterion, related to YHV1. These descriptions take account of previous work by EFSA (2008) and Stentiford et al. (2009) for susceptibility to YHV1. The descriptions to be applied to assess susceptibility to YHV1 in accordance with Chapter 1.5. of the Aquatic Code are thus:

<table>
<thead>
<tr>
<th>A: Replication</th>
<th>B: Viability / Infectivity</th>
<th>C: Pathology / Clinical signs</th>
<th>D: Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of characteristic inclusion bodies and positive labelling of inclusion bodies by ISH or IFAT</td>
<td>Single passage bioassay to a SPF (target pathogen) of any susceptible host species and confirmation of pathogen identification**</td>
<td>Characteristic inclusion bodies, with pyknosis and karyorrhectic nuclei in target tissues and haemocytic infiltration and/or clinical signs***</td>
<td>Haemocytes, heart, peripheral nerves, eye, lymphoid organ and sinuses, connective tissue****</td>
</tr>
<tr>
<td>Presence of virions in inclusion bodies by TEM</td>
<td>Demonstration of increasing copy number over time with RT-qPCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serial passage from individual to SPF individual of the same species*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key:*To demonstrate replication by this approach requires evidence for multiple passages in confirmed target pathogen-free hosts of the same species as being assessed. **To demonstrate viability or infectivity of the target pathogen within the host being assessed, single passage in any known susceptible SPF host is required. ***Clinical signs typical of YHV1 may provide evidence for fulfilment of this category when evidence from histopathology is not available. However, clinical signs according to the Manual chapter may not present equally in all host taxa and may not be specific for infection with YHV1. ****Lymphoid organ not present in most non-penaeid host taxa.
Article 1.5.5. In reference to specific identification of YHV1, the Group noted that as currently written, there are several inconsistencies between the Aquatic Code Chapter 9.2. (Infection with Yellow Head Virus) and the Aquatic Manual Chapter 2.2.8. (Yellow Head Disease). Since only infection with Yellow Head Virus (genotype 1) of the Yellowhead complex of viruses (i.e. that including GAV, etc.) is listed by the OIE, it was recommended that the Code and Manual chapters should be modified to clarify this point. To this end, and for the purposes of defining susceptibility of hosts to the listed pathogen (YHV1), publications were scrutinised to provide specific evidence that the pathogen under study was in fact YHV1 and not (potentially) other viruses within the complex. Where this definition could not be made, poor evidence for fulfilling stage 2 of the approach (see Articles 1.5.3. and 1.5.5.) was noted. In such cases, and where other studies could not be identified to fulfil this evidence gap, a host taxon would not be proposed for listing in the Aquatic Code (Group 1).

Article 1.5.7. In reference to outcomes of the assessment for each host taxon, those hosts for which identification of the agent is confirmed (section 1.5.5.) and where evidence to fulfil either criteria A, or at least two of B, C, D of Article 1.5.6. (see table above) is provided, hosts may be classified as Group 1 (for inclusion in the Code). This outcome is strengthened somewhat where infections are natural rather than experimental (Article 1.5.4.). In cases where evidence to fulfil some of those criteria listed in Article 1.5.6. (see table above) are not achieved, and/or where identification of the pathogen is not sufficient to confirm YHV1 (Article 1.5.5.), or where infection was due to invasive experimental procedures only, hosts may be classified as Group 2 (for inclusion in the Manual).

In all other cases, where evidence for ‘infection’ cannot be demonstrated conclusively (e.g. where only PCR evidence for presence of the pathogen was provided), hosts were classified to a third group, Group 3. Group 3 hosts may potentially include those taxa to be listed in section 2.2.6. of the Manual chapter under the heading of ‘Vector’.

In reviewing susceptibility of aquatic hosts to YHV1 the ad hoc Group considered the taxonomic range over which susceptible hosts exist. Such an approach may be used to better inform on risk related to the importation of potentially susceptible hosts. Evidence for susceptibility to YHV1 is available from only two families (Penaeidae and Palaemonidae) within the Decapod suborder (Dendrobranchiata). Susceptibility was tested (though not demonstrated) in several other families (including Brachyura, the crabs) of the suborder Pleocyemata. An understanding of taxonomic spread in host range is a new concept in addressing susceptibility and will undoubtedly highlight the variation in virulence strategies for the pathogens (including those of fish and molluscs) listed the Aquatic Code and Manual. Where specific evidence does not exist for susceptibility of a given host taxon to YHV1 (or other listed crustacean diseases), a risk analysis based upon taxonomic range should be conducted.

5. Outcome of the analysis

The ad hoc Group recommended to retain the listing of Penaeus monodon as a species susceptible to YHV1 infection. The assessment undertaken by the ad hoc Group against the criteria is provided below:

STAGE 1

Transmission has been obtained naturally (Wijegoonawardane et al., 2008 and Boonyaratpalin et al., 1993) in accordance with Article 1.5.4. The ad hoc Group considered that this criterion was met.

AND STAGE 2

The identity of the pathogenic agent has been confirmed (Wijegoonawardane et al., 2008) in accordance with Article 1.5.5. The ad hoc Group considered that this criterion was met.

AND STAGE 3

There is evidence of infection with the pathogenic agent in the suspect host species (Wijegoonawardane et al., 2008; Boonyaratpalin et al., 1993; Longyant et al., 2006) in accordance with criteria A to D in Article 1.5.6. The ad hoc Group considered that this criterion was met.
Annex 27 (contd)

In addition:

The *ad hoc* Group recommended to list *Penaeus vannamei* as a species susceptible to YHV1 infection. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has been obtained naturally (Songsuk *et al.*, 2011), and by experimental procedures (Lightner *et al.*, 1998) that mimic natural pathways for infection in accordance with Article 1.5.4. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 2**

The identity of the pathogenic agent has been confirmed (Songsuk *et al.*, 2011) in accordance with Article 1.5.5. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 3**

There is evidence of infection with the pathogenic agent in the suspect host species (Lightner *et al.*, 1998; Songsuk *et al.*, 2011) in accordance with criteria A to D in Article 1.5.6. The *ad hoc* Group considered that this criterion was met.

The *ad hoc* Group recommended to list *Penaeus stylirostris* as a species susceptible to YHV1 infection. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has been obtained naturally (Castro-Longoria *et al.*, 2008), in accordance with Article 1.5.4. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 2**

The identity of the pathogenic agent has been confirmed (Castro-Longoria *et al.*, 2008) in accordance with Article 1.5.5. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 3**

There is evidence of infection with the pathogenic agent in the suspect host species (Lu *et al.*, 1994; Castro-Longoria *et al.*, 2008) in accordance with criteria A to D in Article 1.5.6. The *ad hoc* Group considered that this criterion was met.

The *ad hoc* Group recommended to list *Metapenaeus affinis* as a species susceptible to YHV1 infection. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has been obtained by experimental procedures that mimic natural pathways for infection (Longyant *et al.*, 2006) in accordance with Article 1.5.4. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 2**

The identity of the pathogenic agent has been confirmed (Longyant *et al.*, 2006) in accordance with Article 1.5.5. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 3**

There is evidence of infection with the pathogenic agent in the suspect host species (Longyant *et al.*, 2006) in accordance with criteria A to D in Article 1.5.6. The *ad hoc* Group considered that this criterion was met.
The *ad hoc* Group recommended to list *Palaemonetes pugio* as a species susceptible to YHV1 infection. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has been obtained by experimental procedures that mimic natural pathways for infection (Ma *et al.*, 2009) in accordance with Article 1.5.4. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 2**

The identity of the *pathogenic agent* has been confirmed (Ma *et al.*, 2009) in accordance with Article 1.5.5. The *ad hoc* Group considered that this criterion was met.

**AND STAGE 3**

There is evidence of infection with the *pathogenic agent* in the suspect host species (Ma *et al.*, 2009) in accordance with criteria A to D in Article 1.5.6. The *ad hoc* Group considered that this criterion was met.

In addition:

The *ad hoc* Group recommended to remove *Penaeus esculentus* as a species susceptible to YHV1 infection as there is insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has only been obtained by experimental invasive procedures that do not mimic natural pathways for infection in accordance with Article 1.5.4. Further the literature only refers to studies using genotype 2 (GAV) so susceptibility to infection with the listed YHV1 cannot be confirmed (Spann *et al.*, 2000, 2003). The *ad hoc* Group considered that this criterion was not met.

**AND STAGE 2**

The identity of the *pathogenic agent* has not been confirmed in accordance with Article 1.5.5. Specifically, studies have not sufficiently demonstrated use of the agent YHV1 (Spann *et al.*, 2000, 2003). The *ad hoc* Group considered that this criterion was not met.

**AND STAGE 3**

There is not sufficient evidence of infection with the *pathogenic agent* in the suspect host species in accordance with criteria A to D in Article 1.5.6. Specifically, non-specific clinical signs were reported but internal pathology was not characteristic (Spann *et al.*, 2003). The *ad hoc* Group considered that this criterion was not met.

The *ad hoc* Group recommended to remove *Penaeus japonicus* as a species susceptible to YHV1 infection as there is insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3. The assessment undertaken by the *ad hoc* Group against the criteria is provided below.

**STAGE 1**

Transmission has been obtained naturally for the infection (Wang *et al.*, 1996) in accordance with Article 1.5.4. The *ad hoc* Group considered that this criterion was met.
Annex 27 (contd)

AND STAGE 2

The identity of the pathogenic agent has not been confirmed (Wang et al., 1996) in accordance with Article 1.5.5. Specifically, animals obtained from the outbreak under study were coinfected with WSSV and did not display typical clinical signs of YHV1. In addition, no typing of YHV1 in accordance with Section 7 of the appropriate Manual chapter was reported (Wang et al., 1996). The ad hoc Group considered that this criterion was not met.

AND STAGE 3

There is evidence of infection with the pathogenic agent in the suspect host species (Wang et al., 1996) in accordance with criteria A to D in Article 1.5.6. Specifically, although TEM was reported, the authors did not confirm that infection was caused by YHV1 (Wang et al., 1996). The ad hoc Group considered that this criterion was met.

6. Review and finalise report of meeting

The ad hoc Group utilised an existing database of papers and reports pertaining to Yellowhead disease (YHV1, GAV and other known genotypes) (Dr Stentiford, Cefas). In addition, the ad hoc Group searched the CAB Abstracts database (time range 1968 and 2015) (EBSCO host) using the search terms “yellow head virus” (n=386) and “yellow baculovirus” (n=30). We limited our search from 2008 to 2015 to the key words “yellow head disease” (n=115) as the literature had been reviewed previous to 2009 (Stentiford et al., 2009). Over 700 articles in the database were identified using these key words. The initial selection of articles was based on the relevance of the titles to the objectives of the terms of reference (host susceptibility). Selected titles were then evaluated to determine the route of infection, confirmation of the pathogen genotype, and the presence of viable replicating virus in the host. Once we had sufficient papers to assess that a host species was susceptible we did not search for other references pertaining to that crustacean host species. The search was broadened by using the Boolean operators “OR” and “AND”, and other databases, such as Google Scholar and PubMed. Key terms searched included “YHV and Mosquito” (n=2), and “YHV and chinensis” (n=0), and “YHV and indicus” (n=1). A few “backward” searches on relevant articles were also conducted. Approximately 40 papers were downloaded for initial review. In total 18 papers were cited. Three proceedings and two peer-reviewed papers initially selected could not be accessed.

7. Summary

In summary, five crustacean host taxa fulfilled the criteria required for listing a species susceptible to infection with YHV1 according to Article 1.5 of Aquatic Animal Health Code. These Group 1 hosts were: Penaeus monodon, Penaeus vannamei, Penaeus stylirostris, Palaemonetes pugio and Metapenaeus affinis. In addition, a further nine crustacean host taxa fulfilled some of the criteria required for listing as susceptible but evidence was lacking to either confirm the identity of the pathogen under study as YHV1, to demonstrate a natural route of infection, or to definitively confirm an ‘infected’ status. These Group 2 hosts were: Macrobrachium sintangense, Metapenaeus brevicornis, Palaemon serrifer, Palaemon styliferus, Penaeus aztecus, Penaeus duorarum, Penaeus japonicus, Penaeus merguiensis and Penaeus setiferus. Finally, numerous taxa were classified in to a third group when either the viability of the virus within the host was not demonstrated, or where its status as a mechanical vector could not be ruled out. Detailed analysis of evidence pertaining to Group 1, 2 and 3 hosts is provided in Annex IV.
Table 2. Susceptibility of species to yellow head virus Genotype 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Overall status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metapenaeus affinis</td>
<td>1</td>
</tr>
<tr>
<td>Palaemonetes pugio</td>
<td>1</td>
</tr>
<tr>
<td>Penaeus monodon</td>
<td>1</td>
</tr>
<tr>
<td>Penaeus stylirostris</td>
<td>1</td>
</tr>
<tr>
<td>Penaeus vannamei</td>
<td>1</td>
</tr>
<tr>
<td>Macrobrachium sintangense</td>
<td>2</td>
</tr>
<tr>
<td>Metapenaeus brevicomis</td>
<td>2</td>
</tr>
<tr>
<td>Palaemon serrifer</td>
<td>2</td>
</tr>
<tr>
<td>Palaemon styliferus</td>
<td>2</td>
</tr>
<tr>
<td>Penaeus aztecs</td>
<td>2</td>
</tr>
<tr>
<td>Penaeus duorarum</td>
<td>2</td>
</tr>
<tr>
<td>Penaeus japonius</td>
<td>2</td>
</tr>
<tr>
<td>Penaeus merguiensis</td>
<td>2</td>
</tr>
<tr>
<td>Penaeus setiferus</td>
<td>2</td>
</tr>
<tr>
<td>Acetes sp.</td>
<td>3</td>
</tr>
<tr>
<td>Callinectes sapidus</td>
<td>3</td>
</tr>
<tr>
<td>Chelonibia patula</td>
<td>3</td>
</tr>
<tr>
<td>Ergasilus manicatus</td>
<td>3</td>
</tr>
<tr>
<td>Fundulus grandis</td>
<td>3</td>
</tr>
<tr>
<td>Metapenaeus bennetiae</td>
<td>3</td>
</tr>
<tr>
<td>Metapenaeus ensis</td>
<td>3</td>
</tr>
<tr>
<td>Octolasmis muelleri</td>
<td>3</td>
</tr>
<tr>
<td>Penaeus esculentus</td>
<td>3</td>
</tr>
</tbody>
</table>
Annex 27 (contd)

8. Next meeting

Next meeting date to be confirmed after Aquatic Animals Health Standards Commission meeting in March 2015.

9. References


.../Annexes
MEETING OF THE OIE AD HOC GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 10–12 February 2015

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MEETING OF AD HOC GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Paris, 10–12 February 2015

Agenda

1) Welcome and introduction
2) Objectives of the meeting
3) Terms of Reference
4) Discussion of working documents and other relevant documents
5) Outcome of the analysis
6) Review and finalise the report of the meeting
7) Summary
8) Next meeting
AD HOC GROUP ON SUSCEPTIBILITY OF CRUSTACEAN SPECIES TO INFECTION WITH OIE LISTED DISEASES

Background

A new Chapter 1.5, ‘Criteria for listing species as susceptible to infection with a specific pathogen’ was introduced into the 2014 edition of the Aquatic Code. The purpose of this chapter is to provide criteria for determining which host species are listed as susceptible in Article X.X.2. of each disease-specific chapter in the Aquatic Code. The criteria are to be applied progressively to each disease-specific chapter in the Aquatic Code.

These assessments will be undertaken by ad hoc Groups and the assessments will be provided to Member Countries’ for comment prior to any change in the list of susceptible species in Article X.X.2. of the disease-specific chapters in the Aquatic Code.

For species where there is some evidence of susceptibility but insufficient evidence to demonstrate susceptibility through the approach described in Article 1.5.3., information will be included in the relevant disease-specific chapter in the Aquatic Manual.

Purpose

The ad hoc Group on Susceptibility of crustacean species to infection with OIE listed diseases will undertake this task for OIE listed crustacean diseases.

Terms of Reference

1) Consider standards of evidence required to satisfy the criteria in Chapter 1.5.
2) Review relevant literature documenting susceptibility of species
3) Propose susceptible species for OIE listed diseases based on Article 1.5.7.
4) Propose susceptible species for OIE listed diseases based on Article 1.5.8.

Expected outputs of the ad hoc Group

1) Develop a list of susceptible species for inclusion in the relevant Articles of crustacean disease-specific chapters in the Aquatic Code and Manual, starting with Yellow Head Disease, and if time allows other OIE listed crustacean diseases.
2) Draft a report for consideration by the Aquatic Animals Commission at their March 2015 meeting.
Evidence for susceptibility of host species to infection with yellow head virus according to Chapter 1.5. Criteria for listing species as susceptible to infection with a specific pathogen.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Route of transmission</th>
<th>Pathogen id.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Reference</th>
<th>Outcome</th>
<th>Status</th>
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Annex 27 (contd)

Annex IV (contd)

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Route of transmission: Natural (N), Experimental Non-invasive (EN), Experimental Invasive (EI);
## Aquatic Code

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<thead>
<tr>
<th>Task</th>
<th>March 2015</th>
<th>May GS 2015</th>
<th>Sept 2015</th>
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<tr>
<td>1 User’s guide</td>
<td>Review Member Countries comments</td>
<td>Propose for adoption</td>
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<td>2 Glossary</td>
<td>Review Member Countries</td>
<td>Propose for adoption</td>
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<td>3 Article 1.1.5. (Chapter 1.1.)</td>
<td>Review Member Countries</td>
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<td>4 Chapter 1.2 – Criteria for listing</td>
<td>Consider <em>ad hoc</em> group report and circulate for Member Countries comments</td>
<td>Review Member comments</td>
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<td>5 Revision of Section 4 to improve guidance on the control of disease</td>
<td>Develop a plan in light of Aquatic Conf. recommendations.</td>
<td>Agree on priorities in section 4</td>
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<tr>
<td>6 Chapter 4.3. – General recommendations on disinfection</td>
<td></td>
<td>Review AHG draft chapter and circulate for member.</td>
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<tr>
<td>7 Chapter 4.X. – Recommendations for surface disinfection of salmonid eggs</td>
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<tr>
<td>8 Chapter 4.7. – Control of pathogenic agents in aquatic animal feed</td>
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<tr>
<td>9 Chapter 6.6. – Risk analysis for antimicrobial resistance in aquaculture (new)</td>
<td>Review Member Countries comments</td>
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<tr>
<td>10 Listing of susceptible species in disease-specific chapters</td>
<td>Review AHG report and propose amendments to 9.2.2</td>
<td>Review Member Countries’ comments</td>
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<td>11 AHPND</td>
<td>Review Member Countries comments</td>
<td>Propose for adoption</td>
<td>If adopted. AAC to develop new Chapter</td>
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Annex 28 (contd)

Aquatic Manual

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<td>12 Crustacean chapters (YHD, NHP, TSD, IHHN)</td>
<td>Review Member Countries comments</td>
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<td>13 Infection with Perkinsus olseni</td>
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<td>14 Chapter 1.1.3. – Methods for disinfection of aquaculture establishments</td>
<td>Propose deletion if new Code chapter 4.X. is adopted</td>
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<td>15 Listing of susceptible species in disease-specific chapters</td>
<td>Review AHG report and request amendments in section 2.2.1. of chapter 2.2.8. YHD by the author</td>
<td>Review and circulate for Member Countries’ comments</td>
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<td>16 AHPND chapter</td>
<td>Review AHG Report and draft chapter and circulate for Member Countries comments</td>
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<td>17 Test performance</td>
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<td>18 Sections on agent stability (in connection with disinfection)</td>
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Other items

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<td>19 OIE Global Aquatic Animal Health Conference</td>
<td>Discuss conference recommendation for review of work plan</td>
<td>Present conference recommendation</td>
<td>Discuss conference recommendation for establishing work plan</td>
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<td>20 Fish-borne Zoonotic Trematodes (FZT)</td>
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