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

Paris (France), 24–28 February 2014

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at the OIE Headquarters in Paris from 24 to 28 February 2014.

The list of participants and the adopted agenda are presented at [Annexes 1](#) and [2](#).

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts circulated after the Commission's October 2013 meeting: Argentina, Australia, Brazil, Canada, Chile, China (People's Rep. of), Chinese Taipei, Colombia, Cuba, Japan, Mexico, New Zealand, Norway, Singapore, Sri Lanka, Switzerland, Thailand, the United States of America (USA), the Member States of the European Union (EU), the African Union–Interafrican 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 24 January 2014 and amended texts in the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) where appropriate. The amendments are shown in the usual manner by 'double underline' and '~~strike through~~' and may be found in the Annexes to the report. The amendments made at the February 2014 meeting are highlighted with a coloured background in order to distinguish them from those made at the October 2013 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 Aquatic Animals Commission appreciated the work required to prepare and submit comments but would like to remind Member Countries that it is important that they submit comments (for the *Aquatic Code* and *Aquatic Manual*) in the requested format, i.e. specific proposed text changes supported by a scientific rationale. Without this information it is difficult for the Aquatic Animals Commission to address a comment.

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 20](#) are proposed for adoption at the 82nd General Session in May 2014; [Annex 21](#) is presented for Member Countries' comments; [Annexes 22 to 24](#) 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 Annex 21 of this report must reach OIE Headquarters by **1st September 2014** to be considered at the October 2014 meeting of the Aquatic Animals Commission.

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

Texts proposed for adoption:	Annex number
<i>Aquatic Code:</i>	
Glossary	Annex 3
Chapter 1.1. Notification of diseases and epidemiological information	Annex 4
Chapter 1.2. Criteria for listing aquatic animal diseases	Annex 5
Chapter 1.3. Diseases listed by the OIE	Annex 6
Chapter 2.1. Import risk analysis	Annex 7
Chapter 3.1. Quality of Aquatic Animal Health Services	Annex 8
Chapter 5.1. General obligations related to certification	Annex 9
Chapter 5.2. Certification procedures	Annex 10
Chapter 9.4. Necrotising hepatopancreatitis	Annex 11
Model Chapter X.X. (clean text and track changes text)	Annexes 12A and 12 B
Chapter 9.8. Yellow head disease	Annex 13
Chapter 10.5. Infection with infectious salmon anaemia virus	Annex 14
Chapter 10.X. Infection with Salmonid alphavirus (new)	Annex 15
Chapter X.X. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new) (clean text and track changes text)	Annexes 16A and 16B
<i>Aquatic Manual:</i>	
Chapter 2.3.5. Infection with infectious salmon anaemia virus	Annex 17
Chapter 2.4.9. Infection with ostreid herpesvirus 1 microvariants	Annex 18
Chapter 2.3.X. Infection with salmonid alphavirus virus	Annex 19
Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis	Annex 20

Texts for Member Countries' comment:	Annex number
Guide to the Use of the <i>Aquatic Animal Health Code</i>	Annex 21
Annexes for Member Countries' information:	
Article X.X.2.	Annex 22
<i>Ad hoc</i> Group report on Safety of Products Derived from Aquatic Animals (February 2014)	Annex 23
Aquatic Animal Health Standards Commission Work Plan for 2013/2014	Annex 24

1. OIE *Aquatic Animal Health Code*

The Aquatic Animals Commission agreed with a Member Country comment that there is inconsistency in the use of the terms 'disease(s)' and 'diseases of aquatic animals', as well as 'aetiological agents' and 'pathogenic agents' throughout the *Aquatic Code*. The Commission agreed that 'disease(s)' should be preferred to 'diseases of aquatic animals' for the sake of simple wording where no ambiguity may exist with regards to diseases of terrestrial animals. Also, the Commission agreed to avoid use of the term 'aetiological agents' in favor of 'pathogenic agents' which is defined in the glossary.

The Commission noted that it would be a significant task to amend these inconsistencies throughout the *Aquatic Code* and agreed that this will be addressed over time as chapters are revised for other purposes. The Commission paid particular attention to the issue of inconsistencies in Chapter 1.1. Notification of diseases and epidemiological information, Model Chapter X.X., and the proposed Chapter X.X. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents.

1.1. Glossary

Emerging disease

The Aquatic Animals Commission considered the many Member Countries' comments on the proposed definition and the revised definition developed by the OIE Terrestrial Animal Health Standards Commission (the Code Commission) at their February 2014 meeting.

The Aquatic Animals Commission noted that it was important that the definition reflected the criteria for listing an 'emerging disease' described in the current Article 1.2.3. so that these points are retained in the *Aquatic Code* when Article 1.2.3. is deleted. In addition, in response to several Member Countries' comments, the Commission included some text to clarify that an 'emerging disease' is a non-listed disease. The Commission recognised that there are differences between the definitions proposed by the Aquatic Animals Commission and the Code Commission but considered that it is important that the definition in the *Aquatic Code* takes account of the circumstances regarding disease emergence in the aquatic sector.

Susceptible species

In response to several Member Countries' comments, the Aquatic Animals Commission amended the definition to improve clarity.

Veterinarian

Although several Member Countries submitted comments on the proposed definition for 'veterinarian', the Aquatic Animals Commission agreed that this definition should not be further amended to ensure that it remains the same as the *Terrestrial Code* definition.

Risk assessment

The Aquatic Animals Commission accepted the OIE Headquarters' suggestion to add 'scientific' to the definition of risk assessment.

Pathogenic agent

The Aquatic Animals Commission proposed to delete the reference to the *Aquatic Code* in the definition for 'pathogenic agent' as they considered this was too restrictive as this term is used in a broader context than the *Aquatic Code* only.

Notification

The Aquatic Animals Commission agreed with several Member Countries' comments that the term 'Competent Authority' replace 'Veterinary Authority' to ensure alignment with the use of this term in Chapter 1.1.

The revised Glossary is attached as [Annex 3](#) to be presented for adoption at the 82nd General Session in May 2014.

1.2. Notification of diseases and epidemiological information (Chapter 1.1.)

The Aquatic Animals Commission considered the Member Countries' comments received along with the revised chapter developed by the Code Commission at their February 2014 meeting.

The Aquatic Animals Commission incorporated the Code Commission's proposed amendments into the revised chapter, with the only exception of point e) of Article 1.1.3. For this point the Code Commission proposed the use of the words 'unusual host species'. However, the Aquatic Animals Commission proposed to retain the term 'new host species' instead of 'unusual host species' because they considered that the term 'unusual' is subjective. The Commission also considered that approximately 500 different species of aquatic animal species are farmed, globally, with several new species brought to aquaculture every year and that 'new host species' are important for trade and should be reported.

In addition, in response to a Member Country's comment, the Aquatic Animals Commission amended the text, where relevant, replacing the term 'aetiological agent' with the term 'pathogenic agent' as the latter is a defined term in the *Aquatic Code* (see Item 1.1.)

The revised Chapter 1.1. is attached as [Annex 4](#) to be presented for adoption at the 82nd General Session in May 2014.

1.3. Criteria for listing an emerging disease (Chapter 1.2.)

The Aquatic Animals Commission noted that no Member Countries opposed the proposal to delete Article 1.2.3. 'Criteria for listing an emerging aquatic animal disease'. Although some Member Countries' commented on the criteria in Article 1.2.2., the Commission agreed that these comments will be addressed at a future meeting of the Commission.

The revised Chapter 1.2. is attached as [Annex 5](#) to be presented for adoption at the 82nd General Session in May 2014.

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

In line with the proposal to delete Article 1.2.3. 'Criteria for listing an emerging aquatic animal disease' (see Item 1.3.), the Aquatic Animals Commission proposed the consequential deletion of the words: 'or criteria for listing an *emerging aquatic animal disease* (see Article 1.2.3.)' from the Preamble in Chapter 1.3.

1.4.1. Infection with ostreid herpesvirus-1 microvariant

The Aquatic Animals Commission noted that as a consequence of the proposal to delete Article 1.2.3. ‘Criteria for listing an emerging aquatic animal disease’ (see Item 1.3.), infection with ostreid herpesvirus-1 microvariant would be deleted from Article 1.3.2.

The Aquatic Animals Commission highlighted that infection with ostreid herpesvirus-1 microvariant meets the definition for an emerging disease and therefore when Article 1.2.3. is deleted, the obligation to report the occurrence of this disease would remain as per provisions of Chapter 1.1. Notification of diseases and epidemiological information.

1.4.2. Yellow head disease

In line with the OIE approach to move towards disease names in the format of ‘infection with’ in Chapter 9.8. ‘Yellow head disease’ of the *Aquatic Code*, the Aquatic Animals Commission agreed to amend the listed name in Article 1.3.3. to: Infection with yYellow head virus disease (see Item 1.11.).

The revised Chapter 1.3. is attached as Annex 6 to be presented for adoption at the 82nd General Session in May 2014.

1.4.3. Acute hepatopancreatic necrosis disease

The Aquatic Animals Commission reminded Member Countries that it had considered Acute hepatopancreatic necrosis disease (AHPND) for possible listing in accordance with Article 1.2.2. at its October 2013 meeting.

The Aquatic Animals Commission considered Member Countries’ comments and an expert’s opinion to consider the listing of AHPND. In addition, the Commission reviewed new information on AHPND and considered whether the disease satisfies the criteria for listing in Article 1.2.2.

The Aquatic Animals Commission considered that the publicly available information on AHPND is insufficient to propose listing of the disease in accordance with Article 1.2.2. In particular, the Commission reiterated the comments made in its October 2013 meeting report that there is insufficient information to accurately characterise the causative agent of AHPND. A virulent form of *Vibrio parahaemolyticus* is considered to be the causative agent of AHPND; however, this *Vibrio* species is ubiquitous and, if OIE trade standards were to be developed, it is essential that the causative agent of AHPND can be distinguished from other forms of the bacterium. Furthermore, there is currently no specific test available that can be used to detect the causative agent in subclinical infections.

The Aquatic Animals Commission noted that research has been undertaken to characterise the causative agent of AHPND and that specific diagnostic tests are under development. Information on preliminary PCR primers have been made publically available by Drs Flegel and Lo but their analytical specificity remains uncertain. In addition, information was provided to the Commission by Dr Lightner that a PCR test has been developed by the University of Arizona with rights assigned to a commercial party. However, no technical details regarding the performance of this test were provided to the Commission. The lack of information on the analytical specificity of both assays is of concern given the ubiquitous nature of *V. parahaemolyticus*. The Commission encourages Member Countries to provide any relevant information that would assist in the assessment for listing against criteria in Article 1.2.2.

In addition, the Aquatic Animals Commission noted a comment from a Member Country about reluctance they have experienced ‘from current expert laboratories to release biological materials to investigate diagnostic methods’. The Commission encourages Member Countries and their laboratories to share information and materials to allow the development and validation of assays for AHPND, as for any new or emerging disease.

The Aquatic Animals Commission reiterated its opinion that AHPND meets the definition of an emerging disease and that Member Countries should notify the occurrence of the disease in accordance with Chapter 1.1.

An OIE Technical Factsheet on AHPND has been developed and will be updated as new information becomes available. The Aquatic Animals Commission would welcome suggestions to keep the document current. It is available at:

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

1.4.4. Infectious hypodermal and hematopoietic necrosis virus

The Aquatic Animals Commission noted the request from a Member Country to consider the possible delisting of Infectious hypodermal and hematopoietic necrosis virus. The Commission agreed to consider this issue at a future Commission meeting.

1.5. Import risk analysis (Chapter 2.1.)

The Aquatic Animals Commission reviewed amendments proposed by the Terrestrial Code Commission and agreed to make similar amendments in the *Aquatic Code* chapter to ensure alignment, i.e. to delete ‘potential’ from the term ‘potential hazard’ throughout the chapter because this word is inaccurate as a qualifier of hazard.

The Aquatic Animals Commission also accepted Member Countries’ and OIE Headquarters’ editorial comments to improve clarity in Articles 2.1.1., 2.1.5. and 2.1.6.

The revised Chapter 2.1. is attached as Annex 7 to be presented for adoption at the 82nd General Session in May 2014.

1.6. Quality of Aquatic Animal Health Services (Chapter 3.1.)

The Aquatic Animals Commission reviewed amendments proposed by the Code Commission and agreed to make similar amendments in Article 3.1.2. points 7 and 9 in the corresponding chapter in the *Aquatic Code* to ensure alignment.

The revised Chapter 3.1. is attached as Annex 8 to be presented for adoption at the 82nd General Session in May 2014.

1.7. General recommendations on disinfection (Chapter 4.3.)

The Aquatic Animals Commission reviewed Chapter 1.1.3. ‘Methods for disinfection of aquaculture establishments’ in the OIE *Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual)* and agreed that this chapter is misplaced in the *Aquatic Manual* since the scope of the *Manual* is diagnosis. The Commission also agreed that the scope of Chapter 1.1.3. has not been well defined; the chapter covers multiple issues such as routine disinfection procedures, disinfection of eggs, sanitation and biosecurity measures.

The Aquatic Animals Commission recognised that while information on disinfection does not belong in the *Aquatic Manual*, Chapter 4.3. ‘Methods for disinfection of aquaculture establishments’ in the *Aquatic Code* should be revised to encompass the topics currently contained in the *Aquatic Manual* chapter. The Commission agreed that once a revised chapter in the *Aquatic Code* is adopted, the *Aquatic Manual* chapter will be deleted.

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

The Aquatic Animals Commission accepted the OIE Headquarters’ suggestion to reword Article 5.1.2. points 1 and 2 to improve clarity and to align with the corresponding chapter in the *Terrestrial Code*.

The revised Chapter 5.1. is attached as [Annex 9](#) to be presented for adoption at the 82nd General Session in May 2014.

1.9. Certification procedures (Chapter 5.2.)

The Aquatic Animals Commission reviewed a Member Country's comment and amendments proposed by the Terrestrial Code Commission in point 1 of Article 5.2.4. to better describe the procedures for electronic certification. The Commission agreed to make similar amendments in the corresponding article in the *Aquatic Code*.

The revised Chapter 5.2. is attached as [Annex 10](#) to be presented for adoption at the 82nd General Session in May 2014.

1.10. Necrotising hepatopancreatitis (Chapter 9.4.)

The Aquatic Animals Commission disagreed with some Member Countries' comments that the proposed name change from infection with necrotising hepatopancreatitis bacteria to *Candidatus Hepatobacter penaei* should not be made at this time. The Commission emphasised that the proposed name has been published in a peer reviewed journal and that the term 'Candidatus' would be removed from the *Aquatic Code* once the new name is accepted by the International Committee for Taxonomy of Bacteria.

The Aquatic Animals Commission wished to note that once the proposed horizontal amendments are adopted (see Item 1.11.) they would also be made throughout this chapter.

The revised Chapter 9.4. is attached as [Annex 11](#) to be presented for adoption at the 82nd General Session in May 2014.

1.11. Horizontal issues

Model Chapter. X.X.

The Aquatic Animals Commission considered Member Countries' comments and amended the model Chapter X.X., as appropriate.

The Aquatic Animals Commission wished to emphasise that the model chapter provided describes horizontal amendments that will be applied in all disease-specific chapters. However, where there are disease-specific differences between chapters, these differences will be preserved. The Commission wished to highlight the following differences:

In the model chapter the disease name is presented as 'infection with pathogen X'/'disease X'. This takes into account that some disease-specific chapters use a disease name (e.g. 'Crayfish plague') while others use the format 'infection with' (e.g. 'infection with *A. invadans*').

- The proposed amendment to the first paragraph of Article X.1.4. to include the following words: 'areas covered by the shared water bodies' will be made in all disease-specific chapters except the two amphibian disease chapters (8.1. and 8.2.) where the words will remain unchanged as 'covered by *the zone*' as this is more correct for amphibians.
- Point 1 of Articles X.X.4. and X.X.5. in the model chapter will be included in all disease-specific chapters with the exception of the disease-specific chapters for 'Infection with *A. invadans*', 'Viral haemorrhagic septicaemia' and 'Infection with *P. olseni*', as this point does not currently appear in these chapters.
- Point 2 of Article X.X.4. will be applied to all disease-specific chapters including all mollusc disease-specific chapters which currently have slightly different wording. The additional text in all mollusc chapters, '- in all area where the species are present -' and 'not known to be established in wild populations' will be deleted because this detail is described in Chapter 1.4. and applies to all listed diseases.

- Article X.X.5. will be applied to all the disease-specific chapters. However, the numbering of the points in Article 10.3.5. of Chapter 10.3. Infection with *G. salaris* will differ as this Article has 5 points rather than the four because of the consideration of the salinity of the water.
- Article X.X.10. 'Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use from a country, zone or compartment not declared free from 'infection with pathogen X'/disease X' that currently appears in the amphibian and fish disease specific chapters will be added to all the crustacean and mollusc disease specific chapters as the Aquatic Animals Commission considered this article also applies to these host species.
- The time periods for surveillance, indicated by 'X' in the model chapter will remain as they currently appear in each disease specific chapter. The Aquatic Animals Commission noted a number of Member Countries' comments requesting the rationale for the time frames required and agreed that these time frames would not be reconsidered at this time.

The revised model chapter X.X. is attached as [Annex 12](#) (12 A: clean text and 12 B: track changes) to be presented for adoption at the 82nd General Session in May 2014.

1.12. Yellow head disease (Chapter 9.8.)

The Aquatic Animals Commission considered the definition for Yellow head disease in Article 9.8.1. and agreed that the definition is unclear and proposed the following amendments:

For the purposes of the *Aquatic Code*, infection with yellow head disease virus (YHD) means infection with yellow head virus genotype 1 (YHV). YHV and the related gill-associated virus are is classified as a species in the genus *Okavirus*, family *Roniviridae* and order *Nidovirales*. Common synonyms are listed in the corresponding chapter of the *Aquatic Manual*.

The Aquatic Animals Commission noted that this amendment is consistent with the fact that genotype 1 is associated with yellow head disease and clarifies that the case definition in the *Aquatic Code* is in line with the definition in Chapter 2.2.8. 'Yellow head disease' in the *Aquatic Manual*.

In addition, the Aquatic Animals Commission agreed to amend the name of the disease to 'infection with yellow head virus', in line with the current approach used in the *Aquatic Code* to name diseases (see Item 1.4.2.).

The Aquatic Animals Commission wished to note that once adopted the amended name change and proposed horizontal amendments (see Item 1.11) will be made throughout this chapter.

The revised Chapter 9.8. is attached as [Annex 13](#) to be presented for adoption at the 82nd General Session in May 2014.

1.13. Infection with infectious salmon anaemia virus (Chapter 10.5.)

The Aquatic Animals Commission considered Member Countries' comments and amended the draft chapter, as appropriate. The Commission also included horizontal amendments as proposed in the model Chapter X.X. (see Item 1.11.).

The revised Chapter 10.5. is attached as [Annex 14](#) to be presented for adoption at the 82nd General Session in May 2014.

1.14. Infection with Salmonid alphavirus (new chapter 10.X.)

The Aquatic Animals Commission considered Member Countries' comments and amended the draft chapter, as appropriate. The Commission also included horizontal amendments as proposed in the model Chapter X.X. (see Item 1.11.).

The Aquatic Animals Commission considered the February 2014 report of the *ad hoc* Group on Safety of Products Derived from Aquatic Animals (see Annex 23) and agreed with their recommendations regarding the list of commodities in Articles 10.X.3 and 10.X.12, and Article 10.X.13 on importation of disinfected eggs. The Commission agreed that the designations 'under study' in the draft chapter now be deleted.

The revised Chapter X.X. is attached as [Annex 15](#) to be presented for adoption at the 82nd General Session in May 2014.

1.15. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new Chapter X.X.)

The Aquatic Animals Commission considered Member Countries' comments and amended the draft chapter, as appropriate.

The Aquatic Animals Commission considered a Member comment that such a chapter may not be necessary, as no equivalent chapter exists in the *Terrestrial Code*. The Commission noted that approximately 500 different species of aquatic animal species are farmed globally, with several new species brought to aquaculture every year. The Commission agreed that under these circumstances guidance should be provided as to whether a species is susceptible or not, based on a transparent and science based decision making process.

The Aquatic Animals Commission clarified that 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*. Once this Chapter X.X. 'Criteria for determining susceptibility of aquatic animals' is adopted, the criteria will 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*. The Commission emphasised that the decision to list a species as susceptible in Article X.X.2. of each disease specific chapter in the *Aquatic Code* will be based on a finding that the evidence for susceptibility is sufficient.

The Aquatic Animals Commission also clarified that for species where there is evidence for susceptibility but that evidence is insufficient to demonstrate susceptibility through the approach described in Chapter X.X. 'Criteria for determining susceptibility of aquatic animals', information will be included in the relevant disease chapter in the *Aquatic Manual*.

In addition, following adoption of Chapter X.X., and application of the criteria to each listed disease, the current cross reference to the *Aquatic Manual* in Article X.X.2. in each disease specific chapter in the *Aquatic Code* will be removed and text amended appropriately (see [Annex 22](#) for Member Country information).

The revised Chapter X.X. is attached as [Annex 16](#) (16 A: clean text and 16 B: track changes) to be presented for adoption at the 82nd General Session in May 2014.

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

The Aquatic Animals Commission reviewed the 'Guide to the Use of the *Aquatic Animal Health Code*' taking into account the revised 'User's Guide' being proposed for the *Terrestrial Code* and amended the text as appropriate.

The revised 'Guide' is attached as [Annex 21](#) for Member Countries' comments.

1.17. General recommendations: disease prevention and control (Section 4)

The Aquatic Animals Commission reviewed the structure of Section 4. ‘General recommendations: disease prevention and control’ and acknowledged the need to revise these chapters and agreed that this should be done progressively. Given the decisions made regarding Chapter 1.1.3. ‘Methods for disinfection of aquaculture establishments’ in the *Aquatic Manual* (see Item 1.7.), the Commission agreed to prioritise revision of the *Aquatic Code* Chapter 4.3. ‘General recommendations of disinfection’. The Commission also agreed that the next chapters to be revised should be those chapters regarding zoning and compartmentalisation given the demand from Member Countries for improved guidance on these issues. The Commission reminded Member Countries that Chapter 6.1. ‘Control of hazards in aquatic animal feed’, currently under revision, will be moved to this section once finalised.

The Aquatic Animals Commission discussion on zoning and compartmentalization was informed by a report on the workshop ‘Certifying Disease Status for Safe Trade in Aquaculture Workshop’ hosted by the OIE Collaborating Center for Risk Analysis, Animal Disease Surveillance Systems, Epidemiologic Monitoring in Colorado, USA. The Commission acknowledged the usefulness of this workshop and will consider their recommendations in the Commission’s future work on this topic. The Commission noted that information on this workshop is available at: www.cvmb.colostate.edu/aphi/aquaculture.

2. OIE Manual of Diagnostic Tests for Aquatic Animals

2.1. Review of the *Aquatic Manual* chapters

2.1.1. Chapter 2.3.5. Infection with infectious salmon anaemia 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 2.3.5. is attached as [Annex 17](#) for adoption at the 82nd General Session in May 2014.

2.1.2. Chapter 2.4.9. Infection with ostreid herpesvirus 1 microvariants

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

The revised Chapter 2.4.9. is attached as [Annex 18](#) for adoption at the 82nd General Session in May 2014.

2.1.3. Chapter 2.3.X. Infection with salmonid alphavirus

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

The revised Chapter 2.3.X. is attached as [Annex 19](#) for adoption at the 82nd General Session in May 2014.

2.1.4. Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis

The Aquatic Animals Commission considered the issue of virus-like sequences that are incorporated into the host genome and in consultation with the chapter’s authors, amended the text to improve clarity regarding test performance.

The revised chapter 2.2.2 is attached as [Annex 20](#) for adoption at the 82nd General Session in May 2014.

2.2. Other Aquatic Manual issues

2.2.1. Yellow head disease (Chapter 2.3.5.)

The Aquatic Animals Commission noted that the inclusion of *Euphausia superba* (Antarctic krill) in the list of susceptible host species in Chapter 2.4.5. 'Yellow head disease' in the *Aquatic Manual* was a result of a mistake in the referenced scientific paper. The Commission noted that *E. superba* has therefore been removed as a susceptible species from the web version of Chapter 2.3.5. of the *Aquatic Manual*.

Relevant References:

Flegel T.W., Sriurairatana S., Wongterasupaya C., Boonsaeng V., Panyim S. & Withyachumnarnkul B. (1995). Progress in characterization and control of yellow-head virus of *Penaeus monodon*. In: Swimming through Troubled Water, Proceedings of the Special Session on Shrimp Farming, Browdy C.L. & Hopkins J.S., eds. World Aquaculture Society, Baton Rouge, LA, USA, pp 76–83.

Longyant S., Sithigorngul P., Chaivisuthangkura P., Rukpratanporn S., Sithigorngul W. & Menasveta P. (2005). Differences in susceptibility of palaemonid shrimp species to yellow head virus (YHV) infection. *Dis. Aquat. Org.*, 64, 5–12.

2.2.2. Proposal from a workshop on mollusc disease diagnosis

The Aquatic Animals Commission considered recommendations from a workshop on mollusc disease diagnosis provided by a Member Country regarding the levels to which the diagnostic tests in the *Aquatic Manual* had been validated and the 'rating of tests against purpose of use' provided in Table 5.1. in each disease-specific chapter. The Commission fully supported the workshop recommendations and agreed that these are important topics and proposed that they be considered at the Global Conference of the OIE Reference Centres (see Item 4).

2.2.3. Disease specific guidance documents on surveillance for a mollusc and a crustacean disease

The Aquatic Animals Commission noted that the document on 'Surveillance for viral haemorrhagic septicaemia' has been uploaded onto the Commission's webpages and is available at: http://www.oie.int/fileadmin/Home/eng/International_Standard_Setting/docs/pdf/Aquatic_Commission/Surveillance_VHS.pdf

The Aquatic Animals Commission reviewed the document on 'Surveillance for infection with *Bonamia ostreae*' and requested that this be uploaded onto the Commission's webpages. A third document on 'Surveillance for white spot virus' is expected to be uploaded on the Commission's webpages later in the year.

3. OIE Reference Centres

3.1. Applications for OIE Reference Laboratory status and designated expert

The Aquatic Animals Commission reviewed three applications for OIE Reference Laboratory status and a change of designated expert at an existing OIE Reference Laboratory and recommended that the following be approved:

OIE Reference Laboratory for Infection with infectious salmon anaemia virus

Aquaculture Pathology Laboratory, Genetic and Molecular Immunology Laboratory of the Pontificia Catholic University of Valparaíso, Avenida Universidad, 330 Valparaíso, Chile

Designated Reference Expert: Dr Sergio Hernán Marshall González.

OIE Reference Laboratory for White spot disease

No.1, University Road, Tainan City 701, Taiwan, College of 2 Bioscience and Biotechnology, National Cheng Kung University, Chinese Taipei

Designated Reference Expert: Prof. Grace Chu-Fang Lo.

OIE Reference Laboratory for Infection with *salmonid alphavirus*

National Veterinary Institute, P.O. Box 750, Sentrum, 0106 Oslo, Norway

Designated Reference Expert: Dr Torunn Taksdal.

OIE Reference Laboratory for Infection with *Gyrodactylus salaris*

Dr Haakon Hansen to replace Dr Tor Atle Mo as the Designated Reference Expert at the National Veterinary Institute, Norway.

3.2. Review of Annual reports of Reference Centre activities for 2013

Dr Min-Kyung Park, Scientific and Technical Department of the OIE, presented an analysis of the activities of the Reference Laboratories and Collaborating Centres for aquatic animal diseases in 2013. She informed the Aquatic Animals Commission that a new online web-based annual report template had been used by the Reference Laboratories for the first time in 2013.

The Aquatic Animals Commission expressed its on-going appreciation for the enthusiastic support and expert advice given to the OIE by the Reference Centres.

The Aquatic Animals Commission reviewed the responses from Reference Laboratories on implementing and maintaining internationally recognised quality management systems. The Commission recommended that this topic and validation of diagnostic tests be addressed at the Third Global Conference of OIE Reference Centres (see Item 4.).

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

Dr François Diaz, OIE Scientific and Technical Department, presented the draft programme for the Third Global Conference of the OIE Reference Centres. The Aquatic Animals Commission welcomed the inclusion of a parallel session on aquatic animals. The Commission agreed that specific issues of quality management systems, validation of diagnostic tests as well as the rating of diagnostic methods for different purpose should be addressed during this parallel session.

5. Aquatic Animals Commission Work Plan for 2014/2015

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

The detailed Aquatic Animals Commission's Work Plan for 2014/15 is presented at [Annex 24](#) for Member Countries information.

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

The Aquatic Animals Commission, as the Scientific Committee, progressed the development of a draft programme.

7. Next meeting

The next meeting is scheduled 29th September to 3rd October 2014.

.../Annexes

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

Paris, 24–28 February 2014

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

Paris, 24–28 February 2014

Adopted agenda

1. OIE *Aquatic Animal Health Code*
 - 1.1. Glossary
 - 1.2. Notification of diseases and epidemiological information (Chapter 1.1.)
 - 1.3. Criteria for listing an emerging disease (Chapter 1.2.)
 - 1.4. Diseases listed by the OIE (Chapter 1.3.)
 - 1.4.1. Infection with ostreid herpesvirus-1 microvariant
 - 1.4.2. Yellow head disease
 - 1.4.3. Acute hepatopancreatic necrosis syndrome
 - 1.4.4. Infectious hypodermal and hematopoietic necrosis virus
 - 1.5. Import risk analysis (Chapter 2.1.)
 - 1.6. Quality of Aquatic Animal Health Services (Chapter 3.1.)
 - 1.7. General recommendations of disinfection (Chapter 4.3.)
 - 1.8. General obligations related to certification (Chapter 5.1.)
 - 1.9. Certification procedures (Chapter 5.2.)
 - 1.10. Necrotising hepatopancreatitis (Chapter 9.4.)
 - 1.11. Horizontal issues
 - 1.12. Yellow head disease (Chapter 9.8.)
 - 1.13. Infection with infectious salmon anaemia virus (Chapter 10.5.)
 - 1.14. Infection with Salmonid alphavirus (new chapter 10.X.)
 - 1.15. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new Chapter X.X.)
 - 1.16. Guide to the Use of the *Aquatic Animal Health Code*
 - 1.17. General recommendations: disease prevention and control (Section 4)

Annex 2 (contd)

2. OIE *Manual of Diagnostic Tests for Aquatic Animals*
 - 2.1. Review of the *Aquatic Manual* chapters
 - 2.1.1. Chapter 2.3.5. Infection with infectious salmon anaemia virus
 - 2.1.2. Chapter 2.4.9. Infection with ostreid herpesvirus 1 microvariants
 - 2.1.3. Chapter 2.3.X. Infection with salmonid alphavirus virus
 - 2.1.4. Chapter 2.2.2. Infectious hypodermal and haematopoietic necrosis
 - 2.2. Other *Aquatic Manual* issues
 - 2.2.1. Yellow head disease (Chapter 2.3.5.)
 - 2.2.2. Proposal from a workshop on mollusc disease diagnosis
 - 2.2.3. Disease-specific guidance documents on surveillance for a mollusc and a crustacean disease
3. OIE Reference Centres
 - 3.1. Applications for OIE Reference Laboratory status and designated expert
 - 3.2. Review of Annual reports of Reference Centre activities for 2013
4. Third Global Conference of the OIE Reference Centres, Seoul (Rep. of Korea), 14–16 October 2014
5. Aquatic Animals Commission Work Plan for 2014/2015
6. OIE Global Conference on Aquatic Animal Health: ‘Riding the wave to the future’
7. Next meeting

GLOSSARY

Emerging disease

means a disease, other than listed diseases, newly recognised *infection* which has a significant impact on aquatic animal or public health resulting from: the evolution or

≡ a change of an known existing pathogenic agent, a known infection or its spreading to a new geographic area or species population; or

≡ a newly previously unrecognised or suspected pathogenic agent or disease diagnosed for the first time and which has a significant impact on aquatic animal or public health.

Susceptible species

means a species of *aquatic animal* in which *infection* has been demonstrated by the occurrence of natural cases or by experimental exposures to the *pathogenic agent* that mimics the natural transmission pathways for infection. Each disease chapter in the Aquatic Code and the Aquatic Manual contains a list of currently known susceptible species.

Notification

means the procedure by which:

- a) the Veterinary Competent Authority informs the Headquarters,
- b) the Headquarters inform Veterinary Competent Authorities of Member Countries

of the occurrence of a *disease*, according to the provisions of Chapter 1.1.

Pathogenic agent

means an organism that causes or contributes to the development of a *disease* referred to in the Aquatic Code.

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*.

Veterinarian

means a person with appropriate education, registered or licensed by the relevant *veterinary statutory body* of a country to practise veterinary medicine/science in that country.

— Text deleted.

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 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 Article 1.1.3. **and Article 1.1.3.bis.**
- 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. **This means that the detection of an infectious agent of a listed disease in an aquatic animal should be reported, even in the absence of clinical signs of disease.**
- 5) In addition to notifying findings in accordance with Article 1.1.3. **and 1.1.3.bis.** Member Countries shall also provide information on the measures taken to prevent the spread of *diseases*; **information shall include, including 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 a the final report that declared ~~an~~ the outbreak ended;

Annex 4 (contd)

- c) first occurrence of a new strain of a *pathogenic agent* of a *listed disease* new to in a country, a *zone* or a *compartment*;
- d) a sudden and unexpected change increase in the distribution; or increase in incidence or virulence of, or morbidity or mortality of caused by the aetiological pathogenic agent of a *listed disease*, prevalent present within a country, a *zone* or a *compartment*;
- e) ~~evidence of change in the epidemiology~~ first occurrence of a *listed disease*; in a new unusual host species (including host range, pathogenicity, strain) in particular if there is a zoonotic impact;
- f) ~~an emerging disease with significant morbidity or mortality, or zoonotic potential;~~

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; ~~in any for each event notified case~~, 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.

~~Although Member Countries are only required to notify *listed diseases* and *emerging diseases* according to points 1 to 4 above, they are encouraged to inform the OIE of other animal health events of epidemiological significance.~~

Article 1.1.3.bis

Veterinary 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 for an *emerging disease*, as described under point 1. These should continue until:
 - a) the *disease* has been eradicated; or
 - b) the situation becomes sufficiently stable; or
 - c) sufficient scientific information is available to determine whether it meets the criteria for listing.

Article 1.1.4.

- 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*.

Annex 4 (contd)

- 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 1.1.5.

- 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 1.1.4. and other relevant information.

— Text deleted.

CHAPTER 1.2.

CRITERIA FOR LISTING AQUATIC ANIMAL DISEASES

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' efforts to prevent the transboundary spread of important *diseases* of *aquatic animals* through transparent and consistent reporting.

For the *diseases* listed in accordance with Article 1.2.2., the corresponding *disease*-specific chapters in the *Aquatic Code* provide standards for safe *international trade* in *aquatic animals* and their products.

~~The purpose of listing *diseases* in accordance with Article 1.2.3. is to recognise important *emerging diseases* and collect relevant epidemiological information. This information is collected to enable later consideration of listing of the *disease* in accordance with Article 1.2.2. *Diseases* listed in accordance with Article 1.2.3. do not have a corresponding *disease*-specific chapter in the *Aquatic Code* and thus no specific standards for *international trade*. Member Countries should only institute *disease*-specific trade requirements where these are justified by a science-based *risk assessment*.~~

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

Article 1.2.2.

Criteria for listing an aquatic animal disease

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

No.	Criteria for listing	Explanatory notes
A. Consequences		
1.	The <i>disease</i> has been shown to cause significant production losses at a national or multinational (zonal or regional) level.	There is a general pattern that the <i>disease</i> 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 <i>disease</i> is linked to its morbidity, mortality and effect on product quality.
2.	Or	The <i>disease</i> has been shown to or scientific evidence indicates that it is likely to cause significant morbidity or mortality in wild aquatic animal populations.
3.	Or	The agent is of public health concern.
And B. Spread		
4.	Infectious aetiology of the <i>disease</i> is proven.	

Annex 5 (contd)

5.	Or	An infectious agent is strongly associated with the <i>disease</i> , but the aetiology is not yet known.	Infectious <i>diseases</i> of unknown aetiology can have equally high-risk implications as those <i>diseases</i> where the infectious aetiology is proven. Whilst <i>disease</i> occurrence data are gathered, research should be conducted to elucidate the aetiology of the <i>disease</i> and the results be made available within a reasonable period of time.
6.	And	Likelihood of international spread, including via live aquatic animals, their products or fomites.	International trade in aquatic animal species susceptible to the <i>disease</i> exists or is likely to develop and, under international trading practices, the entry and establishment of the <i>disease</i> is likely.

7.	And	Several countries or countries with zones may be declared free of the <i>disease</i> based on the general surveillance principles outlined in Chapter 1.4.	Free countries/zones could still be protected. Listing of <i>diseases</i> that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a <i>disease</i> can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread <i>diseases</i> , or the protection of the last remaining free zones from a widespread <i>disease</i> .
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And C. Diagnosis

8.		A repeatable and robust means of detection/diagnosis exists.	A diagnostic test should be widely available and preferably has undergone a formal standardisation and validation process using routine field samples (See <i>Aquatic Manual</i> .) or a robust case definition is available to clearly identify cases and allow them to be distinguished from other pathologies.
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Article 1.2.3.

Criteria for listing an emerging aquatic animal disease

An *emerging disease* may be proposed for listing if it meets the criteria 1 or 2, and 3 or 4. Such proposals should be accompanied by a case definition for the *disease* under consideration.

No.	Criteria for listing	Explanatory notes
1.	Infectious aetiology of the disease is proven.	
Or		
2.	An infectious agent is strongly associated with the disease, but the aetiology is not yet known.	Infectious diseases of unknown aetiology can have equally high risk implications as those diseases where the infectious aetiology is proven. Whilst disease occurrence data are gathered, research should be conducted to elucidate the aetiology of the disease and the results be made available within a reasonable period of time.
And		
3.	The agent is of public health concern.	
Or		
4.	Significant spread in naive populations of wild or cultured aquatic animals.	The disease has exhibited significant morbidity, mortality or production losses at a zone, compartment or country level. 'Naive' means animals previously unexposed either to a new disease or a new form of a known disease.

 — Text deleted.

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

Preamble: The following *diseases* are listed by the OIE according to the criteria for listing an *aquatic animal disease* (see Article 1.2.2.) ~~or criteria for listing an *emerging aquatic animal disease* (see Article 1.2.3.).~~

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

Article 1.3.1.

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

- Epizootic haematopoietic necrosis
- Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)
- Infection with *Gyrodactylus salaris*
- Infection with HPR-deleted or HPR0 infectious salmon anaemia virus
- Infection with salmonid alphavirus
- Infectious haematopoietic necrosis
- Koi herpesvirus disease
- Red sea bream iridoviral disease
- Spring viraemia of carp
- Viral haemorrhagic septicaemia.

Article 1.3.2.

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

- Infection with abalone herpesvirus
- Infection with *Bonamia ostreae*
- Infection with *Bonamia exitiosa*
- Infection with *Marteilia refringens*
- ~~Infection with ostreid herpesvirus 1 microvariant¹~~
- Infection with *Perkinsus marinus*
- Infection with *Perkinsus olseni*
- Infection with *Xenohaliotis californiensis*.

Article 1.3.3.

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

- Crayfish plague (*Aphanomyces astaci*)
- ~~Infection with yellow head virus disease~~
- Infectious hypodermal and haematopoietic necrosis
- Infectious myonecrosis
- Necrotising hepatopancreatitis
- Taura syndrome
- White spot disease
- White tail disease.

Annex 6 (contd)

Article 1.3.4.

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

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

- Text deleted.

1 ~~Listed according to Article 1.2.3.~~

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).

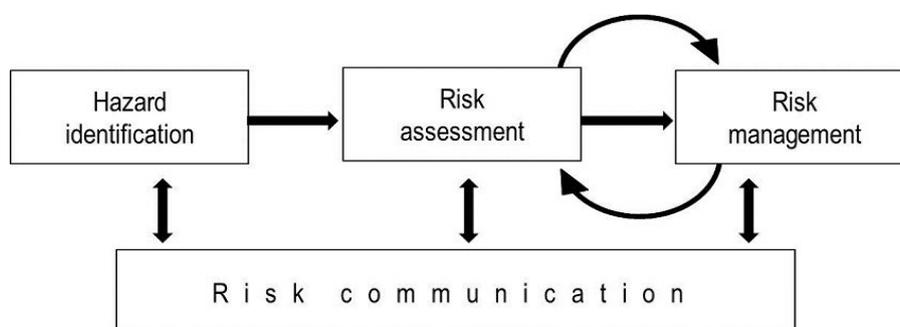


Fig. 1. The four components of risk analysis

The *risk assessment* is the component of the analysis that estimates the *risks* associated with a *hazard*. *Risk assessments* may be qualitative or quantitative. For many *diseases*, particularly for those *diseases* listed in the *Aquatic Code* where there are well developed internationally agreed standards, there is broad agreement concerning the likely *risks*. In such cases it is more likely that a qualitative assessment is all that is required. Qualitative assessment does not require mathematical modelling skills to carry out and so is often the type of assessment used for routine decision-making. No single method of import *risk assessment* has proven applicable in all situations, and different methods may be appropriate in different circumstances.

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

Annex 7 (contd)

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.

Article 2.1.4.

Risk assessment steps1. 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 7 (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.~~ 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 ~~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 ~~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 7 (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.

— Text deleted.

CHAPTER 3.1.

QUALITY OF AQUATIC ANIMAL HEALTH SERVICES

Article 3.1.1.

The quality of *Aquatic Animal Health Services* depends on a set of factors, which include fundamental principles of an ethical, organisational, legislative, regulatory and technical nature. The *Aquatic Animal Health Services* shall conform to these fundamental principles, regardless of the political, economic or social situation of their country.

Compliance with these fundamental principles by a Member Country's *Aquatic Animal Health Service* is important in the establishment and maintenance of confidence in its *aquatic animal health status* and *international aquatic animal health certificates* provided by the *Aquatic Animal Health Service* of other Member Countries.

These fundamental principles are presented in Article 3.1.2. Other factors to consider when evaluating *Aquatic Animal Health Services* are described in the *Aquatic Code* (*notification, principles of certification, etc.*).

The ability of *Aquatic Animal Health Services* to deliver appropriate services, monitor and control *aquatic animal diseases* based on Member Countries' *aquatic animal health* legislation and regulations, can be measured through an evaluation or audit whose general principles are described in Articles 3.1.3. and 3.1.4.

A procedure for evaluating *Aquatic Animal Health Services* by OIE experts, on a voluntary basis, is described in Article 3.1.5.

Article 3.1.2.

Fundamental principles of quality

Aquatic Animal Health Services should comply with the following principles to ensure the quality of their activities:

1. Professional judgement

Aquatic Animal Health Services should ensure that personnel have the relevant qualifications, scientific expertise and experience to give them the competence to make sound professional judgements.

2. Independence

Care should be taken to ensure that the *Aquatic Animal Health Service* personnel are free from any commercial, financial, hierarchical, political or other pressures which may inappropriately influence their judgement or decisions.

3. Impartiality

Aquatic Animal Health Services should be impartial. In particular, all the parties affected by their activities have a right to expect their services to be delivered under reasonable and non-discriminatory conditions.

4. Integrity

Aquatic Animal Health Services are responsible for ensuring that the work of each of their personnel is of a consistently high level of integrity. Any fraud, corruption or falsification should be identified, documented and corrected.

Annex 8 (contd)5. Objectivity

Aquatic Animal Health Services should conduct themselves, in an objective, transparent and non-discriminatory manner.

6. Aquatic animal health legislation and regulations

Aquatic animal health legislation and regulations are a fundamental element that supports good governance and provides the legal framework for all key activities of the *Aquatic Animal Health Service*.

Legislation and regulations should be suitably flexible to allow for judgements of equivalence and efficient responses to changing situations. In particular, they should define and document the responsibilities and structure of the organisations in charge of traceability and control of *aquatic animal* movements, *aquatic animal disease* control and reporting systems, epidemiological *surveillance* and communication of epidemiological information.

7. General organisation

Aquatic Animal Health Services should be able to demonstrate that they are in a position able to anticipate the requirements for and have control of the establishment and application of *aquatic animal* health measures, and of international *aquatic animal* health certification activities. This should be demonstrated by means of appropriate legislation and regulations, sufficient financial resources and effective organisation.

Aquatic Animal Health Services should have at their disposal effective systems for *aquatic animal disease surveillance, diagnosis* and *notification* of disease problems that may occur in the national territory, in accordance with the provisions of the *Aquatic Code*. They should at all times endeavour to improve their performance in terms of *aquatic animal* health information systems and *aquatic animal disease* control.

Aquatic Animal Health Services should define and document the responsibilities and structure of the organisation (in particular the chain of command) in charge of issuing *international aquatic animal health certificates*.

Each position within the *Aquatic Animal Health Services* that has an impact on their quality should be described. These job descriptions should include the requirements for education, training, technical knowledge and experience.

8. Quality policy

Aquatic Animal Health Services should define and document their policy and objectives for, and commitment to, quality, and should ensure that this policy is understood, implemented and maintained at all levels in the organisation. Where conditions allow, they may implement a quality system corresponding to their areas of activity and appropriate for the type, range and volume of work that they have to perform. The recommendations provided in this chapter describe a suitable reference system, which should be used if a Member Country chooses to adopt a quality system.

9. Procedures and standards

Aquatic Animal Health Services should develop and document appropriate procedures and standards for all providers of relevant activities and associated facilities. These procedures and standards may for example relate to:

- a) programming and management of activities, including international *aquatic animal* health certification activities;
- b) prevention, control and *notification* of *disease outbreaks*;
- c) *risk analysis, epidemiological surveillance* and zoning;

- d) emergency preparedness for disasters which could have an impact on aquatic animal health and welfare;
- de) inspection and sampling techniques;
- ef) diagnostic tests for *aquatic animal diseases*;
- fg) preparation, production, registration and control of *biological products* for use in the *diagnostic* or prevention of *diseases*;
- gh) border controls and import regulations;
- hi) *disinfection*;
- ij) treatments intended to inactivate pathogens in *aquatic animal* products.

Where there are standards in the *Aquatic Code* or in the *Aquatic Manual*, *Aquatic Animal Health Services* should comply with these standards when applying *aquatic animal* health measures and when issuing *international aquatic animal health certificates*.

10. Information, complaints and appeals

Aquatic Animal Health Services should undertake to reply to requests from *Aquatic Animal Health Services* of other Member Countries or any other authority, in particular ensuring that any requests for information, complaints or appeals that are presented are dealt with in a timely manner.

A record should be maintained of all complaints and appeals and of the relevant action taken by *Aquatic Animal Health Services*.

11. Documentation

Aquatic Animal Health Services should have at their disposal a reliable and up-to-date documentation system suited to their activities.

12. Self-evaluation

Aquatic Animal Health Services should undertake periodical self-evaluation especially by documenting achievements against goals, and demonstrating the effectiveness of their organisational components and resource adequacy.

A procedure for evaluating *Aquatic Animal Health Services* by OIE experts, on a voluntary basis, is described in Article 3.1.5.

13. Communication

Aquatic Animal Health Services should have effective internal and external systems of communication covering administrative and technical staff and parties affected by their activities.

14. Human and financial resources

Responsible authorities should ensure that adequate resources are made available to implement effectively the above activities.

Annex 8 (contd)

Article 3.1.3.

For the purposes of the *Aquatic Code*, every Member Country should recognise the right of another Member Country to undertake, or request it to undertake, an evaluation of its *Aquatic Animal Health Services* where the initiating Member Country is an actual or a prospective importer of *aquatic animal commodities* and/or where the evaluation is to be a component of a *risk analysis* process that is to be used to determine or review *sanitary measures* which apply to such trade.

A Member Country has the right to expect that the evaluation of its *Aquatic Animal Health Services* will be conducted in an objective and transparent manner. A Member Country undertaking an evaluation should be able to justify any measure taken as a consequence of its evaluation.

Article 3.1.4.

A Member Country which intends to conduct an evaluation of another Member Country's *Aquatic Animal Health Services* should provide notice in writing, and allow sufficient time for the other Member Country to comply with the request. This notice should define the purpose of the evaluation and details of the information required.

On receipt of a formal request for information to enable an evaluation of its *Aquatic Animal Health Services* by another Member Country, and following bilateral agreement of the evaluation process and criteria, a Member Country should expeditiously provide the Member Country requesting the evaluation with meaningful and accurate information of the type requested.

The evaluation process should take into account the fundamental principles and other factors of quality laid down in Article 3.1.1. and in Article 3.1.2. It should also take into consideration the specific circumstances regarding quality, as described in Article 3.1.1., prevailing in the countries concerned.

The outcome of an evaluation conducted by a Member Country should be provided in writing as soon as possible, and in any case within four months of receipt of the relevant information, to the Member Country which has undergone the evaluation. The evaluation report should detail any findings that affect trade prospects. The Member Country which conducts the evaluation should clarify in detail any points of the evaluation on request.

In the event of a dispute between two Member Countries over the conduct or the conclusions of the evaluation of *Aquatic Animal Health Services*, the matter should be dealt with having regard to the procedures set out in Article 3.1.3.

Article 3.1.5.

Evaluation facilitated by OIE experts under the auspices of the OIE

The OIE has established procedures for the evaluation of *Aquatic Animal Health Services* of Member Countries. Member Countries can make a request to the OIE for an evaluation of their *Aquatic Animal Health Services*.

The World Assembly of OIE Delegates may endorse a list of approved experts to facilitate the evaluation process. Under these procedures, the Director General of the OIE recommends an expert(s) from that list.

The expert(s) facilitate(s) the evaluation of the *Aquatic Animal Health Services* of the Member Country using the OIE *Performance of Veterinary Services and/or Aquatic Animal Health Services (OIE PVS Tool: Aquatic)*. The expert(s) produce(s) a report in consultation with the Veterinary Services of the Member Country.

The report is submitted to the Director General of the OIE and, with the consent of the Member Country, published by the OIE.

— Text deleted.

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.

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

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

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

Article 5.1.2.

Responsibilities of the importing country

- 1) The import requirements included in the *international aquatic animal health certificate* should assure that *commodities* introduced into the *importing country* comply with OIE standards. *Importing countries* should restrict their requirements to those recommended in the relevant standards of the OIE necessary to achieve the national appropriate level of protection. If there are no such standards or if the country wishes to establish more trade restrictive measures, if these are stricter than the OIE standards, they these should be based on an import *risk analysis*.
- 2) The *international aquatic animal health certificate* should not include requirements for the exclusion of *pathogenic agents* or *aquatic animal diseases* that are present in the *importing country* and are not subject to any official control programme, except when the strain of the *pathogenic agent* in the *exporting country* is of significantly higher pathogenicity and/or has a larger host range. The measures imposed on imports to manage the *risks* posed by a *pathogenic agent* or *aquatic animal disease* should not be more trade restrictive require a higher level of protection than the that provided by measures applied as part of the official control programme operating within the *importing country*.
- 3) The *international aquatic animal health certificate* should not include measures against *pathogenic agents* or *diseases* that are not OIE listed, unless the *importing country* has demonstrated through an import *risk analysis*, carried out in accordance with Section 2, that the *pathogenic agent* or *disease* poses a significant *risk* to the *importing country*.

Annex 9 (contd)

- 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.

Annex 9 (contd)

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

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

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

— Text deleted.

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 documentation sent directly from the *Competent Authority* of the *exporting country* to the *Competent Authority* of the *importing country*. ~~Normally, such systems also 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 information such as laboratory results and aquatic animal identification data.~~
 - 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 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).~~
- 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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NOTE: Horizontal changes proposed in Annex 12 will be applied to this chapter as for all disease-specific chapters once adopted. For clarity, only the proposed pathogen name amendment is shown here.

CHAPTER 9.4.

NECROTISING HEPATOPANCREATITIS

Article 9.4.1.

For the purposes of the *Aquatic Code*, necrotising hepatopancreatitis (NHP) means *infection with Candidatus *Hepatobacter penaei* necrotising hepatopancreatitis bacteria (NHP-B)*. This obligate intracellular bacterium is a member of the order α -Proteobacteria.

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

Article 9.4.2.

Scope

The recommendations in this chapter apply to: Pacific white shrimp (*Penaeus vannamei*), blue shrimp (*P. stylirostris*), northern white shrimp (*P. setiferus*) and northern brown shrimp (*P. aztecus*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

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

Article 9.4.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from necrotising hepatopancreatitis

- 1) *Competent Authorities* should not require any NHP related conditions, regardless of the NHP status of the *exporting country, zone or compartment* when authorising the importation or transit of the following *aquatic animals and aquatic animal products* from the species referred to in Article 9.4.2. intended for any purpose and complying with Article 5.4.1.:
 - a) heat sterilised hermetically sealed crustacean products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
 - b) cooked crustacean products that have been subjected to heat treatment at 100°C for at least 3 minutes (or any time/temperature equivalent which has been demonstrated to inactivate the *Candidatus H. penaei* NHP-B);
 - c) pasteurised crustacean products that have been subjected to heat treatment at 63°C for at least 30 minutes (or any time/temperature equivalent which has been demonstrated to inactivate the *Candidatus H. penaei* NHP-B);
 - d) crustacean oil;
 - e) crustacean *meal*; and
 - f) chemically extracted chitin.

Annex 11 (contd)

- 2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 9.4.2., other than those referred to in point 1 of Article 9.4.3., *Competent Authorities* should require the conditions prescribed in Articles 9.4.7. to 9.4.11. relevant to the NHP status of the *exporting country, zone or compartment*.
- 3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* from an *exporting country, zone or compartment* not declared free of NHP of a species not covered in Article 9.4.2. but which could reasonably be expected to pose a *risk* of transmission for NHP, *Competent Authorities* should conduct a *risk analysis* in accordance with the recommendations in the *Aquatic Code*. The *exporting country* should be informed of the outcome of this assessment.

Article 9.4.4.

Necrotising hepatopancreatitis free country

A country may make a *self-declaration of freedom* from NHP if it meets the conditions in points 1, 2, 3 or 4 below.

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

- 1) A country where none of the *susceptible species* referred to in Article 9.4.2. is present may make a *self-declaration of freedom* from NHP when *basic biosecurity conditions* have been continuously met in the country for at least the past two years.

OR

- 2) A country where the *susceptible species* referred to in Article 9.4.2. are present but there has been no observed occurrence of the *disease* for at least the past ten years despite conditions that are conducive to its clinical expression, as described in the corresponding chapter of the *Aquatic Manual*, may make a *self-declaration of freedom* from NHP when *basic biosecurity conditions* have been continuously met in the country for at least the past two years.

OR

- 3) A country where the last observed occurrence of the *disease* was within the past ten years or where the *infection* status prior to *targeted surveillance* was unknown (e.g. because of the absence of conditions conducive to its clinical expression as described in the corresponding chapter of the *Aquatic Manual*) may make a *self-declaration of freedom* from NHP when:

- a) *basic biosecurity conditions* have been continuously met for at least the past two years; and
- b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of Candidatus *H. penaei* NHP B.

OR

- 4) A country that has previously made a *self-declaration of freedom* from NHP but in which the *disease* is subsequently detected may make a *self-declaration of freedom* from NHP again when 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 (see *Aquatic Manual*) have been completed; and

Annex 11 (contd)

- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of Candidatus *H. penaei* NHP-B; and
- d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least the past two years.

In the meantime, part of the non-affected area may be declared a free *zone* provided that such part meets the conditions in point 3 of Article 9.4.5.

Article 9.4.5.

Necrotising hepatopancreatitis free zone or free compartment

A *zone* or *compartment* within the *territory* of one or more countries not declared free from NHP may be declared free by the *Competent Authority(ies)* of the country(ies) concerned if the *zone* or *compartment* meets the conditions referred to in points 1, 2, 3 or 4 below.

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

- 1) A *zone* or *compartment* where none of the *susceptible species* referred to in Article 9.4.2. is present may be declared free from NHP when *basic biosecurity conditions* have been continuously met in the *zone* or *compartment* for at least the past two years.

OR

- 2) A *zone* or *compartment* where the *susceptible species* referred to in Article 9.4.2. are present but in which there has not been any observed occurrence of the *disease* for at least the past ten years despite conditions that are conducive to its clinical expression, as described in the corresponding chapter of the *Aquatic Manual*, may be declared free from NHP when *basic biosecurity conditions* have been continuously met in the *zone* or *compartment* for at least the past two years.

OR

- 3) A *zone* or *compartment* where the last observed occurrence of the *disease* was within the past ten years or where the *infection* status prior to *targeted surveillance* was unknown (e.g. because of the absence of conditions conducive to its clinical expression as described in the corresponding chapter of the *Aquatic Manual*) may be declared free from NHP when:

- a) *basic biosecurity conditions* have been continuously met for at least the past two years; and
- b) *targeted surveillance*, as described in Chapter 1.4., has been in place, through the *zone* or *compartment*, for at least the past two years without detection of Candidatus *H. penaei* NHP-B.

OR

- 4) A *zone* previously declared free from NHP but in which the *disease* is subsequently detected may again be declared free from NHP when 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 (see *Aquatic Manual*) have been completed; and
- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of Candidatus *H. penaei* NHP-B; and

Annex 11 (contd)

- d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least the past two years.

Article 9.4.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from NHP following the provisions of points 1 or 2 of Articles 9.4.4. or 9.4.5. (as relevant) may maintain its status as NHP free provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from NHP following the provisions of point 3 of Articles 9.4.4. or 9.4.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as IMN free provided that conditions that are conducive to clinical expression of NHP, as described in the corresponding chapter of the *Aquatic Manual*, exist, and *basic biosecurity conditions* are continuously maintained.

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

Article 9.4.7.

Importation of live aquatic animals from a country, zone or compartment declared free from necrotising hepatopancreatitis

When importing live *aquatic animals* of species referred to in Article 9.4.5. from a country, *zone* or *compartment* declared free from NHP, the *Competent Authority* of the *importing country* should require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* attesting that, on the basis of the procedures described in Articles 9.4.4. or 9.4.5. (as applicable), the place of production of the *aquatic animal* is a country, *zone* or *compartment* declared free from NHP.

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

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

Article 9.4.8.

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

- 1) When importing, for *aquaculture*, live *aquatic animals* of species referred to in Article 9.4.2. from a country, *zone* or *compartment* not declared free from NHP, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:
 - a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and
 - b) the treatment of all effluent and waste materials in a manner that ensures inactivation of Candidatus *H. penaei* NHP-B.
- 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:

Annex 11 (contd)

- a) identify stock of interest (cultured or wild) in its current location;
 - b) evaluate stock health/disease history;
 - c) take and test samples for Candidatus *H. penaei* NHP-B, pests and general health/disease status;
 - d) import and quarantine of a founder (F-0) population in a secure facility;
 - e) produce F-1 generation from the F-0 stock in *quarantine*;
 - f) culture F-1 stock and at critical times in its development (life cycle) sample and test for Candidatus *H. penaei* NHP-B and perform general examinations for pests and general health/disease status;
 - g) if Candidatus *H. penaei* NHP-B 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 NHP free or specific pathogen free (SPF) for Candidatus *H. penaei* NHP-B;
 - h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the *country, zone or compartment*.
- 4) With respect to point 3e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

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

Article 9.4.9.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from necrotising hepatopancreatitis

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

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 9.4.3., or products described in point 1 of Article 9.4.11., or other products authorised by the *Competent Authority*; and
- 2) all effluent and waste materials from the processing are treated in a manner that ensures inactivation of Candidatus *H. penaei* NHP-B or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 9.4.10.

Importation of aquatic animal products from a country, zone or compartment declared free from necrotising hepatopancreatitis

When importing *aquatic animal products* of species referred to in Article 9.4.2. from a *country, zone or compartment* declared free from NHP, the *Competent Authority* of the *importing country* should require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* attesting that, on the basis of the procedures described in Articles 9.4.4. or 9.4.5. (as applicable), the place of production of the consignment is a *country, zone or compartment* declared free from NHP.

Annex 11 (contd)

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

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

Article 9.4.11.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from necrotising hepatopancreatitis

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

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

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

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

— Text deleted.

Model amphibian, crustacean, fish and mollusc disease chapter (Clean text)

Article X.X.1.

[...]

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

[...]

Article X.X.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from 'infection with pathogen X'/'disease X'

[...]

- 3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article X.X.2. but which could reasonably be expected to pose a *risk* of spread of 'infection with pathogen X'/'disease X', the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in *Chapter 2.1*. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.

[...]

Article X.X.4.

Country free from 'Infection with pathogen X'/'Disease X'

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

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from 'infection with pathogen X'/'disease X' if:

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

OR

- 2) any of the *susceptible species* referred to in Article X.X.2. are present and the following conditions have been met:
- a) there has been no observed occurrence of the *disease* for at least the last 'X' years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*), and
 - b) *basic biosecurity conditions* have been continuously met for at least the last 'X' years.

OR

- 3) the *disease* status prior to *targeted surveillance* is unknown but the following conditions have been met:
- a) *basic biosecurity conditions* have been continuously met for at least the last 'X' years; and
 - b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last 'X' years without detection of 'infection with pathogen X'/'disease X'.

Annex 12A (contd)

OR

- 4) it previously made a *self-declaration of freedom* from 'infection with pathogen X'/disease X' and subsequently lost its *disease free status* due to the detection of 'infection with pathogen X'/disease X' 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 (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 'X' years without detection of 'infection with pathogen X'/disease X'.

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

Article X.X.5.

Zone or compartment free from 'infection with pathogen X'/disease X'

If a *zone* or *compartment* extends over more than one country, it can only be declared a zone or compartment free from 'infection with pathogen X'/disease X' 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 pathogen X'/disease X' 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 X.X.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last 'X' years.

OR

- 2) the *susceptible species* referred to in Article X.X.2. are present in the *zone* or *compartment* but the following conditions have been met:
 - a) there has not been any observed occurrence of the *disease* for at least the last 'X' years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*), and
 - b) *basic biosecurity conditions* have been continuously met for at least the last 'X' years.

OR

- 3) the disease status prior to *targeted surveillance* is unknown but the following conditions have been met:
 - a) *basic biosecurity conditions* have been continuously met for at least the last 'X' years; and
 - b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last 'X' years without detection of 'infection with pathogen X'/'disease X'.

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from 'infection with pathogen X'/disease X' and subsequently lost its *disease free* status due to detection of 'infection with pathogen X'/disease X' in the *zone* but the following conditions have been met:
- a) on detection of the *disease*, the affected area was declared an *infected zone* and a *protection zone* was established; and
 - b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the *risk* of further spread of the *disease*, and the appropriate *disinfection* procedures (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 'X' years without detection of 'infection with pathogen X'/disease X'.

[...]

Article X.X.7.

Importation of live aquatic animals from a country, zone or compartment declared free from 'infection with pathogen X'/disease X'

When importing live *aquatic animals* of the species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from 'infection with pathogen X'/disease X', the *Competent Authority* of the *importing country* should require 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* is a country, *zone* or *compartment* declared free from 'infection with pathogen X'/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.9.

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

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article X.X.2. from a country, *zone* or *compartment* not declared free from 'infection with pathogen X'/disease X', the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article X.X.3., or products described in point 1 of Article X.X.12., or other products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 'Pathogen X' or is disposed in a manner that prevents contact of waste with *susceptible species*.

Annex 12A (contd)

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

[...]

Article X.X.10. **(Add this article to mollusc and crustacean disease-specific chapters)**

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

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

- 1) the consignment is delivered directly to and held in *quarantine* facilities for slaughter and processing into products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 'Pathogen X'. This Article does not apply to *commodities* referred to in point 1 of Article X.X.3.

[...]

Article X.X.11. **(in fish and amphibian disease-specific chapters)** and

Article X.X.10. **(in mollusc and crustacean disease-specific chapters)**

Importation of aquatic animal products from a country, zone or compartment declared free from 'infection with pathogen X'/disease X'

When importing *aquatic animal products* of the species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from 'infection with pathogen X'/disease X', the *Competent Authority* of the *importing country* should require 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 *commodity* is a country, *zone* or *compartment* declared free from 'infection with pathogen X'/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.

[...]

— Text deleted.

Model amphibian, crustacean, fish and mollusc disease chapter (Track Changes)

Article X.X.1.

[...]

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

[...]

Article X.X.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from 'Infection with pathogen X'/'Disease X'

[...]

- 3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* ~~from an exporting country, zone or compartment not declared free from 'Infection with pathogen X'/'Disease X'~~ of a species not covered in Article X.X.2. but which could reasonably be expected to pose a *risk of transmission spread* of 'infection with pathogen X'/'disease X', the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The Competent Authority of the *exporting country* should be informed of the outcome of this assessment.

[...]

Article X.X.4.

'Disease X' free Country free from 'Infection with pathogen X'/'Disease X'

~~A country may make a self-declaration of freedom from crayfish plague if it meets the conditions in points 1, 2, 3 or 4 below.~~

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

As described in Article 1.4.6., a country may make a self-declaration of freedom from 'infection with pathogen X'/'disease X' if:

- 1) ~~A country where~~ none of the *susceptible species* referred to in Article X.X.2. ~~are~~ is present ~~may make a self-declaration of freedom from crayfish plague when~~ and basic biosecurity conditions have been continuously met in the *country* for at least the last 'X' years.

OR

- 2) ~~A country where~~ any of the susceptible species referred to in Article X.X.2. are present and but the following conditions have been met:

- a) there has been no observed occurrence of the *disease* for at least the last 'X' years despite conditions that are conducive to its clinical expression; ~~(as described in the corresponding chapter of the Aquatic Manual), may make a self-declaration of freedom from 'Disease X' and~~
- b) ~~when~~ basic biosecurity conditions have been continuously met ~~in the country~~ for at least the last 'X' years.

OR

Annex 12B (contd)

3. ~~A country where the last observed occurrence of the disease was within the past 'X' years or where the disease status prior to targeted surveillance was is unknown, (e.g. because of the absence of conditions conducive to its clinical expression (as described in the corresponding chapter of the Aquatic Manual) may make a self-declaration of freedom from 'Disease X' when but the following conditions have been met:~~
- a) ~~basic biosecurity conditions have been continuously met for at least the least 'X' years; and~~
 - b) ~~targeted surveillance, as described in Chapter 1.4., has been in place for at least the least 'X' years without detection of 'infection with pathogen X'/disease X'.~~

OR

4. ~~A country that has it previously made a self-declaration of freedom from 'Infection with pathogen X'/disease X' and subsequently lost its disease free status due to the detection of infection with pathogen X'/disease X' in which the disease is subsequently detected may make a self-declaration of freedom from 'Disease X' again when 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 (described in the see 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~~
 - ed) ~~targeted surveillance, as described in Chapter 1.4., has been in place for at least the least 'X' years without detection of 'infection with pathogen X'/disease X'; and~~
 - d) ~~previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place for at least the past 'X' years.~~

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

Article X.X.5.

Zone or compartment free from 'Infection with pathogen X'/Disease X' free zone or free compartment

If a zone or compartment extends over more than one country, it can only be declared a 'infection with pathogen X'/disease X' free zone or compartment if all the relevant Competent Authorities confirm that all relevant conditions have been met.

As described in Article 1.4.6., a zone or compartment within the territory of one or more countries not declared free from 'Infection with pathogen X'/Disease X' may be declared free by the Competent Authority(ies) of the country(ies) concerned if, the zone or compartment meets the conditions referred to in points 1, 2, 3 or 4 below.

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

- 4) ~~A zone or compartment where none of the susceptible species referred to in Article X.X.2. is are present in the zone or compartment may be declared free from 'Disease X' when and basic biosecurity conditions have been continuously met in the zone or compartment for at least the least 'X' years.~~

OR

2. ~~A zone or compartment where the susceptible species referred to in Article X.X.2. are present in the zone or compartment but the following conditions have been met:~~

Annex 12B (contd)

- a) ~~in which~~ there has not been any observed occurrence of the *disease* for at least the past 'X' years despite conditions that are conducive to its clinical expression; ~~(as described in the corresponding chapter of the *Aquatic Manual*).~~ may be declared free from 'Disease X' when and
- b) *basic biosecurity conditions* have been continuously met ~~in the zone or compartment~~ for at least the past 'X' years.

OR

3. ~~A zone or compartment where the last observed occurrence of the disease was within the past 'X' years or where the infection the *disease* status prior to targeted surveillance was is unknown (e.g. because of the absence of conditions conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*)) may be declared free from 'Disease X' when but the following conditions have been met:~~
- a) *basic biosecurity conditions* have been continuously met for at least the past 'X' years; and
- b) *targeted surveillance*, as described in Chapter 1.4., has been in place, ~~through in~~ in the *zone* or *compartment*, for at least the past 'X' years and no *infection* with without detection of 'infection with pathogen X'/'disease X'.

OR

4. ~~A zone it previously made a self-declaration of freedom for a zone from 'Infection with pathogen X'/'Disease X' declared free from 'Disease X' but and subsequently lost its disease free status due to the detection of 'infection with pathogen X'/'disease X' in the zone in which the disease is subsequently detected may again be declared free from 'Disease X' when 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 (described in the see *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
- ed) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past 'X' years without detection of 'infection with pathogen X'/'disease X'; and
- d) ~~previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least the past 'X' years.~~

[...]

Article X.X.7.

Importation of live aquatic animals from a country, zone or compartment declared free from 'Infection with pathogen X'/'Disease X'

When importing live *aquatic animals* of the species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from 'infection with pathogen X'/'disease X', the *Competent Authority* of the *importing country* should require 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* is a country, *zone* or *compartment* declared free from 'infection with pathogen X'/'disease X'.

Annex 12B (contd)

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.9.

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

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article X.X.2. from a country, *zone* or *compartment* not declared free from 'Infection with pathogen X'/'Disease X', the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article X.X.3., or products described in point 1 of Article X.X.12., or other products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 'Pathogen X' or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

[...]

Article X.X.10. **(This article will be added to mollusc and crustacean disease-specific chapters)**

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

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

- 1) the consignment is delivered directly to and held in *quarantine* facilities for slaughter and processing into products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 'Disease X'.

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

[...]

Article X.X.11. **(In fish and amphibian disease-specific chapters)** and

Article X.X.10. **(In mollusc and crustacean disease-specific chapters this article will become X.X.11 due to addition of the article above)**

Importation of aquatic animal products from a country, zone or compartment declared free from 'Infection with pathogen X'/'Disease X'

When importing *aquatic animal products* of the species referred to in Article X.X.2. from a country, *zone* or *compartment* declared free from 'infection with pathogen X'/'disease X', the *Competent Authority* of the *importing country* should require 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 *commodity* is a country, *zone* or *compartment* declared free from 'infection with pathogen X'/'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.

[...]

— Text deleted.

CHAPTER 9.8.

INFECTION WITH YELLOW HEAD DISEASE VIRUS

Article 9.8.1.

For the purposes of the *Aquatic Code*, infection with yellow head disease virus (YHD) means *infection* with yellow head virus genotype 1 (YHV). YHV and the related gill-associated virus are ~~is~~ classified as a species in the genus *Okavirus*, family *Roniviridae* and order *Nidovirales*. Common synonyms are listed in the corresponding chapter of the *Aquatic Manual*.

[...]

— Text deleted.

CHAPTER 10.5.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

Article 10.5.1.

For the purposes of the *Aquatic Code*, infection with infectious salmon anaemia virus (ISAV) means *infection* with HPR0 (non-deleted highly polymorphic region) or HPR-deleted ISAV of the genus *Isavirus* of the family *Orthomyxoviridae*. Both genotypes should be notified in accordance with the *Aquatic Code*.

There is a link between non-pathogenic HPR0 ISAV and pathogenic HPR-deleted ISAV, with some *outbreaks* potentially occurring as a result of the emergence of HPR-deleted from HPR0.

The provisions in this chapter are provided in recognition of three possible levels of *disease* status with respect to ISAV:

- 1) HPR0 ISAV and HPR-deleted ISAV free;
- 2) HPR0 ISAV endemic (but HPR-deleted ISAV free);
- 3) HPR0 ISAV and HPR-deleted ISAV endemic.

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

Article 10.5.2.

Scope

The recommendations in this chapter apply to: Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*) and rainbow trout (*Onchorynchus mykiss*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 10.5.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to ISAV are for any detectable ISAV, including HPR0 ISAV.

- 1) *Competent Authorities* should not require any conditions related to infection with ISAV, regardless of the ISAV status of the *exporting country, zone or compartment* when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 10.5.2. intended for any purpose and complying with Article 5.4.1.:
 - a) heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
 - b) pasteurised fish products that have been subjected to a heat treatment at 90°C for at least 10 minutes (or to any time/temperature equivalent which has been demonstrated to inactivate ISAV);
 - c) mechanically dried, eviscerated fish (i.e. a heat treatment at 100°C for 30 minutes or any time/temperature equivalent which has been demonstrated to inactivate ISAV);
 - d) fish oil;
 - e) fish *meal*; and
 - f) fish skin leather.

Annex 14 (contd)

- 2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 10.5.2., other than those referred to in point 1 of Article 10.5.3., *Competent Authorities* should require the conditions prescribed in Articles 10.5.10. to 10.5.17. relevant to the ISAV status of the *exporting country, zone or compartment*.
- 3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* ~~from an exporting country, zone or compartment not declared free from infection with ISAV~~ of a species not covered in Article 10.5.2. but which could reasonably be expected to pose a *risk of transmission spread of infection with ISAV*, ~~the~~ *Competent Authorities* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. the Aquatic Code. The Competent Authority of the exporting country should be informed of the outcome of this assessment.

Article 10.5.4.

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.

~~A country may make a self-declaration of freedom from infection with ISAV if it meets the conditions in points 1, 2 or 3 below.~~

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 10.5.6.).

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

- 1) ~~A country where~~ none of the *susceptible species* referred to in Article X.X.2. ~~are~~ ~~is~~ present ~~may make a self-declaration of freedom from crayfish plague when~~ and basic biosecurity conditions have been continuously met ~~in the country~~ for at least the past two years.

OR

- 2) ~~A country where~~ any of the *species* referred to in Article 10.5.2. are present ~~but~~ ~~and~~ there has been no detectable occurrence of infection with ISAV ~~may make a self-declaration of freedom from infection with ISAV when:~~ but the following conditions have been met:

- a) *basic biosecurity conditions* have been continuously met for at least the past two years; and
- b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of infection with ISAV.

OR

- 3) ~~A country that has~~ it previously made a *self-declaration of freedom* from infection with ISAV ~~and subsequently lost its disease free status due to the detection of~~ ~~but in which~~ infection with ISAV is subsequently detected ~~may make a self-declaration of freedom from infection with ISAV again when~~ but the following conditions have been met:

- a) on detection of the disease any 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 ISAV, and the appropriate *disinfection* procedures (described in the see Aquatic Manual) have been completed; and

Annex 14 (contd)

- 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
- ed) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of infection with ISAV;
- d) ~~previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place for at least two years following since eradication of the disease.~~

In the meantime, part or all of the non-affected area may be declared a free zone provided that such part meets the conditions in point 3 of Article 10.5.6.

The pathway for self-declaration of freedom from infection with ISA 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 10.5.5.

Country free from infection with HPR-deleted infectious salmon anaemia virus

In this article, all statements refer to a country free from infection with HPR-deleted ISAV but not necessarily free from infection with HPR0 ISAV.

~~A country may make a self-declaration of freedom from infection with HPR deleted ISAV if it meets the conditions in points 1, 2, or 3 or 4 below.~~

If a country shares a zone with one or more other countries, it can only make a *self-declaration of freedom* from infection with HPR-deleted ISAV if all the areas covered by the shared water bodies are declared countries or zones free from infection with HPR-deleted ISAV (see Article 10.5.7.).

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

- 1) ~~A country where none of the susceptible species is present may make a self-declaration of freedom from infection with HPR deleted ISAV when basic biosecurity conditions have been continuously met in the country for at least the past two years.~~

OR

- 12) A country where any of the susceptible species referred to in Article 10.5.2. are present and but the following conditions have been met:

- a) there has been no observed occurrence of infection with HPR-deleted ISAV for at least the past ten years despite conditions that are conducive to clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); ~~may make a self-declaration of freedom from infection with HPR deleted ISAV when and~~

- b) *basic biosecurity conditions* have been continuously met in the country for at least the past ten years.

OR

Annex 14 (contd)

23) ~~A country where the last observed occurrence of infection with HPR-deleted ISAV was within the past ten years or where the disease status prior to targeted surveillance is was unknown (e.g. because of the absence of conditions conducive to clinical expression as described in the corresponding chapter of the Aquatic Manual) may make a self-declaration of freedom from infection with HPR-deleted ISAV when but the following conditions have been met:~~

- a) ~~basic biosecurity conditions~~ have been continuously met for at least the ~~last~~ past two years; and
- b) ~~targeted surveillance~~, as described in Chapter 1.4., has been in place for at least the ~~last~~ past two years without detection of infection with HPR-deleted ISAV.

OR

34) ~~A country that has it previously made a self-declaration of freedom from infection with HPR-deleted ISAV and subsequently lost its disease free status due to the detection of but in which infection with HPR-deleted ISAV is subsequently detected may make a self-declaration of freedom from infection with HPR-deleted ISAV again when but~~ the following conditions have been met:

- a) on detection of infection with HPR-deleted 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 HPR-deleted ISAV, and the appropriate *disinfection* procedures (~~described in the see~~ *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.~~
- ed) ~~targeted surveillance~~, as described in Chapter 1.4., has been in place for at least the ~~last~~ past two years without detection of infection with HPR-deleted ISAV; ~~and.~~
- d) ~~previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place for at least two years following eradication of the disease.~~

In the meantime, part, ~~or all~~, of the non-affected area may be declared a free *zone* provided that such part meets the conditions in point 3 of Article 10.5.7.

Article 10.5.6.

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 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; the zone or compartment meets the conditions referred to in points 1, 2 or 3 below.~~

- 1) ~~A zone or compartment where~~ none of the *susceptible species* ~~referred to in Article 10.5.2. are is~~ present may be declared free from infection with ISAV when ~~in the zone or compartment and~~ *basic biosecurity conditions* have been continuously met ~~in the zone or compartment~~ for at least the ~~last~~ past two years.

OR

- 2) ~~A zone or compartment where~~ the species referred to in Article 10.5.2. are present ~~but and~~ there has been no detectable occurrence of infection with ISAV ~~may be declared free from infection with ISAV when:~~ but the following conditions have been met:
- a) *basic biosecurity conditions* have been continuously met for at least the ~~l~~past two years; and
 - b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the ~~l~~past two years without detection of infection with ISAV.

OR

- 3) ~~it A zone~~ 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~~ but in which any infection with ISAV in the zone is detected may be declared free from infection with ISAV again when the ~~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 ISAV, and the appropriate *disinfection* procedures (described in the see 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
 - ed) targeted surveillance, as described in Chapter 1.4., has been in place for at least the ~~l~~past two years without detection of infection with ISAV; ~~and~~
 - d) previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place for at least two years following eradication of the disease.

Article 10.5.7.

Zone or compartment free from infection with HPR-deleted infectious salmon anaemia virus

In this article, all statements refer to a *zone* or *compartment* free from infection with HPR-deleted ISAV but not necessarily free from infection with 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 HPR-deleted ISAV if all the relevant Competent Authorities confirm that all relevant conditions have been met.

As described in Article 1.4.6., a A zone or compartment within the territory of one or more countries not declared free from infection with HPR-deleted ISAV may be declared free by the Competent Authority(ies) of the country(ies) concerned if: the zone or compartment meets the conditions referred to in points 1, 2, or 3 or 4 below.

- 1) ~~A zone or compartment where none of the susceptible species is present may be declared free from infection with HPR-deleted ISAV when basic biosecurity conditions have been continuously met in the zone or compartment for at least the past two years.~~

OR

- 12) ~~A zone or compartment where~~ the susceptible species referred to in Article 10.5.2. are present in the zone or compartment ~~but the following conditions have been met:~~

Annex 14 (contd)

a) there has been no observed occurrence of infection with HPR-deleted ISAV for at least the past ten years despite conditions that are conducive to its clinical expression, (as described in the corresponding chapter of the *Aquatic Manual*); and

b) ~~may be declared free from infection with HPR-deleted ISAV when~~ basic biosecurity conditions have been continuously met in the zone or compartment for at least the ~~past~~ ten years.

OR

23) ~~A zone or compartment where the last observed occurrence of infection with HPR-deleted ISAV was within the past ten years or where the disease status prior to targeted surveillance is was unknown (e.g. because of the absence of conditions conducive to clinical expression as described in the corresponding chapter of the Aquatic Manual) may be declared free from infection with HPR-deleted ISAV when: but the following conditions have been met:~~

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

b) targeted surveillance, as described in Chapter 1.4., has been in place for at least the ~~past~~ two years without detection of infection with HPR-deleted ISAV.

OR

34) ~~it A zone previously made a self-declaration of freedom for a zone from infection with HPR-deleted ISAV and subsequently lost its disease free status due to the detection of but in which infection with HPR-deleted ISAV in the zone is detected may be declared free from infection with HPR-deleted ISAV again when but the following conditions have been met:~~

a) on detection of infection with HPR-deleted 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 HPR-deleted ISAV~~, and the appropriate *disinfection* procedures (~~described in the see 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.~~

ed) targeted surveillance, as described in Chapter 1.4., has been in place for at least the ~~past~~ two years without detection of infection with HPR-deleted ISAV; and.

d) ~~previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place for at least two years following eradication of the disease.~~

Article 10.5.8.

Maintenance of free status for infection with infectious salmon anaemia virus

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

A country, zone or compartment that is declared free from infection with ISAV following the provisions of point 1 of Articles 10.5.4. or 10.5.6. (as relevant) may maintain its status as free from infection with ISAV provided that *basic biosecurity conditions* are continuously maintained.

A country, zone or compartment that is declared free from infection with ISAV following the provisions of point 2 of Articles 10.5.4. or 10.5.6. (as relevant) may maintain its status as free from infection with ISAV provided that *targeted surveillance* is continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*, and *basic biosecurity conditions* are continuously maintained.

Article 10.5.9.

Maintenance of free status for infection with HPR-deleted infectious salmon anaemia virus

In this article, all statements refer to a country, *zone* or *compartment* free from infection with HPR-deleted ISAV, but not necessarily free from infection with HPR0 ISAV.

A country, *zone* or *compartment* that is declared free from infection with HPR-deleted ISAV following the provisions of points 1 or 2 of Articles 10.5.5. or 10.5.7. (as relevant) may maintain its status as free from infection with ISAV provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with HPR-deleted ISAV following the provisions of point 3 of Articles 10.5.5. or 10.5.7. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions that are conducive to clinical expression, as described in the corresponding chapter of the *Aquatic Manual*, exist and *basic biosecurity conditions* are continuously maintained.

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

Article 10.5.10.

Importation of live aquatic animals from a country, zone or compartment declared free from infection with infectious salmon anaemia virus

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

When importing live *aquatic animals* of the species referred to in Article 10.5.2. from a country, *zone* or *compartment* declared free from infection with ISAV, the *Competent Authority* of the *importing country* should require 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 10.5.4. or 10.5.6. (as applicable) and 10.5.8., the place of production of the *aquatic animal* is a country, *zone* or *compartment* declared free from infection with ISAV.

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 10.5.3.

Article 10.5.11.

Importation of live aquatic animals from a country, zone or compartment declared free from infection with HPR-deleted infectious salmon anaemia virus

In this article, all statements refer to a country, *zone* or *compartment* free from infection with HPR-deleted ISAV, but not necessarily free from infection with HPR0 ISAV.

When importing live *aquatic animals* of the species referred to in Article 10.5.2. from a country, *zone* or *compartment* declared free from infection with HPR-deleted ISAV, the *Competent Authority* of the *importing country* should require 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 10.5.5. or 10.5.7. (as applicable) and 10.5.9., the place of production of the *aquatic animal* is a country, *zone* or *compartment* declared free from infection with HPR-deleted ISAV.

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

Annex 14 (contd)

This Article does not apply to *commodities* referred to in point 1 of Article 10.5.3.

Article 10.5.12.

Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

- 1) When importing, for *aquaculture*, live *aquatic animals* of the species referred to in Article 10.5.2. from a country, *zone* or *compartment* not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:
 - a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and
 - b) water used in transport and the treatment of all effluent and waste materials in a manner that ensures inactivation of ISAV.
- 2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.
- 3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: <http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx>) may be summarised to the following points:
 - a) identify stock of interest (cultured or wild) in its current location;
 - b) evaluate stock health/*disease* history;
 - c) take and test samples for ISAV, pests and general health/*disease* status;
 - d) import and quarantine in a secure facility of a founder (F-0) population;
 - 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 ISAV and perform general examinations for pests and general health/*disease* status;
 - g) if ISAV 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 ISAV free or specific pathogen free (SPF) for ISAV;
 - h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.
- 4) With respect to point 3e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* referred to in point 1 of Article 10.5.3.

Article 10.5.13.

Importation of aquatic animals and aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 10.5.2. from a country, zone or compartment not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 10.5.3., or products described in point 1 of Article 10.5.16., or other products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of ISAV or is disposed in a manner that prevents contact of waste with *susceptible species*.

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

Article 10.5.14.

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

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

When importing, for use in animal *feed*, or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of the species referred to in Article 10.5.2. from a country, zone or compartment not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to and held in *quarantine* for slaughter and processing into products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of ISAV.

This Article does not apply to *commodities* referred to in point 1 of Article 10.5.3.

Article 10.5.15.

Importation of aquatic animal products from a country, zone or compartment declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

When importing *aquatic animal products* of the species referred to in Article 10.5.2. from a country, zone or compartment declared free from infection with ISAV, the *Competent Authority* of the *importing country* should require 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 10.5.4., ~~10.5.5.~~, ~~10.5.6.~~ or 10.5.7~~6~~. (as applicable) and 10.5.8. the place of production of the *commodity* is a country, zone or compartment declared free from infection with ISAV.

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

Annex 14 (contd)

This Article does not apply to *commodities* referred to in point 1 of Article 10.5.3.

Article 10.5.15.bis**Importation of aquatic animal products from a country, zone or compartment declared free from infection with HPR-deleted infectious salmon anaemia virus**

In this article, all statements refer to a country, zone or compartment free from infection with HPR-deleted ISAV, but not necessarily free from infection with HPR0 ISAV.

When importing *aquatic animal products* of the species referred to in Article 10.5.2. from a country, zone or compartment declared free from infection with HPR-deleted ISAV, the *Competent Authority* of the *importing country* should require 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 10.5.5. or 10.5.7. (as applicable) and 10.5.9., the place of production of the *commodity* is a country, zone or compartment declared free from infection with HPR-deleted ISAV.

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 10.5.3.

Article 10.5.16.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

- 1) *Competent Authorities* should not require any conditions related to infection with ISAV, regardless of the ISAV status of the *exporting country, zone or compartment*, when authorising the importation or transit of **fish fillets or steaks (frozen or chilled)** the following ~~*commodities*~~ which have been prepared and packaged for retail trade and which comply with Article 5.4.2.:
 - a) **fish fillets or steaks (frozen or chilled).**

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

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

- 2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of the species referred to in Article 10.5.2. from a country, zone or compartment not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Article 10.5.17.

Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with infectious salmon anaemia virus

In this article, all statements referring to infection with ISAV are for any detectable ISAV, including HPR0 ISAV.

Annex 14 (contd)

- 1) When importing disinfected eggs of the species referred to in Article 10.5.2. for *aquaculture*, from a country, zone or compartment not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should assess the *risk* associated with at least:
 - a) the ISAV status of the water to be used during the *disinfection* of the eggs;
 - b) the level of *infection* with ISAV in broodstock (ovarian fluid and milt); and
 - c) the temperature and pH of the water to be used for *disinfection*.

- 2) If the *Competent Authority* of the *importing country* concludes that the importation is acceptable, it should apply the following *risk* mitigation measures including:
 - a) the eggs should be disinfected prior to importing, according to the methods described in Chapter 1.1.3. of the *Aquatic Manual* (under study) or those specified by the *Competent Authority* of the *importing country*; and
 - b) between *disinfection* and the import, eggs should not come into contact with anything which may affect their health status.

Member Countries may wish to consider internal measures, such as renewed *disinfection* of the eggs upon arrival in the *importing country*.

- 3) When importing disinfected eggs of the species referred to in Article 10.5.2. for *aquaculture*, from a country, zone or compartment not declared free from infection with ISAV, the *Competent Authority* of the *importing country* should require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* attesting that the procedures described in point 2 of this article have been fulfilled.

— Text deleted.

CHAPTER 10.X.

INFECTION WITH SALMONID ALPHAVIRUS

Article 10.X.1.

General provisions

For the purposes of the *Aquatic Code*, infection with *Salmonid alphavirus* (SAV) means *infection* with any subtype of SAV of the genus *Alphavirus* of the family *Togaviridae*.

Information on methods for diagnostic tests is provided in the *Aquatic Manual*.

Article 10.X.2.

Scope

The recommendations in this chapter apply to: Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*) and rainbow trout (*Onchorynchus mykiss*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 10.X.3.

Importation or transit of aquatic animals and aquatic animal products for any purpose from a country, zone or compartment not declared free from infection with Salmonid alphavirus

- 1) *Competent Authorities* should not require any conditions related to infection with SAV, regardless of the SAV status of the *exporting country, zone or compartment* when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 10.X.2. intended for any purpose and complying with Article 5.4.1.:
 - a) heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
 - b) pasteurised fish products that have been subjected to a heat treatment at 90°C for at least 10 minutes (or to any time/temperature equivalent which has been demonstrated to inactivate SAV);
 - c) mechanically dried, eviscerated fish (i.e. a heat treatment at 100°C for 30 minutes or any time/temperature equivalent which has been demonstrated to inactivate SAV);
 - d) fish oil;
 - e) fish *meal*; and
 - f) fish skin leather] under study
- 2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 10.X.2., other than those referred to in point 1 of Article 10.X.3., *Competent Authorities* should require the conditions prescribed in Articles 10.X.7. to 10.X.12. relevant to the SAV status of the *exporting country, zone or compartment*.
- 3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* from an *exporting country, zone or compartment* not declared free from infection with SAV of a species not covered in Article 10.X.2. but which could reasonably be expected to pose a *risk of spread transmission* for *infection with* SAV, *the Competent Authorities* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. the Aquatic Code. The Competent Authority of the *exporting country* should be informed of the outcome of this assessment.

Annex 15 (contd)

Article 10.X.4.

Country free from infection with Salmonid alphavirus

~~A country may make a self-declaration of freedom from infection with SAV if it meets the conditions in points 1, 2, 3 or 4 below.~~

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with SAV if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with SAV (see Article 10.X.5.).

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

- 1) ~~A country where none of the susceptible species referred to in Article 10.X.2. are present may make a self-declaration of freedom from infection with SAV when and basic biosecurity conditions have been continuously met in the country for at least the past two years.~~

OR

- 2) ~~A country where the any of the susceptible species referred to in Article 10.X.2. are present and the following conditions have been met:~~

- a) there has been no observed occurrence of the *disease* for at least the past ten years despite conditions that are conducive to clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and
- b) ~~may make a self-declaration of freedom from infection with SAV when~~ *basic biosecurity conditions* have been continuously met ~~in the country~~ for at least the past ten years.

OR

- 3) ~~A country where the disease status prior to targeted surveillance was is unknown may make a self-declaration of freedom from infection with SAV when but the following conditions have been met:~~

- a) *basic biosecurity conditions* have been continuously met for at least the past two years; and
- b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the past two years without detection of infection with SAV.

OR

- 4) ~~A country that has it previously made a self-declaration of freedom from infection with SAV and but in which the disease is subsequently lost its disease free status due to the detected ion may make a self-declaration of freedom from of infection with SAV again but when the following conditions have been met:~~

- a) on detection of the disease SAV, 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 (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 past two years without detection of infection with SAV.

Annex 15 (contd)

- d) ~~previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place since eradication of the disease.~~

In the meantime, part or all of the non-affected area may be declared a free zone provided that such part meets the conditions in point 3 of Article 10.X.5.

Article 10.X.5.

Zone or compartment free from infection with salmonid alphavirus

~~In this article, all statements refer to a zone or compartment free from infection with SAV.~~

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

~~As described in Article 1.4.6., a A zone or compartment within the territory of one or more countries not declared free from infection with SAV may be declared free by the Competent Authority(ies) of the country(ies) concerned if: the zone or compartment meets the conditions referred to in points 1, 2, 3 or 4 below.~~

- 1) ~~A zone or compartment where none of the susceptible species referred to in Article 10.X.2. are present in the zone or compartment and basic biosecurity conditions have been continuously met in the zone or compartment for at least the past two years.~~

OR

- 2) ~~A zone or compartment where the susceptible species referred to in Article 10.X.2. are present in the zone or compartment but the following conditions have been met:~~

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

~~b) may be declared free from infection with SAV when basic biosecurity conditions have been continuously met in the zone or compartment for at least the past ten years.~~

OR

- 3) ~~A zone or compartment where the disease status prior to targeted surveillance was is unknown but the following conditions have been met:~~

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

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

OR

- 4) ~~if A zone 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 but in which infection with SAV in the zone is detected may be declared free from infection with SAV again when but the following conditions have been met:~~

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

Annex 15 (contd)

- b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the *risk* of further spread of the disease SAV, and the appropriate *disinfection* procedures (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
- ed) targeted surveillance, as described in Chapter 1.4., has been in place for at least the past two years without detection of infection with SAV.
- d) previously existing basic biosecurity conditions have been reviewed and modified as necessary and have continuously been in place since eradication of the disease.

Article 10.X.6.

Maintenance of free status for infection with salmonid alphavirus

A country, *zone* or *compartment* that is declared free from infection with SAV following the provisions of points 1 or 2 of Articles 10.X.4. or 10.X.5. (as relevant) may maintain its status as free from infection with SAV provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with SAV following the provisions of point 3 of Articles 10.X.4. or 10.X.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions that are conducive to clinical expression, as described in the corresponding chapter of the *Aquatic Manual*, exist and *basic biosecurity conditions* are continuously maintained.

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

Article 10.X.7.

Importation of live aquatic animals from a country, zone or compartment declared free from infection with salmonid alphavirus

When importing live *aquatic animals* of the species referred to in Article 10.X.2. from a *country, zone* or *compartment* declared free from infection with SAV, the *Competent Authority* of the *importing country* should require 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 10.X.4. or 10.X.5. (as applicable) and 10.X.6., the place of production of the *aquatic animal* is a *country, zone* or *compartment* declared free from infection with SAV.

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 10.X.3.

Article 10.X.8.

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

- 1) When importing, for *aquaculture*, live *aquatic animals* of the species referred to in Article 10.X.2. from a *country, zone* or *compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, apply the following *risk* mitigation measures:

Annex 15 (contd)

- a) the direct delivery to and lifelong holding of the consignment in biosecure facilities for continuous isolation from the local environment; and
 - b) the treatment of all effluent and waste materials in a manner that ensures inactivation of SAV.
- 2) If the intention of the introduction is the establishment of a new stock, relevant aspects of the Code of Practice on the Introductions and Transfers of Marine Organisms of the International Council for the Exploration of the Seas (ICES) should be considered.
- 3) For the purposes of the *Aquatic Code*, relevant aspects of the ICES Code (full version see: <http://www.ices.dk/publications/our-publications/Pages/Miscellaneous.aspx>) may be summarised to the following points:
- a) identify stock of interest (cultured or wild) in its current location;
 - b) evaluate stock health/*disease* history;
 - c) take and test samples for SAV, pests and general health/*disease* status;
 - d) import and quarantine in a secure facility of a founder (F-0) population;
 - 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 SAV and perform general examinations for pests and general health/*disease* status;
 - g) if SAV is not detected, pests are not present, and the general health/*disease* status of the stock is considered to meet the *basic biosecurity conditions* of the *importing country, zone or compartment*, the F-1 stock may be defined as free from infections with SAV or specific pathogen free (SPF) for SAV;
 - h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone or compartment*.
- 4) With respect to point 3e), *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 a low *infection* level.

This Article does not apply to *aquatic animals* referred to in point 1 of Article 10.X.3.

Article 10.X.9.

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

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 10.X.2. from a *country, zone or compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

- 1) the consignment is delivered directly to and held in *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 10.X.3., or products described in point 1 of Article 10.X.12., or other products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of SAV or is disposed in a manner that prevents contact of waste with *susceptible species*.

Annex 15 (contd)

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

Article 10.X.10.

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

When importing, for use in animal *feed*, or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of the species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should require that:

- 1) the consignment is delivered directly to and held in *quarantine* for slaughter and processing into products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of SAV.

This Article does not apply to *commodities* referred to in point 1 of Article 10.X.3.

Article 10.X.11.

Importation of aquatic animal products from a country, zone or compartment declared free from infection with salmonid alphavirus

When importing *aquatic animal products* of the species referred to in Article 10.X.2. from a country, *zone* or *compartment* declared free from infection with SAV, the *Competent Authority* of the *importing country* should require 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 10.X.4. or 10.X.5, (as applicable) and 10.X.6., the place of production of the *commodity* is a country, *zone* or *compartment* declared free from infection with SAV.

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 10.X.3.

Article 10.X.12.

Importation of aquatic animals and aquatic animal products for retail trade for human consumption from a country, zone or compartment not declared free from infection with salmonid alphavirus

- 1) *Competent Authorities* should not require any conditions related to infection with SAV, regardless of the SAV status of the *exporting country, zone* or *compartment*, when authorising the importation or transit of **fish fillets or steaks (frozen or chilled) the following commodities** which have been prepared and packaged for retail trade and which comply with Article 5.4.2.:

[a] — fish fillets or steaks (frozen or chilled)] under study.

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

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

Annex 15 (contd)

- 2) When importing *aquatic animals* or *aquatic animal products*, other than those referred to in point 1 above, of the species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Article 10.X.13.

Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with salmonid alphavirus

- 1) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should assess the *risk* associated with at least:
- a) the SAV status of the water to be used during the *disinfection* of the eggs;
 - b) the level of *infection* with SAV in broodstock (ovarian fluid and milt); and
 - c) the temperature and pH of the water to be used for *disinfection*.
- 2) If the *Competent Authority* of the *importing country* concludes that the importation is acceptable, it should apply the following *risk* mitigation measures including:
- a) the eggs should be disinfected prior to importing, according to the methods described in Chapter 1.1.3. of the *Aquatic Manual* (under study) or those specified by the *Competent Authority* of the *importing country*; and
 - b) between *disinfection* and the import, eggs should not come into contact with anything which may affect their health status.

Member Countries may wish to consider internal measures, such as renewed *disinfection* of the eggs upon arrival in the *importing country*.

- 3) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with SAV, the *Competent Authority* of the *importing country* should require an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* or a *certifying official* approved by the *importing country* attesting that the procedures described in point 2 of this article have been fulfilled. ~~under study.~~

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CLEAN TEXT

CHAPTER X.X

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

Article X.X.1.

The purpose of this chapter is to provide criteria for determining which species are listed as susceptible in Article X.X.2. of each *disease-specific* chapter in the *Aquatic Code*.

Article X.X.2.

Scope

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

The decision to list a species as susceptible should be based on a finding that the evidence is definite. However, possible susceptibility of a species is also important information and this should also be included in Section 2.2.1. of the *disease* chapter of the *Aquatic Manual*.

Article X.X.3.

Approach

A three stage approach is outlined in this chapter to assess susceptibility of a species to *infection* with a specified *pathogenic agent* and is based on:

- 1) criteria to determine whether the route of transmission is consistent with natural pathways for the *infection* (as described in Article X.X.4.);
- 2) criteria to determine whether the *pathogenic agent* has been adequately identified (as described in Article X.X.5.);
- 3) criteria to determine whether the evidence indicates that presence of the *pathogenic agent* constitutes an *infection* (as described in Article X.X.6.).

Article X.X.4.

Stage 1: criteria to determine whether the route of transmission is consistent with natural pathways for the infection

The evidence should be classified as transmission through:

- 1) natural occurrence; includes situations where *infection* has occurred without experimental intervention e.g. *infection* in wild or farmed populations; or
- 2) non-invasive experimental procedures; includes cohabitation with infected hosts, infection by immersion or ingestion; or
- 3) invasive experimental procedure; includes injection, exposure to unnaturally high loads of pathogen, or exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment.

Annex 16A (contd)

Consideration needs to be given to whether experimental procedures (e.g. inoculation, infectivity load) mimic natural pathways for *disease* transmission. Consideration should also be given to environmental factors as these may affect host resistance or transmission of the pathogen.

Article X.X.5.

Stage 2: criteria to determine whether the pathogenic agent has been adequately identified

The *pathogenic agent* should be identified and confirmed in accordance with the methods described in Section 7 (corroborative diagnostic criteria) of the relevant *disease* chapter in the *Aquatic Manual*, or other methods that have been demonstrated to be equivalent.

Article X.X.6.

Stage 3: criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection

A combination of the following criteria should be used to determine *infection* (see Article X.X.7):

- A. the *pathogenic agent* is multiplying in the host, or developing stages of the *pathogenic agent* are present in or on the host;
- B. viable *pathogenic agent* is isolated from the proposed *susceptible species*, or infectivity is demonstrated by way of transmission to naive individuals;
- C. clinical or pathological changes are associated with the *infection*;
- D. the specific location of the pathogen corresponds with the expected target tissues.

The type of evidence to demonstrate *infection* will depend on the *pathogenic agent* and potential host species under consideration.

Article X.X.7.

Outcomes of the assessment

The decision to list a species as susceptible should be based on a finding of definite evidence. Evidence should be provided for the following:

- 1) Transmission has been obtained naturally or by experimental procedures that mimic natural pathways for the *infection* in accordance with Article X.X.4.;

AND

- 2) the identity of the *pathogenic agent* has been confirmed in accordance with Article X.X.5;

AND

- 3) there is evidence of *infection* with the *pathogenic agent* in the suspect host species in accordance with criteria A to D in Article X.X.6. Evidence to support criterion A alone is sufficient to determine *infection*. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D would be required to determine *infection*.

Article X.X.8.

Species for which there is incomplete evidence for susceptibility

The decision to list a species as susceptible in Article X.X.2. of each *disease-specific* chapter should be based on a finding that the evidence is definite.

However, where there is insufficient evidence to demonstrate susceptibility through the approach described in Article X.X.3 because transmission does not mimic natural pathways of *infection*, or the identity of the *pathogenic agent* has not been confirmed, or *infection* is only partially supported, information will be included in the relevant *disease-specific* chapter in the *Aquatic Manual*.

If there is insufficient evidence to demonstrate susceptibility of a species, the *Competent Authority* should assess the risk of spread of the pathogen under consideration, in accordance with the recommendations in Chapter 2.1., prior to the implementation of import health measures.

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TRACK CHANGES

CHAPTER X.X

~~CRITERIA FOR LISTING SPECIES AS SUSCEPTIBLE TO INFECTION WITH A SPECIFIC PATHOGEN DETERMINING SUSCEPTIBILITY OF AQUATIC ANIMALS TO SPECIFIC PATHOGENIC AGENTS~~

Article X.X.1.

The purpose of this chapter is to provide criteria for determining which ~~which susceptible~~ species that are listed as susceptible in Article X.X.2. of each *disease-specific* chapter in the *Aquatic Code* ~~and Article 2.2.1. of each disease specific chapter in the Aquatic Manual.~~

Article X.X.2.

Scope

~~This chapter provides criteria to determine which species should be listed as susceptible to infection with the aetiological agent of listed diseases. Susceptibility may include clinical or non-clinical infection. This chapter but does not provide criteria for identifying include~~ mechanical vectors (i.e. species that may carry the pathogen aetiological pathogenic agent without replication).

~~The decision to list a species as susceptible should be based on a finding that the evidence is definite. However, possible susceptibility of a species is also important information and this should also be included in Section 2.2.1. of the disease chapter of the Aquatic Manual.~~

The decision to list a species as susceptible should be based on a finding that the evidence is definite. However, possible susceptibility of a species is also important information and this should also be included in Section 2.2.1. of the disease chapter of the Aquatic Manual.

Article X.X.3.

Approach

~~There~~ Are three stages approach is outlined in this chapter to assessing susceptibility of a species to *infection* with a specified aetiological pathogenic agent and is based on:

- 1) criteria to determine whether the route of infection used transmission is consistent with natural pathways for the *infection* (as described in Article X.X.4.);
- 2) criteria to determine whether the aetiological pathogenic agent has been adequately identified using a technique (as described in Article X.X.5.);
- 3) criteria to determine whether the evidence indicates that presence of the aetiological pathogenic agent constitutes an *infection* (as described using the criteria in Article X.X.6.).

Article X.X.4.

Stage 1: criteria to determine whether the route of transmission infection used is consistent with natural pathways for the infection for transmission of infection

The evidence should be classified as transmission through: a) natural occurrence, b) non-invasive experimental procedure, or c) invasive experimental procedure.

Annex 16B (contd)

- a) natural occurrence; includes all situations where infection has arisen occurred without direct experimental intervention e.g. infection arising in wild or farmed populations; or
- b) non-invasive experimental procedures; includes cohabitation with infected hosts, infection by immersion or ingestion; or
- c) invasive experimental procedure; includes injection, exposure to unnaturally high loads of pathogen, or exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment.

Consideration needs to be given to whether experimental procedures (e.g. inoculation, infectivity load, host stress) mimic natural pathways for disease transmission. Consideration should also be given to environmental factors as these may affect host resistance or transmission of the pathogen.

Article X.X.5.

Stage 2: criteria to determine whether the aetiological pathogenic agent has been adequately identified for identification of the aetiological agent

The aetiological pathogenic agent should be identified and confirmed in accordance with the methods described in Section 7 (corroborative diagnostic criteria) of the relevant disease chapter in the *Aquatic Manual*, or other methods that have been demonstrated to be equivalent.

~~Under some circumstances the presumptive identification of the aetiological agent has been made but not confirmed in accordance with the *Aquatic Manual*.~~

Article X.X.6.

Stage 3: criteria to determine whether the evidence indicates that presence of the aetiological pathogenic agent constitutes an infection to determine infection

A combination of the following criteria should be used to determine infection (see Article X.X.7):

- A. the aetiological pathogenic agent is multiplying in the host, or that developing or latent stages of the aetiological pathogenic agent are present in or on the host;
- B. viable aetiological pathogenic agent is isolated from the proposed susceptible species, or viability infectivity is demonstrated via by way of transmission to naive individuals (by natural routes);
- C. clinical and/or pathological changes are associated with the infection;
- D. the specific location of the pathogen corresponds with the expected target tissues.

The type of evidence to demonstrate infection will depend on the aetiological pathogenic agent and potential host species under consideration.

Article X.X.7.

Outcomes of the assessment

The decision to list a species as susceptible should be based on a finding that the of definite evidence is definite. Evidence should be provided for the following:

~~Susceptible species can be classified as 1) Possible or 2) Definite~~

4- Definite susceptible species:

- 1a) Transmission has been obtained by naturally or by experimental procedures that mimic natural pathways of for the infection in accordance with Article X.X.4.;

AND

- 2b) the identity of the **aetiological pathogenic agent** has been confirmed in accordance with Article X.X.5;

AND

- 3e) there is evidence of *infection* with the **aetiological pathogenic agent** in the suspect host species in accordance with criteria A to D in Article X.X.6. Evidence to support criterion A alone is sufficient to determine *infection*. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D would be required to determine *infection*.

Article X.X.8.

Species for which there is incomplete evidence for susceptibility

The decision to list a species as susceptible in Article X.X.2 of each disease-specific chapter should be based on a finding that the evidence is definite.

However, ~~where there evidence exists but~~ is insufficient evidence to demonstrate susceptibility through the approach described in Article X.X.3 of a species because either transmission does not mimic natural pathways of *infection*, or the identity of the **aetiological pathogenic agent** has not been confirmed, or *infection* is only partially supported, ~~the~~ information will be included in the relevant disease-specific chapter in the *Aquatic Manual*.

Where these species could reasonably be expected to pose a risk of transmission for the pathogen under consideration, *Competent Authorities* should conduct a *risk analysis* in accordance with the recommendations in the *Aquatic Code*. If there is insufficient evidence to demonstrate susceptibility of a species, the *Competent Authority* should assess the risk of spread of the pathogen under consideration, in accordance with the recommendations in Chapter 2.1., prior to the implementation of import health measures.

2- Possible susceptible species:-

- a) ~~The presumptive identification of the aetiological agent has been made but may not have been confirmed in accordance with Article X.X.5.;~~

AND

- b) ~~there is evidence of *infection* with the aetiological agent in the suspect species in accordance with Article X.X.6. At least one of criteria A, B, C or D in Article X.X.6. is required.~~

Article X.X.8.

Taxonomic relationship of susceptible species

~~Defining species as possible susceptible on the basis of a taxonomic relationship at levels higher than genus requires solid evidence that the pathogen has a very wide host range.~~

~~For aetiological agents with a wide host range, the taxonomic relationship of a species to other known *susceptible species* may be used to assume susceptibility. Species can be classified as 'possible' *susceptible species* if they reside in a genus that includes at least two *susceptible species* and in which there is no strong evidence of resistance to *infection*.~~

~~Evidence of resistance would include the following:~~

Annex 16B (contd)

- 1) ~~Appropriate testing reveals no evidence of *infection* when animals are exposed to the pathogen in natural setting where the pathogen is known to be present and to cause *disease* in *susceptible species*.~~

- 2) ~~Appropriate testing reveals no evidence of *infection* when animals are exposed through controlled challenges by natural routes.~~

— Text deleted.

CHAPTER 2.3.5.

INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

1. Scope

For the purpose of this chapter, infection with infectious salmon anaemia virus (ISAV) means infection with highly polymorphic region (HPR)-deleted ISAV or HPR0 ISAV (with a non-deleted HPR) of the genus *Isavirus* of the family *Orthomyxoviridae*.

Infection with HPR-deleted ISAV may cause infectious salmon anaemia (ISA) in Atlantic salmon (*Salmo salar*), which is a generalised and lethal condition characterised by severe anaemia, and variable haemorrhages and necrosis in several organs. The disease course is prolonged with low daily mortality (0.05–0.1%) typically only in a few cages. Cumulative mortality may become very high for a period lasting several months if nothing is done to limit disease dissemination (Rimstad *et al.*, 2011).

Detection of HPR0 ISAV has never been associated with ISA in Atlantic salmon. This virus genotype **replicates is known to cause transiently subclinical infection** and has mainly been **detected** localised to the gills. **There is evidence of a Δ link** between non-pathogenic HPR0 ISAV and pathogenic HPR-deleted ISAV, with some outbreaks potentially occurring as a result of the emergence of HPR-deleted ISAV from HPR0 ISAV **has been suggested** (Cunningham *et al.*, 2002; Mjaaland, *et al.*, 2002).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

ISAV is an enveloped virus, 100–130 nm in diameter, with a genome consisting of eight single-stranded RNA segments with negative polarity. The virus has haemagglutinating, receptor-destroying and fusion activity (Falk *et al.*, 1997; Mjaaland *et al.*, 1997; Rimstad *et al.*, 2011).

The morphological, physiochemical and genetic properties of ISAV are consistent with those of the *Orthomyxoviridae*, and ISAV has been classified as the type species of the genus *Isavirus* (Kawaoka *et al.*, 2005) within this virus family. The nucleotide sequences of all eight genome segments, encoding at least ten proteins, have been described (Clouthier *et al.*, 2002; Cottet *et al.*, 2014; Rimstad *et al.*, 2011), including the 3' and 5' non-coding sequences (Kulshreshtha *et al.*, 2010). Four major structural proteins have been identified, including a 68 kDa nucleoprotein, a 22 kDa matrix protein, a 42 kDa haemagglutinin-esterase (HE) protein responsible for receptor-binding and receptor-destroying activity, and a 50 kDa surface glycoprotein with putative fusion (F) activity, encoded by genome segments 3, 8, 6 and 5, respectively. Segment 1, 2, and 4 encode the viral polymerases PB2, PB1 and PA. The two smallest genomic segments, segments 7 and 8, each contain two open reading frames (ORF). The ORF1 of segment 7 encodes a protein with type I interferon antagonistic properties, while ORF2 has been suggested to encode for a nuclear export protein (NEP). Whether the ORF1 gene product is nonstructural or a structural component of the virion remains to be determined. The smaller ORF1 of segment 8 encodes the matrix protein, while the larger ORF2 encodes an RNA-binding structural protein also with type I interferon antagonistic properties.

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas. According to sequence differences in the 5'-region of the HE gene, ISAV isolates have been divided into two major groups, one European and one North American group. In the HE gene, a small HPR near the transmembrane domain has been identified. This region is characterised by the presence of gaps rather than single-nucleotide substitutions (Cunningham *et al.*, 2002; Mjaaland *et al.*, 2002). A full-length gene (HPR0) has been suggested to represent a precursor from which all ISAV HPR-deleted (pathogenic) variants of ISAV originate. The presence of non-pathogenic HPR0 ISAV **genome** has been reported in both apparently healthy wild and farmed Atlantic salmon, but has not been detected in diseased fish with clinical disease and pathological signs consistent with ISA (Christiansen *et al.*, 2011; Cunningham *et al.*, 2002; Lyngstad *et al.*, 2012; Markussen *et al.*, 2008; McBeath *et al.*, 2009; Nylund *et al.*, 2007). A mixed infection of HPR-deleted and HPR0 ISAV variants has been reported (Kibengo *et al.*, 2009). Recent studies show that HPR0

Annex 17 (contd)

ISAV variants occur frequently in sea-reared Atlantic salmon. The HPR0 ISAV strain seems to be more seasonal and transient in nature and displays a tissue tropism with high prevalence in gills (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011). To date there has been no direct evidence linking the presence of HPR0 ISAV to a subsequent clinical ISA outbreak. The risk of emergence of pathogenic HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low but not negligible (Christiansen *et al.*, 2011; EFSA, 2012; Lyngstad *et al.*, 2012).

In addition to the variations seen in the HPR of the HE gene, other gene segments may also be of importance for development of ISA. A putative virulence marker has been identified in the fusion (F) protein. Here, a single amino acid substitution, or a sequence insertion, near the protein's putative cleavage site has been found to be a prerequisite for virulence (Kibenge *et al.*, 2007; Markussen *et al.*, 2008). Aside from insertion/recombination, ISAV also uses gene segment reassortment in its evolution, with potential links to virulence (Devold *et al.*, 2006; Markussen *et al.*, 2008; Mjaaland *et al.*, 2005).

2.1.2. Survival outside the host

ISAV has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater sampled at farming sites with ISAV-positive Atlantic salmon (Kibenge *et al.*, 2004). It is difficult to estimate exactly how long the virus may remain infectious in the natural environment because of a number of factors, such as the presence of particles or substances that may bind or inactivate the virus. Exposing cell culture-propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (Falk *et al.*, 1997).

2.1.3. Stability of the agent (effective inactivation methods)

ISAV is sensitive to UV irradiation (UVC) and ozone. A 3-log reduction in infectivity in sterile fresh water and seawater was obtained with a UVC dose of approximately 35 Jm⁻² and 50 Jm⁻², respectively, while the corresponding value for ISAV in wastewater from a fish-processing plant was approximately 72 Jm⁻². Ozonated seawater (4 minutes with 8 mg ml⁻¹, 600–750 mV redox potential) may inactivate ISAV completely. Incubation of tissue homogenate from diseased fish at pH 4 or pH 12 for 24 hours inactivated ISAV infectivity. Incubation in the presence of chlorine (100 mg ml⁻¹) for 15 minutes also inactivated virus infectivity (Rimstad *et al.*, 2011). Cell culture-isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes of exposure at 56°C (Falk *et al.*, 1997).

2.1.4. Life cycle

The main infection route is most likely through the gills for both HPR0 and HPR-deleted ISAV, but infection via the intestine or skin cannot be excluded. HPR-deleted ISAV has been used in the studies referred to below. Endothelial cells lining blood vessels seem to be the primary target cells for ISAV as demonstrated by electron microscopy immunohistochemistry and *in-situ* hybridisation. Virus replication has also been demonstrated in leukocytes, and sinusoidal macrophages in kidney tissue stain positive for ISAV using immunohistochemistry (IHC). As endothelial cells are the target cells (see Section 2.2.4), virus replication may occur in any organ (Aamelfot *et al.*, 2012; Rimstad *et al.*, 2011).

The haemagglutinin-esterase (HE) molecule of ISAV, like the haemagglutinin (HA) of other orthomyxoviruses (influenza A, B and C viruses), is essential for binding of the virus to sialic acid residues on the cell surface. In the case of ISAV, the viral particle binds to glycoprotein receptors containing 4-O-acetylated sialic acid residues, which also functions as a substrate for the receptor-destroying enzyme. Further uptake and replication seem to follow the pathway described for influenza A viruses, indicated by demonstration of low pH-dependent fusion, inhibition of replication by actinomycin D and α -amanitin, early accumulation of nucleoprotein followed by matrix protein in the nucleus and budding of progeny virions from the cell surface (Cottet *et al.*, 2011; Rimstad *et al.*, 2011).

The route of shedding of ISAV from infected fish may be through natural excretions/secretions.

The HPR0 variant has ~~hitherto~~ not been isolated in cell culture, which hampers *in-vivo* and *in-vitro* studies of characteristics and the life cycle of this virus variant.

2.2. Host factors

2.2.1. Susceptible host species

Natural outbreaks of ISA have only been recorded in farmed Atlantic salmon, and in Coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge *et al.*, 2001). Subclinically infected feral Atlantic salmon, brown trout and sea trout (*S. trutta*) have been identified by RT-PCR (Kibenge *et al.*, 2004; Plarre *et al.*, 2005). In marine fish, detection of ISAV by RT-PCR has been reported in tissues of pollock (*Pollachius virens*) and cod (*Gadus morhua*), but only in fish collected from cages with Atlantic salmon exhibiting ISA (MacLean SA *et al.*, 2003 reviewed in Kibenge *et al.*, 2004). Following experimental infection by bath immersion, ISAV has been detected by RT-PCR in Rainbow trout (*Oncorhynchus mykiss*) (Biacchesi *et al.*, 2007) and herring (*Clupea harengus*), the latter in and a subsequent transmission to Atlantic salmon. Attempts have been made to induce infection or disease in pollock, *Pollachius virens*, but with negative results. Replication of ISAV has also been demonstrated in several salmonid species but only after intraperitoneal injection of ISAV infected material (reviewed in Rimstad *et al.*, 2011).

2.2.2. Susceptible stages of the host

In Atlantic salmon, disease outbreaks are mainly reported in seawater cages, and only a few cases have been reported in the freshwater stage, including one case in yolk sac fry (Rimstad *et al.*, 2011). ISA has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater. Genetics may also play an important role in the susceptibility of Atlantic salmon to ISA, as differences in susceptibility among different family groups have been observed.

2.2.3. Species or subpopulation predilection (probability of detection)

ISA is primarily a disease of Atlantic salmon.

2.2.4. Target organs and infected tissue

For fish that have developed ISA: endothelial cells in all organs become infected (gills, heart, liver, kidney, spleen and others) (Aamelfot *et al.*, 2012). HPR0 ISAV variants seem primarily to target the gills, but this variant has also been detected in kidney and heart (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011).

2.2.5. Persistent infection with lifelong carriers

Persistent infection in lifelong carriers has not been documented in Atlantic salmon, but at the farm level, infection may persist in the population by continuous infection of new individuals that do not develop clinical signs of disease. This may include infection with the HPR0 ISAV variants, which seems to be only transient in nature (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011). Experimental infection of rainbow trout and brown trout with ISAV indicate that persistent infection in these species could be possible (Rimstad *et al.*, 2011).

2.2.6. Vectors

Passive transfer of ISAV by salmon lice (*Lepeophtheirus salmonis*) has been demonstrated under experimental conditions. Although natural vectors have not been identified, several different vector groups could be possible vectors under certain defined conditions (reviewed in Rimstad *et al.*, 2011).

2.2.7. Known or suspected wild aquatic animal carriers

Wild Atlantic salmon (*Salmo salar*), brown trout and sea trout (*S. trutta*) may be carriers of ISAV (Rimstad *et al.*, 2011). The importance of wild marine fish (see Section 2.2.1) as virus carriers needs to be clarified. The results from a study from the Faroe Islands point to the potential presence of an unknown marine reservoir for this virus (Christiansen *et al.*, 2011).

2.3. Disease pattern

2.3.1. Transmission mechanisms

Studies of recurrent epidemics of ISA in different salmon-producing areas conclude that the virus spreads locally between adjoining sites. Proximity to sites with ISA outbreaks is a risk of primary importance, and the risk for a susceptible farm increases the nearer it is to an infected farm. Sequence analysis of ISAV from ISA outbreaks in Norway shows a high degree of similarity between viruses isolated from neighbouring ISA affected sites, further supporting ISAV transmission between proximate sites. The risk of transmission of ISAV is dependent on the level of biosecurity measures in place. Suggested pathways for ISAV transmission are through sea water, shipment of live fish, transmission through sea lice, and via infected wild salmonids (Aldrin *et al.*, 2011; Gustafson *et al.*, 2007; Lyngstad *et al.*, 2011; Mardones *et al.*, 2011; Rimstad *et al.*, 2011).

Many ISA outbreaks in Norway appear to be isolated in space and time from other outbreaks with unknown sources of infection (Aldrin *et al.*, 2011). A suggested hypothesis for disease emergence is occasional transition of HPR0 ISAV into HPR-deleted ISAV variants causing solitary outbreaks or local epidemics through local transmission (Lyngstad *et al.*, 2011; 2012). The risk of emergence of HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low but not negligible (EFSA, 2012). A direct link between HPR0 variants and HPR-deleted ISAV remains to be demonstrated.

As ISA has also been reported from smolt-producing sites with Atlantic salmon, transmission of ISAV from parent to progeny cannot be excluded. Even though there is no evidence of true vertical transmission, eggs and embryos could be a risk of transmission if ISAV biosecurity measures are not adequate (Mardones *et al.*, 2014; Rimstad *et al.*, 2011).

2.3.2. Prevalence

In a net pen containing diseased fish, the prevalence of HPR-deleted ISAV may vary widely, while in adjacent net pens ISAV may be difficult to detect, even by the most sensitive methods. Therefore, for diagnostic investigations it is important to sample from net pens containing diseased fish.

There is increasing evidence that the prevalence of the non-pathogenic HPR0 ISAV genotype may be high in Atlantic salmon production areas. HPR0 variants in Atlantic salmon appear to be a seasonal and transient infection (Christiansen *et al.*, 2011). HPR0 variants of ISAV have also been detected in wild salmonids (reviewed in Rimstad *et al.*, 2011).

2.3.3. Geographical distribution

Initially reported in Norway in the mid-1980s (Thorud & Djupvik, 1988), ISA in Atlantic salmon has since then been reported in Canada (New Brunswick in 1996; Mullins *et al.*, 1998), the United Kingdom (Scotland in 1998), the Faroe Islands (2000), the USA (Maine in 2001) and in Chile (2007) (Cottet *et al.*, 2011; Rimstad *et al.*, 2011). The presence of the HPR0 ISAV variant has been reported in all countries where ISA has occurred.

2.3.4. Mortality and morbidity

During ISA outbreaks, morbidity and mortality may vary greatly within and between different net pens in a seawater fish farm, and between different fish farms. Morbidity and mortality within a net pen may start at very low levels. Typically, daily mortality ranges from 0.5 to 1% in affected cages. Without intervention, mortality increases and seems to peak in early summer and winter. The range of cumulative mortality during an outbreak is from insignificant to moderate, but in severe cases, cumulative mortality exceeding 90% may be recorded during several months. Initially, an outbreak of ISA may be limited to one or two net pens over a long time period. In such cases, if net pens with clinical ISA are slaughtered immediately, further development of clinical ISA at the site may be prevented. In outbreaks where smolts have been infected in well boats during transport, simultaneous outbreaks may occur.

HPR0 ISAV has not been associated with ISA in Atlantic salmon.

2.3.5. Environmental factors

Generally, outbreaks of ISA tend to be seasonal with most outbreaks in late spring and late autumn; however outbreaks can occur at any time of the year. Handling of fish (e.g. sorting or treatment, splitting or moving of cages) may initiate disease outbreaks on infected farms, especially if long-term undiagnosed problems have been experienced in advance (Lyngstad *et al.*, 2008).

2.4. Control and prevention

2.4.1. Vaccination

Vaccination against ISA has been carried out in North America since 1999 and the Faroe Islands since 2005. In Norway vaccination against ISA was carried out for the first time in 2009 in a region with a high rate of ISA outbreaks. Chile started vaccinating against ISA in 2010. However, the currently available vaccines do not seem to offer complete protection in Atlantic salmon.

2.4.2. Chemotherapy

Most recently, it has been demonstrated that the broad-spectrum antiviral drug Ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is effective in inhibiting ISAV replication both *in vitro* and *in vivo* (Rivas-Aravena *et al.*, 2011).

2.4.3. Immunostimulation

Not applicable.

2.4.4. Resistance breeding

Differences in susceptibility among different family groups of Atlantic salmon in fresh water have been observed in challenge experiments and in field tests, indicating the potential for resistance breeding (Gjøen *et al.*, 1997).

2.4.5. Restocking with resistant species

Not applicable.

2.4.6. Blocking agents

Not applicable.

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs according to standard procedures is suggested as an important control measure (see Chapter 1.1.3 *Methods for disinfection of aquaculture establishments*).

2.4.8. General husbandry practices

The incidence of ISA may be greatly reduced by implementation of legislative measures or husbandry practices regarding the movement of fish, mandatory health control, transport and slaughterhouse regulations. Specific measures including restrictions on affected, suspected and neighbouring farms, enforced sanitary slaughtering, generation segregation ('all in/all out') as well as disinfection of offal and wastewater from fish slaughterhouses and fish processing plants may also contribute to reducing the incidence of the disease. The experience from the Faroe Islands, where the prevalence of HPR0 is high, demonstrates that the combination of good biosecurity and husbandry reduces the risk of ISA outbreaks substantially.

Annex 17 (contd)**3. Sampling****3.1. Selection of individual specimens**

The following is primarily for verification of suspected cases based on clinical signs and gross pathology or positive RT-PCR for HPR-deleted ISAV.

For detection of HPR0 ISAV, gill tissue should be sampled in randomly selected individuals at different points of time through the production cycle. Only detection using RT-PCR is possible for this genotype.

3.2. Preservation of samples for submission

Haematology:	Heparin or EDTA (ethylene diamine tetra-acetic acid)
Cell culture:	Virus transport medium
Histology and immunohistochemistry:	Fixation in neutral phosphate-buffered 10% formalin
Immunofluorescence (smears):	Either submitted dried, or dried and fixed in 100% acetone
Molecular biology (RT-PCR and sequencing):	Appropriate medium for preservation of RNA

3.3. Pooling of samples

Pooling of samples may be acceptable under some circumstances, however, the impact on sensitivity and design prevalence must be considered is not recommended for verification of ISAV as it is usually of interest to compare results from the various examinations for each individual. For surveillance purposes, pooling of samples for virological examination (PCR and/or cell culture) may be accepted. However, the number of fish to be pooled may depend on the suggested prevalence of ISAV in the population and of the method used.

3.4. Best organs or tissues**3.4.1. Detection of HPR-deleted ISAV**

Blood is preferred for non-lethal sampling. Generally, as ISA is a generalised infection, internal organs not exposed to the environment should be used for diagnostic testing.

Virological examination (cell culture and PCR): heart (should always be included) and mid-kidney;

Histology (prioritised): mid-kidney, liver, heart, pancreas/intestine, spleen;

Immunofluorescence (smears): mid-kidney;

Immunohistochemistry: mid-kidney, heart (including valves and bulbus arteriosus).

3.4.2. Detection of HPR0 ISAV

Gills should be tested by RT-PCR

4. Diagnostic methods**4.1. Field diagnostic methods****4.1.1. Clinical signs**

The most prominent external signs of ISA are pale gills (except in the case of blood stasis in the gills), exophthalmia, distended abdomen, blood in the anterior eye chamber, and sometimes skin haemorrhages especially of the abdomen, as well as scale pocket oedema.

Generally, naturally infected Atlantic salmon naturally infected with HPR-deleted ISAV appear lethargic and may keep close to the wall of the net pen.

Affected fish are generally in good condition. Nutritional status is usually quite normal, but diseased fish have no feed in the digestive tract.

4.2. Pathological evaluation

4.2.1. Gross pathology

Fish infected with HPR-deleted ISAV may show a range of pathological changes, from none to severe, depending on factors such as infective dose, virus strain, temperature, age and immune status of the fish. No lesions are pathognomonic to ISA, but anemia and circulatory disturbances are always present. The following findings have been described to be consistent with ISA, though all changes are seldom observed in one single fish.

- Yellowish or blood-tinged fluid in peritoneal and pericardial cavities.
- Oedema of the swim bladder.
- Small haemorrhages of the visceral and parietal peritoneum.
- Focal or diffusely dark red liver. A thin fibrin layer may be present on the surface.
- Swollen, dark red spleen with rounded margins.
- Dark redness of the intestinal wall mucosa in the blind sacs, mid- and hind-gut, without blood in the gut lumen of fresh specimens.
- Swollen, dark red kidney with blood and liquid effusing from cut surfaces.
- Pinpoint haemorrhages of the skeletal muscle.

4.2.2. Clinical chemistry

- Haematocrit <10 in end stages (25–30 often seen in less advanced cases). Haematocrit <10 should always be followed up by investigation for ISA in sea-water reared Atlantic salmon.
- Blood smears with degenerate and vacuolised erythrocytes and the presence of erythroblasts with irregular nuclear shape. Differential counts show a reduction in the proportion of leucocytes relative to erythrocytes, with the largest reduction being among lymphocytes and thrombocytes.

Liver pathology will lead to increased levels of liver enzymes in the blood.

4.2.3. Microscopic pathology

Histological changes in clinically diseased Atlantic salmon are variable, but can include the following:

- Numerous erythrocytes in the central venous sinus and lamellar capillaries where erythrocyte thrombi also form in the gills.
- Multifocal to confluent haemorrhages and/or hepatocyte necrosis at some distance from larger vessels in the liver. Focal accumulations of erythrocytes in dilated hepatic sinusoids.
- Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria.
- Spleen stroma distended by erythrocyte accumulation.
- Slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney.
- Erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

Annex 17 (contd)**4.2.4. Wet mounts**

Not applicable.

4.2.5. Smears

See Section 4.3.1.1.2

4.2.6 Fixed sections

See Section 4.3.1.1.3

4.2.7. Electron microscopy/cytopathology

Virus has been observed in endothelial cells and leukocytes by electron microscopy of tissue preparations, but this method has not been used for diagnostic purposes.

4.2.8. Differential diagnoses

Other anaemic and haemorrhagic conditions, including erythrocytic inclusion body syndrome, winter ulcer and septicaemias caused by infections with *Moritella viscosa*. Disease cases in Atlantic salmon with haematocrit values below 10 is not a unique finding for ISA, however cases with such low haematocrit values without any obvious explanation should always be tested for the presence of ISAV.

4.3. Agent detection and identification methods**4.3.1. Direct detection methods**

With the exception of molecular techniques (see 4.3.1.2.3), these direct detection methods are only recommended for fish with clinical signs of infection with HPR-deleted ISAV.

4.3.1.1. Microscopic methods*4.3.1.1.1. Wet mounts*

Not applicable.

*4.3.1.1.2. Smears**4.3.1.1.2.1 Indirect fluorescent antibody test*

An indirect fluorescent antibody test (IFAT) using validated monoclonal antibodies (MAbs) against ISAV haemagglutinin-esterase (HE) on kidney smears (imprints) or on frozen tissue sections of kidney, heart and liver has given positive reactions in both experimentally and naturally infected Atlantic salmon. Suspected cases (see Section 7.1) may be confirmed with a positive IFAT.

i) Preparations of tissue smears (imprints)

A small piece of the mid-kidney is briefly blotted against absorbent paper to remove excess fluid, and several imprints in a thumbnail-sized area are fixed on poly-L-lysine-coated microscope slides. The imprints are air-dried, fixed in chilled 100% acetone for 10 minutes and stored either at 4°C for a few days or at -80°C until use.

ii) Staining procedure

After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 30 minutes, the preparations are incubated for 1 hour with an appropriate dilution of anti-ISAV MAb, followed by three washes. For the detection of bound antibodies, the preparations are incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig for 1 hour. PBS with 0.1% Tween 20 is used for washing. All incubations are performed at room temperature.

4.3.1.1.2. Fixed sections

4.3.1.1.3.1 Immunohistochemistry (IHC)

Polyclonal antibody against ISAV nucleoprotein is used on paraffin sections from formalin-fixed tissue. This IHC staining has given positive reactions in both experimentally and naturally infected Atlantic salmon. Preferred organs are mid-kidney and heart (transitional area including all three chambers and valves). Suspected cases due to pathological signs are verified with a positive IHC. Histological sections are prepared according to standard methods.

i) Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 5 µm thick sections (for IHC sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for pathomorphology and IHC as described below.

ii) Staining procedure for IHC

All incubations are carried out at room temperature on a rocking platform, unless otherwise stated.

- a) Antigen retrieval is done by boiling sections in 0.1 M citrate buffer pH 6.0 for 2 × 6 minutes followed by blocking with 5% non-fat dry milk and 2% goat serum in 50 mM TBS (TBS; Tris/HCl 50 mM, NaCl 150 mM, pH 7.6) for 20 minutes.
- b) Sections are then incubated overnight with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein) diluted in TBS with 1% non-fat dry milk, followed by three washes in TBS with 0.1% Tween 20.
- c) For detection of bound antibodies, sections are incubated with Alkaline phosphatase-conjugated antibodies to rabbit IgG for 60 minutes. Following a final wash, Fast Red (1 mg ml⁻¹) and Naphthol AS-MX phosphate (0.2 mg ml⁻¹) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) is added to develop for 20 minutes. Sections are then washed in tap water before counterstaining with Harris haematoxylin and mounted in aqueous mounting medium. ISAV positive and ISAV negative tissue sections are included as controls in every setup.

iii) Interpretation

Negative control sections should not have any significant colour reactions. Positive control sections should have clearly visible red-coloured cytoplasmic and intranuclear staining of endothelial cells in blood vessels or heart endocardium. A test sample section should only be regarded as positive if clear, intranuclear red staining of endothelial cells is found. The intranuclear localisation is particular to the orthomyxovirus nucleoprotein during a stage of virus replication. Concurrent cytoplasmic staining is often dominant. Cytoplasmic and other staining patterns without intranuclear localisation must be considered as nonspecific or inconclusive.

The strongest positive staining reactions are usually obtained in endothelial cells of heart and kidney. Endothelial staining reactions within very extensive haemorrhagic lesions can be slight or absent, possibly because of lysis of infected endothelial cells.

Annex 17 (contd)**4.3.1.2. Agent isolation and identification***4.3.1.2.1. Cell culture*

ASK cells (Devold *et al.*, 2000) are recommended for primary ISAV isolation, but other susceptible cell lines, such as SHK-1 (Dannevig *et al.*, 1995), may be used. However, strain variability and the ability to replicate in different cell lines should be taken into consideration. The ASK cells seem to support isolation and growth of the hitherto known virus isolates. A more distinct cytopathic effect (CPE) may appear in ASK cells. Both the SHK-1 and ASK cell lines appear to lose susceptibility for ISAV with increasing passage level.

The SHK-1 and ASK cells are grown at 20°C in Leibovitz's L-15 cell culture medium supplemented with fetal bovine serum (5% or 10%), L-glutamine (4 mM), gentamicin (50 µg ml⁻¹) and 2-mercaptoethanol (40 µM) (this latter may be omitted).

For virus isolation, cells grown in 25 cm² tissue culture flasks or multi-well cell culture plates, which may be sealed with parafilm or a plate sealer to stabilise the pH of the medium, may be used. Cells grown in 24-well plates may not grow very well into monolayers, but this trait may vary between laboratories and according to the type of cell culture plates used. Serially diluted ISAV-positive controls should be inoculated in parallel with the tissue samples as a test for cell susceptibility to ISAV (this should be performed in a separate location from that of the test samples).

i) Inoculation of cell monolayers

Prepare a 2% suspension of tissue homogenate using L-15 medium without serum or other medium with documented suitability. Remove growth medium from actively growing monolayers (1–3 day old cultures or cultures of 70–80 % confluency) grown in 25 cm² tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers (25 cm² tissue culture flasks) with 1.5 ml of the 2% tissue homogenate. Adjust volume to the respective surface area in use. Allow 3–4 hours incubation at 15°C followed by removal of the inoculum, and addition of fresh, L-15 medium supplemented with 2–5% FCS. Alternatively, a 1/1000 dilution and direct inoculation without medium replacement can be used.

When fish samples come from production sites where infectious pancreatic necrosis virus (IPNV) is regarded as endemic, the tissue homogenate supernatant should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

ii) Monitoring incubation

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of CPE. Typical CPE due to ISAV appears as vacuolated cells that subsequently round up and loosen from the growth surface. If CPE consistent with that described for ISAV or IPNV appears, an aliquot of the medium for virus identification, as described below, must be collected. In the case of an IPNV infection, re-inoculate cells with tissue homogenate supernatant that has been incubated with a lower dilution of IPNV antisera. If no CPE has developed after 14 days, subculture to fresh cell cultures.

iii) Subcultivation procedure

Aliquots of medium (supernatant) from the primary cultures are collected 14 days (or earlier when obvious CPE appears) after inoculation. Supernatants from wells inoculated with different dilutions of identical samples may be pooled for surveillance purposes.

Supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 3–4 hours before addition of fresh medium. Alternatively, add supernatants (final dilutions 1/10 and higher) directly to cell cultures with growth medium.

Annex 17 (contd)

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures with no CPE should always be examined for the presence of ISAV by immunofluorescence (IFAT), haemadsorption or by PCR because virus replication may occur without development of apparent CPE.

The procedure described below has been successful for isolation of HPR-deleted ISAV from fish with clinical signs or from suspected cases. HPR0 has hitherto not been isolated in cell culture.

4.3.1.2.2. *Antibody-based antigen detection methods*

4.3.1.2.2.1 *Virus identification by IFAT*

All incubations are carried out at room temperature unless otherwise stated.

- i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well or 24-well plates), in slide flasks or on cover-slips dependent on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). SHK-1 cells grow rather poorly on glass cover-slips. The necessary monolayers for negative and positive controls must be included.
- ii) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 7 days or, if CPE appears, for a shorter time.
- iii) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. The fixed cell cultures may be stored dry for less than 1 week at 4°C or at -20°C for longer storage.
- iv) Incubate the cell monolayers with anti- ISAV MAb in an appropriate dilution in PBS for 1 hour. and rinse twice with PBS/0.05% Tween 20. If unspecific binding is observed, incubate with PBS containing 0.5% dry skimmed milk.
- v) Incubate with FITC-conjugated goat anti-mouse immunoglobulin for 1 hour (or if antibody raised in rabbits is used as the primary antibody, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity, FITC-conjugated goat anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with the described rinsing in between the additional step. Rinse once with PBS/0.05% Tween 20, as described above. The nuclei can be stained with propidium iodide (100 µg ml⁻¹ in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in dark until examination. For long periods of storage (more than 2–3 weeks) a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution. .

4.3.1.2.3. *Molecular techniques*

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

The primers described below for RT-PCR and real-time RT-PCR will detect both European and North-American HPR-deleted ISAV, and also HPR0 ISAV.

RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.4). The real-time RT-PCR for the detection of ISAV is recommended as it increases the specificity and, probably, also the sensitivity of the test. Though several primer sets for ISAV real-time RT-PCR have been reported, recommended primer sets are presented in the table below. The primer sets derived from genomic segment 8 and segment 7 have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in apparently healthy carrier fish

Annex 17 (contd)

With the widespread occurrence of HPR0 ISAV variants, it is essential to follow up any positive PCR results based on segment 7 or 8 primer sets by sequencing the HPR of segment 6 in order to determine the ISAV HPR variant present (HPR-deleted or HPR0 or both). Adequate primers, designed and validated by the OIE Reference Laboratory are given in the table below. Validation of the HPR primer set for the North American isolates is restricted by the limited sequence data available in the Genbank for the 3' end of ISAV segment 6.

The primers for segment 7 and 8 as well as sequencing primers for segment 6 HPR, are listed below and may also be used for conventional RT-PCR if necessary.

Real-time RT-PCR: Primer and probe sequences	Named	Genomic segment	Product size	Reference
5'-CAG-GGT-TGT-ATC-CAT-GGT-TGA-AAT-G-3' 5'-GTC-CAG-CCC-TAA-GCT-CAA-CTC-3' 5'-6FAM-CTC-TCT-CAT-TGT-GAT-CCC-MGBNFQ-3'	forward primer reverse primer Taqman@probe	7	155 nt	Snow <i>et al.</i> , 2006
5'-CTA-CAC-AGC-AGG-ATG-CAG-ATG-T-3' 5'-CAG-GAT-GCC-GGA-AGT-CGA-T-3' 5'-6FAM-CAT-CGT-CGC-TGC-AGT-TC-MGBNFQ-3'	forward primer reverse primer Taqman@probe	8	104 nt	Snow <i>et al.</i> , 2006
5'-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3' 5'-GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG-TA-3'	forward primer reverse primer	6 (HPR)	304 nt if HPR0	Designed by OIE Ref. Lab.

4.3.1.2.4. Agent purification

ISAV propagated in cell culture can be purified by sucrose gradient centrifugation (Falk *et al.*, 1997) or by affinity purification using immunomagnetic beads coated with anti-ISAV MAb.

4.3.2. Serological methods

Both Atlantic salmon and rainbow trout develop a humoral immune response to the ISAV infection. Enzyme-linked immunosorbent assays (ELISAs) with either purified virus or lysates from ISAV-infected cell cultures have been established for detection of ISAV-specific antibodies. ELISA titres can be very high and appear to be quite specific for the nucleoprotein in Western blots (K. Falk, pers. comm.). The test is not standardised for surveillance or diagnostic use, but may be used as a supplement to direct virus detection and pathology in obscure cases. Furthermore, the level and distribution of seroconversion in an ISAV-infected population may give some information about the spread of infection, particularly in cases where vaccination is not practised, and in wild fish.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance for infection with HPR-deleted ISAV and diagnosis of ISA are listed in Table 5.1. For surveillance of infection with HPR0 ISAV, real-time RT-PCR followed by sequencing is the only recommended method (not included in the table). The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance for infection with HPR-deleted ISAV				Presumptive diagnosis	Confirmatory diagnosis
	Fry Larvae	Parr PLs	Smolt Juveniles	Adults		
Gross signs	d	d	d	d	c	b
Histopathology	d	d	d	b	b	b
IFAT on kidney imprints	d	d	d	d	b	a
Immunohistochemistry	d	d	d	d	b	a
Isolation in cell culture with virus identification	a	a	a	a	a	a
RT-PCR or real-time RT-PCR followed by sequencing	a	a	a	a	b	a

*As the diagnosis of ISA is not based on the results of a single method, the information in this Table should be used with care. See Section 7 for the criteria for ISA diagnosis.

PLs = postlarvae; IFAT = indirect fluorescent antibody test; EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infectious salmon anaemia virus

Regular health inspections combined with investigation for ISA when increased mortality is associated with one of the given clinical signs and/or pathological changes consistent with ISA is an efficient way of obtaining data on the occurrence of ISA in farmed populations. In addition to regular health inspections, testing for HPR-deleted ISAV, preferentially by PCR-based methodology, at certain intervals may be carried out. However, due to **the expected low prevalence in apparently healthy populations and** the uneven spread of infection within a farm, **statistically appropriate large** numbers of samples need to be tested. The significance of positive findings of ISAV by PCR alone for the risk of developing ISA disease is not clear, and therefore any positive findings would have to be followed up by either further testing and/or surveillance of the production site.

Because of the transient nature of HPR0 ISAV, **statistically appropriate large** sample sizes need to be tested at **multiple** time points through the production cycle to be able to document freedom of this infection.

7. Corroborative diagnostic criteria

Reasonable grounds to suspect fish of being infected with ISAV (HPR-deleted or HPR0) are outlined below. The Competent Authority should ensure that, following the suspicion of fish infected with ISAV on a farm, an official investigation to confirm or rule out the presence of the disease will be carried out as quickly as possible, applying inspection and clinical examination, as well as collection and selection of samples and using the methods for laboratory examination as described in Section 4.

7.1. Definition of suspect case (HPR-deleted ISAV)

ISA or infection with HPR-deleted ISAV would be suspected if at least one of the following criteria is met:

- i) Clinical signs consistent with ISA **and/or** pathological changes consistent with ISA (Section 4.2) whether or not the pathological changes are associated with clinical signs of disease;

Annex 17 (contd)

- ii) Isolation and identification of ISAV in cell culture from a single sample (targeted or routine) from any fish on the farm, as described in Section 4.3.1.2.1;
- iii) Evidence for the presence of ISAV from two independent laboratory tests such as RT-PCR (Section 4.3.1.2.3) and or IFAT on tissue imprints (Section 4.3.1.1.2.1) or IHC (Section 4.3.1.1.3.1)

7.2. Definition of confirmed case (HPR-deleted ISAV)**7.2.1. Definition of confirmed ISA**

The following criteria should be met for confirmation of ISA: Mortality, clinical signs and pathological changes consistent with ISA (Section 4.2), and detection of ISAV in tissue preparations by means of specific antibodies against ISAV (IHC on fixed sections [Section 4.3.1.1.3.1] or IFAT on tissue imprints [Section 4.3.1.1.2] or fixed sections as described in Section 4.3.1.1.3) in addition to either:

- i) Isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm, as described in Section 4.3.1.2.1

or

- ii) Detection of ISAV by RT-PCR by the methods described in Section 4.3.1.2.3;

7.2.2 Definition of confirmed HPR-deleted ISAV infection

The criteria given in i) or ii) should be met for the confirmation of infection with HPR-deleted ISAV.

- i) Isolation and identification of ISAV in cell culture from at least two independent samples (targeted or routine) from any fish sample on the farm tested on separate occasions as described in Section 4.3.1.2.1.
- ii) Isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm with corroborating evidence of ISAV in tissue preparations using either RT-PCR (Section 4.3.1.2.3) or IFAT/IHC (Sections 4.3.1.1.2 and 4.3.1.1.3).

7.3. Definition of confirmed infection with HPR0 ISAV**7.3.1. Definition of confirmed infection with HPR0 ISAV**

The criteria given in i) and ii) should be met for the confirmation of HPR0 ISAV infection.

i) An absence of clinical signs consistent with ISA disease or mortality (= apparently healthy fish);

- ii) Detection of ISAV by RT-PCR followed by independent amplification and sequencing of the HPR region of segment 6 to confirm the presence of HPR0 only.

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NB: There is an OIE Reference Laboratory for Infection with infectious salmon anaemia virus (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>). Please contact the OIE Reference Laboratory for any further information on Infection with infectious salmon anaemia virus

CHAPTER 2.4.9

INFECTION WITH OSTREID HERPESVIRUS 1 MICROVARIANTS

1. Scope

For the purpose of this chapter, infection with ostreid herpesvirus 1 microvariants is considered a viral infection of bivalve molluscs caused by ostreid herpesvirus 1 microvariants. Microvariants of OsHV-1 (ostreid herpesvirus 1) are genotypes of OsHV-1 that have sequence variations defined by a deletion in a microsatellite locus upstream from ORF4 (Renault *et al.*, 2012; Segarra *et al.*, 2010) when compared with the reference type OsHV-1. μ Var is a microvariant. Ostreid herpesvirus 1 μ Var is strictly defined by a 12 bp deletion in a microsatellite locus upstream of the ORF4 and additional mutations in ORF4 and ORF42/43 ORF2/43 when compared with the reference sequence (accession number AY509253), however, the scope of this chapter also includes any related variants with a deletion of around 12 base pairs in this region. The term OsHV-1 microvariants is used in this chapter to refer to the OsHV-1 μ Var microvariant and these related variants. The term μ Var is used to define a single variant presenting all the mutations reported by Segarra *et al.* (2010).

Until now, Mortality associated with OsHV-1 microvariants has only been reported in the Pacific cupped oyster *Crassostrea gigas* and in the Portuguese cupped oyster *C. angulata*. Infection with OsHV-1 microvariant mainly affects the Pacific cupped oyster, *Crassostrea gigas*.

2. Disease information

2.1. Agent factors

OsHV-1 is the aetiological agent of a contagious viral disease of Pacific cupped oysters, *Crassostrea gigas*, also affecting other bivalve species. The genome of the virus was sequenced from infected Pacific oyster larvae collected in France in 1995 (Davison *et al.*, 2005). As this specimen was the first to be described (through complete genome sequencing, accession number AY509253), it can be considered as the reference type.

2.1.1. Aetiological agent, agent strains

OsHV-1 particles have been purified from French *C. gigas* larvae (Le Deuff & Renault, 1999) and were observed by transmission electron microscopy to be enveloped icosahedral with electron dense cores and a diameter around 120 nm. The intranuclear location of the virus particles, their size and ultrastructure are characteristic of members of the *Herpesvirales*.

The genome structure and sequence, and the capsid morphology (Davison *et al.*, 2005) have been further studied in order to assess OsHV-1 phylogenetic status in relation to vertebrate herpesviruses. The entire virus DNA was sequenced (GenBank accession number AY509253) and OsHV-1 capsids appear structurally similar to those of other herpes viruses that have been studied (Davison *et al.*, 2005). The virus was classified under the name *Ostreid herpesvirus 1* (OsHV-1) as the first known species in the family *Malacoherpesviridae* (Davison *et al.*, 2009).

A variant of OsHV-1 has been identified in France (Arzul *et al.*, 2001b) in *C. gigas*, *Ruditapes philippinarum* and *Pecten maximus*. Friedman *et al.* (2005) and Moss *et al.* (2007) also described differences in the sequences of OsHV-1 from California and Asia, respectively. Moss *et al.* (2007) suggested that there are at least two strains in Japan, one in South Korea and two in China (the People's Rep. of). One of the strains that occurred in China and South Korea was similar in sequence to the OsHV-1 strain from California described by Friedman *et al.* (2005), and the other strain from China was similar to OsHV-1 from France.

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More recently, polymerase chain reactions (PCRs) using different primer sets and PCR product sequencing enabled the detection of a variant called μ Var and other related Microvariants in association with high mortality events have been reported in Europe, Australia, New Zealand, and Asia (Dundon *et al.*, 2011; Hwang *et al.*, 2013; Jenkins *et al.*, 2013; Lynch *et al.*, 2012; Martenot *et al.*, 2011; Paul-Pont *et al.*, 2013a; 2013b; Peeler *et al.*, 2012; Renault *et al.*, 2012; Segarra *et al.*, 2010, Shimata *et al.*, 2012). The term microvariant is used to define a single variant presenting all the mutations reported by Segarra *et al.* (2010) in two different virus genome areas. The term OsHV-1 microvariants is used to refer to OsHV-1 microvariant and these related variants.

Although the aetiological agent is represented by all specimens or variants of OsHV-1 (Arzul *et al.*, 2001b; Davison *et al.*, 2005; Martenot *et al.*, 2011; Moss *et al.*, 2007; Renault *et al.*, 2012; Segarra *et al.*, 2010; Shimahara *et al.*, 2012), increased mortality outbreaks recently reported in Europe, Australia and New Zealand among *C. gigas* spat in association with the variant OsHV-1 microvariant or related viral variants suggested differences in terms of virulence among OsHV-1 variants. However, the detection of the variant microvariant or related variants have also been reported in absence of mortality events (Dundon *et al.*, 2011; EFSA, 2010; Shimahara *et al.*, 2012) suggesting that viral infection is influenced by both host and environmental factors.

2.1.2. Survival outside the host

Maximum survival time outside the host is unknown.

Schikorski *et al.* (2011a; 2011b) presented data on detection by real-time PCR of OsHV-1 μ Var DNA in seawater following cohabitation experiments. The copy numbers of virus DNA in the water in the first 48 hours after injecting spat with virus reached $1 \times 10^5 \text{ ml}^{-1}$, and reached a maximum of $1 \times 10^6 \text{ ml}^{-1}$ following infection of cohabiting oysters. The amount of infectious virus is unknown.

2.1.3. Stability of the agent (effective inactivation methods)

The lack of cell cultures for OsHV-1 has meant that *in vitro* studies on the stability of the virus with regard to infectivity have not been done. As an alternative, extracted viral DNA was seeded into seawater and $10 \text{ pg } \mu\text{l}^{-1}$ was detected for 16, 9 and 1 day at 4, 11 and 20°C respectively, and in a second experiment, $100 \text{ pg } \mu\text{l}^{-1}$ was detected after 51 days at each temperature.

The longest time for DNA detection in OsHV-1 released from macerated larvae and seeded into seawater was 22 days at 4°C and 12 days at 20°C (Vigneron *et al.*, 2004). However, the relationship between detection of DNA in the PCR and infectivity of the virus is unknown. As a general rule, the survival of many aquatic animal viruses outside the host is greatest at lower temperatures.

As a herpesvirus, OsHV-1 may be assumed to be fragile outside its hosts. High temperature, chemicals or sunlight (UV) may destroy its lipid-containing envelope, capsid or DNA. However, it has been demonstrated that individual herpesvirus species may have different levels of stability to inactivation treatment. Inorganic salts such as Na_2SO_4 present in seawater may stabilise herpesviruses (Wallis & Melnick, 1965).

2.1.4. Life cycle

Transmission The life cycle is direct from host to host (Le Deuff *et al.*, 1994; Schikorski *et al.*, 2011a; 2011b). Recently, it was hypothesised that OsHV-1 is transmitted by vector particles in the water (Paul-Pont *et al.*, 2013a).

2.2. Host factors

2.2.1. Susceptible host species

OsHV-1 has been reported from the Pacific oyster, *C. gigas*, Portuguese oyster, *C. angulata*, suminoe oyster, *C. ariakensis*, European flat oyster, *O. edulis*, Manila clam, *R. philippinarum*, carpet shell clam, *R. decussatus*, and scallops, *P. maximus* (Arzul *et al.*, 2001a; 2001b; Renault *et al.*, 2000). Until now, Mortality attributable to OsHV-1 microvariants Ostreid herpesvirus-1 μ Var (Renault *et al.*, 2012; Segarra *et al.*, 2010) has been mainly reported as affecting in the Pacific oyster, *Crassostrea gigas* and in the Portuguese cupped oyster *Crassostrea angulata* (Arzul *et al.*, 2013; Batista *et al.*, pers. comm.).

2.2.2. Susceptible stages of the host

Although OshV-1 microvariants the virus can be detected in all oyster stages, mortality due to OshV-1 microvariants only mainly concern spat and juveniles in a lesser extent.

~~OshV-1 infection may cause mortality in larvae and juveniles of several bivalve species. The virus can be found in adult bivalves most often in absence of mortality.~~

2.2.3. Species or subpopulation predilection (probability of detection)

~~Crassostrea gigas and O. edulis, R. philippinarum, R. decussates and P. maximus C. angulata~~ are naturally infected by microvariants. Young stages including larvae, spat and juveniles seem to be more susceptible to the infection. The virus is easier to detect in moribund animals than in apparently healthy ones animals.

2.2.4. Target organs and infected tissue

The infection-associated changes lesions in spat/juveniles are mainly observed in connective tissues of all organs in which fibroblastic-like cells may exhibit enlarged nuclei with perinuclear chromatin (Arzul *et al.*, 2002; Lipart & Renault, 2002; Renault *et al.*; 1995; Schikorski *et al.*, 2011a).

2.2.5. Persistent infection with lifelong carriers

Apparently healthy oysters, including adults, have been shown to be PCR-positive for OshV-1 (Arzul *et al.*, 2002; Moss *et al.*, 2007; Sauvage *et al.*, 2009). Pépin *et al.* (2008) showed that DNA copy numbers mg^{-1} tissue were high (up to 10^7) in oysters from populations with abnormal mortalities and low (lowest number detected 10^1) in populations with no abnormal mortalities. A threshold viral copy number of 10^4 mg^{-1} oyster tissue was proposed by Oden *et al.* (2011) as being associated with mortality, an observation supported by Paul-Pont *et al.* (2013b). Determining the levels of viral DNA in oysters by quantitative real-time PCR (qPCR)—might be a way summation—to differentiate between mechanical carriage of virus and low level of infection.

As the virus (DNA, protein or particles) has been detected in tissues of adult oysters, including the gonad (Arzul *et al.*, 2002; Lipart & Renault, 2002), adults may be a source of infection for larvae or spat, particularly under stressful conditions, e.g. from high temperature (Le Deuff *et al.*, 1996). However, what is not certain is whether true vertical transmission (transmission within the gametes) occurs or whether transmission is horizontal (Barbosa-Solomieu *et al.*, 2005).

2.2.6. Vectors

~~No vectors are required~~—The life cycle is direct. In a cohabitation challenge model using filtered seawater, no vector is required (Schikorski *et al.*, 2011a; 2011b). A recent paper hypothesises that a microvariant is also transmitted by vector particles in the water column (Paul-Pont *et al.*, 2013a).

2.2.7. Known or suspected wild aquatic animal carriers

Several bivalve species may act as subclinical and healthy carriers (see Section 2.2.3).

OshV-1 microvariant DNA has been recently detected in France and in Ireland in blue mussel, *Mytilus edulis*, and in *Donax trunculus* (Renault *et al.*, comm. pers.). However, in these cases, it remains unknown if these bivalve species are susceptible, resistant or may act as vector species.

2.3. Disease pattern

2.3.1. Transmission mechanisms

~~OshV-1 DNA has been detected by qPCR in the water around diseased Pacific oysters (Sauvage *et al.*, 2009) and the disease induced by the variant OshV-1 microvariant OshV-1 DNA (μVar) was detected by real-time PCR in the water surrounding dying Pacific oysters in the field—can be experimentally transmitted horizontally via the water (Schikorski *et al.*, 2011a), which presumably is the main natural mode of OshV-1 transmission.~~

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The first published report (Le Deuff *et al.*, 1994) described rapid transmission of the virus from an extract of diseased larvae to axenic larvae of *C. gigas*. Inter species transmission from infected axenic larvae of *C. gigas* to axenic larvae of *C. rivularis* and *Ostrea edulis* was demonstrated experimentally (Arzul *et al.*, 2001b). A suspension of OsHV-1 from *R. philippinarum* was shown to infect axenic larvae of *C. gigas*, and a virus suspension from *C. gigas* was shown to infect axenic larvae of *C. angulata* (Arzul *et al.*, 2001b).

Experimental transmission of OsHV-1- μ Var has been described by Schikorski *et al.* (2011a; 2011b). The disease can be transmitted to Spat can be infected at 22°C following intramuscular injection of a filtered homogenate an extract of naturally infected oysters, and also by cohabiting injected oysters with healthy oysters. Based on real-time qPCR detection, results suggest that the virus may enter the digestive gland and haemolymphatic system, following which the virus was disseminated to other organs.

2.3.2. Prevalence

Reported mortality rates and OsHV-1 microvariants prevalence vary considerably between sites and countries and depend on a range of factors including the age of affected stocks (Lynch *et al.*, 2012; Martenot *et al.*, 2011; Peeler *et al.*, 2012; Renault *et al.*, 2012; Segarra *et al.*, 2010). To better understand the implication of OsHV-1 in *C. gigas* spat mortality outbreaks regularly reported both in the field and in nurseries in France, samples were collected yearly through the French National Network for Surveillance of Mollusc Health between 1997 and 2006 (Garcia *et al.*, 2011). Analyses were carried out by PCR for OsHV-1 detection. Virus DNA was frequently detected in samples collected during mortality events with OsHV-1 detection frequency varying from 9 to 65% depending on the year. Data also demonstrated a particular seasonality and topography of spat oyster mortalities associated with OsHV-1 detection. In the field, mortality outbreaks appeared in summer, preferentially in sheltered environments.

More recently, increased mortality notifications (from 40 to 100%) were reported in 2008–2011 in Europe affecting Pacific oysters. These increased mortalities were associated with the detection of OsHV-1 microvariant or related variants depending of geographical locations (Lynch *et al.*, 2012; Martenot *et al.*, 2011; Peeler *et al.*, 2012; Renault *et al.*, 2012; Segarra *et al.*, 2010).

2.3.3. Geographical distribution

OsHV-1 has been reported from Europe (France, Ireland, Italy, Netherlands, Portugal, Spain, Sweden, United Kingdom), Australia, Brazil, China (People's Rep. of), Korea, Japan, Morocco, Tunisia, Mexico, New Zealand and United States of America. OsHV-1 Microvariants have been reported associated with Pacific oyster mass mortalities in Europe (France, Ireland, Italy, The Netherlands, Spain, UK), Australia, New Zealand, and Korea, but is known to occur elsewhere in the absence of oyster mortalities (e.g. Japan).

2.3.4. Mortality and morbidity

Infection by all strains is often lethal for *C. gigas* spat and juveniles. Death usually occurs 1 week after infection, during or shortly after the warmest annual water temperatures (Friedman *et al.*, 2005; Garcia *et al.*, 2011; Renault *et al.*, 1994b). Infected larvae show a reduction in feeding and swimming activities, and mortality can reach 100% in a few days.

2.3.5. Environmental factors

Mortality outbreaks associated with the detection of OsHV-1 microvariants are more frequent during summer, which might suggest a link between seawater temperature and OsHV-1 microvariants viral infection. The temperature influence on OsHV-1 detection and virus expression was demonstrated for *C. gigas* larvae (Le Deuff *et al.*, 1996) and strongly suspected for *C. gigas* spat (Burge *et al.*, 2007; Friedman *et al.*, 2005; Renault *et al.*, 1995; Sauvage *et al.*, 2009). A temperature threshold related to enhanced OsHV-1 expression or mortality appears difficult to define precisely. In the literature, according to the site, the temperature threshold was variable: 22°C to 25°C on the west coast of the USA (Friedman *et al.*, 2005; Burge *et al.*, 2007) and 18 to 20°C in France (Samain *et al.*, 2007; Soletchnik *et al.*, 1999). High seawater temperatures appear to be one of the potential factors influencing OsHV-1 infection. However, microvariants associated mortality does not occur consistently when water temperature is permissive. In Australia the seasonal risk factors are less certain and the temperature effects appear to be different to those in Europe (Paul-Pont *et al.*, 2013b).

Moreover, stressful conditions particularly rearing techniques seem to favour viral-OsHV-1 infection. In France, During summer, many if oyster transfers occur this may result in virus and might also amplify OsHV-1 transmission.

~~Spat mortality outbreaks associated with OsHV-1 detection generally presented a patchy distribution in the field (Garcia *et al.*, 2011). This particular pattern could be partly explained by the nature of the virus. Herpes viruses are enveloped and are assumed to be relatively labile in their environment. Thus, their transmission relies generally on direct contact. These data suggest that when OsHV-1 is excreted by oysters, it would mainly infect nearby oysters. The probable limited dissemination of OsVH-1 in seawater could partly explain the observation of the patchy mortality distribution rather than a uniform distribution as observed in nurseries. In nurseries, oysters are reared at high densities, are very close together and the seawater is often sequentially renewed.~~

2.4. Control and prevention

2.4.1. Vaccination

Not applicable, although Pacific oysters are known to have an inducible antiviral immune response capability (Green & Montagnani, 2013; Renault *et al.*, 2011).

2.4.2. Chemotherapy

None

2.4.3. Immunostimulation

Not applicable

2.4.4. Resistance breeding

Based on recent data, it has been demonstrated that Pacific cupped oyster families are less susceptible to OsHV-1 including the variant OsHV-1 microvariants μ Var can be obtained (Degremont, 2011; Sauvage *et al.*, 2009).

2.4.5. Restocking with resistant species

In France, A project of restocking with selected Pacific oysters is ongoing in France and first results showed less mortality when these oysters were placed in the field (Degremont *et al.*, com pers.). In Australia, restocking with Sydney rock oysters and flat oysters is occurring.

2.4.6. Blocking agents

None

2.4.7. Disinfection of eggs and larvae

None

2.4.8. General husbandry practices

Biosecurity practices may be successfully applied in confined and controlled facilities such as hatcheries and nurseries in order to protect the facility and the surrounding environment from the introduction of the virus.

As a herpesvirus, OsHV-1 may be assumed to be fragile outside its hosts. High temperature, chemicals or sunlight (UV) may destroy its lipid containing envelope, capsid or DNA. However, it has been demonstrated that individual herpesvirus species may have different levels of stability to inactivation treatment. Inorganic salts such as Na_2SO_4 present in seawater may stabilise herpesviruses (Wallis & Melnick, 1965).

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In ~~artificial controlled~~ rearing conditions (mollusc hatchery/nursery), OsHV-1 outbreaks may therefore be controlled through quarantine and hygiene measures including virus inactivation through ~~adapted~~ treatments such as ~~water filtration and~~ ultraviolet irradiation (~~between 3 and 30 mJ cm⁻²~~) of the ~~recirculating water and water filtration technologies~~. However, it is necessary to keep in mind that reduction of virus load depends on the initial titre and the virus reduction capacity of the techniques used for inactivation. ~~If there was an initial concentration of 1 million viruses per litre and the inactivation method used allowed inactivation of 100,000 viruses per litre, there would still be numerous infective particles in the treated product.~~

Moribund and dead oysters should be removed and destroyed whenever feasible. Equipment used in an infected zone should not be sent and used in a non-affected zone without adequate cleaning and disinfection.

3. Sampling

3.1. Selection of individual specimens

Live or moribund individuals should be sampled.

3.2. Preservation of samples for submission

For histology, the best preservative is Davidson's AFA, but 10% buffered formalin, 10% seawater formalin or other standard histology fixatives are also acceptable. For PCR assays, samples must be preserved in 95–100% ethanol, a suitable nucleic acid preservation reagent or kept frozen (–80°C).

3.3. Pooling of samples

~~Pooling of small spat is acceptable for PCR/qPCR analyses. However, the effect of pooling samples on PCR/qPCR sensitivity has not been evaluated. Samples may be acceptable under some circumstances, however, the impact on sensitivity and design prevalence must be considered.~~

3.4. Best organs or tissues

For histology, ~~a 2-µm thick sections~~ through the visceral mass that include digestive gland, gill and mantle are used. For PCR, a section of mantle tissue or combined gill and mantle tissue is best.

3.5. Samples/tissues that are not suitable

Gonad tissues may be not reliable for PCR assays because of the presence of inhibitors.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Infection by OsHV-1 microvariants may cause an acute disease. Animals are likely to die within a few days of manifesting clinical signs of the disease. Clinical signs may be dead or gaping bivalves but these are not specific to infection with OsHV-1 microvariants.

4.1.2. Behavioural changes

Infected hosts may be slow to close their valves when disturbed but these behavioural changes are not specific to infection with OsHV-1.

4.2. Clinical methods

4.2.1. Gross pathology

Clinical signs may be dead or gaping bivalves but these clinical signs are not specific to infection with OsHV-1.

4.2.2. Clinical chemistry

None

4.2.3. Microscopic pathology

See Section 4.2.6. Fixed sections

4.2.4. Wet mounts

Not applicable

4.2.5. Smears

Not applicable

4.2.6. Fixed sections

The most consistent features of infection with OsHV-1 are nuclear changes including hypertrophy, nuclear margination and pycnosis. The infection-associated lesions in spat are mainly observed in connective tissues in which fibroblastic-like cells exhibit enlarged nuclei with perinuclear chromatin. Highly condensed nuclei (apoptosis features) were also reported in other cells interpreted as haemocytes. These cellular abnormalities are not associated with massive haemocyte infiltration.

Histological examination of the animal is not sufficient to identify infection with herpesvirus. Whilst Cowdry type A inclusions (eosinophilic intranuclear inclusions with perinuclear chromatin) are typical of many herpesvirus infections they are not a diagnostic feature of herpesvirus infections of oysters (Arzul *et al.*, 2002). Cowdry type A inclusions have never been reported following histological examination of infected Pacific cupped oysters in France (Renault *et al.*, 1994a; 1994b). Moreover, intranuclear inclusion bodies were not observed, although there was other cellular/nuclear pathology, in association with OsHV-1 infections in oysters in Mexico (Vásquez-Yeomans *et al.*, 2010) or USA (California) (Friedman *et al.*, 2005).

4.2.7. Electron microscopy/cytopathology

See Section 4.3.1.1.4.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Not applicable

4.3.1.1.2. Smears

Not applicable

Annex 18 (contd)4.3.1.1.3. *Fixed sections*

Samples to be taken: live or moribund oysters.

Technical procedure: Sections of tissue that include mantle, digestive gland, gills and adductor muscle should be fixed for 24 hours in 10% formaldehyde fixatives such as Davidson's AFA or other suitable fixative followed by normal processing for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications up to $\times 400$.

Positive controls: These are recommended and are available from the Genetics and Pathology Laboratory, Ifremer, La Tremblade, France. Positive controls are tissue sections from μ Var any OsHV-1-infected oysters mollusc.

Levels of validation:

- *Specificity and sensitivity:* Whatever is the genotype, specificity is very low, and sensitivity is good for moderate- to high-intensity infections, but low for low-intensity infections.
- *Gold standard:* None

Interpretation of results:

- A positive result is the occurrence of cell abnormalities in tissue sections: Fibroblastic-like cells exhibiting enlarged nuclei with perinuclear chromatin. Highly condensed nuclei are also reported in other cells interpreted as haemocytes. These cellular abnormalities are not associated with massive haemocyte infiltration.
- In susceptible host species, within the known range for OsHV-1 microvariants, a positive result is presumptive evidence of OsHV-1-viral infection only and should be confirmed by species-specific PCR, *in-situ* hybridisation (ISH) and/or DNA sequencing.

Availability of commercial tests: No commercially available tests

4.3.1.1.4. *Electron microscopy/cytopathology*

Transmission electron microscopy can be used to confirm the presence of viral particles in infected animals.

Tissue samples (containing connective tissue such as mantle) for examination by electron microscopy should be fixed using 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide, washed in 0.1 M cacodylate buffer (3 \times 10 minutes), dehydrated in a graded series of ethanol (70%, 1 \times 10 minutes; 95%, 2 \times 15 minutes; 100%, 3 \times 20 minutes), washed in propylene oxide (2 \times 15 minutes), pre-infiltrated in 50% propylene oxide/50% Epon resin (1 hour), infiltrated in 100% Epon resin (1 hour) and then embedded in Epon resin.

Virus replication mainly takes place in fibroblastic-like cells throughout connective tissues especially in mantle, labial palps, gills and digestive gland (Renault *et al.*, 1994b; 1995; Schikorski *et al.*, 2011a). Virogenesis begins in the nucleus of infected cells where capsids and nucleocapsids are observed. Viral particles then pass through the nuclear membrane into the cytoplasm and enveloped particles are released at the cell surface. Intranuclear and cytoplasmic capsids present a variety of morphological types including electron lucent capsids, toroidal core-containing capsids, and brick-shaped core-containing capsids.

4.3.1.2. Agent isolation and identification4.3.1.2.1. *Cell culture/artificial media*

To date, attempts to culture the virus in both vertebrate and invertebrate cell lines and in primary oyster cell cultures have been unsuccessful.

4.3.1.2.2. *Antibody-based antigen detection methods*

Specific antibodies have been developed (Arzul *et al.*, 2002). However, they are not currently available for diagnostic purposes.

4.3.1.2.3. Molecular techniques

At present there are a number of different PCR methods available for the detection of OsHV-1. These include both conventional and real-time PCRs (Martenot *et al.*, 2010; Pépin *et al.*, 2008; Renault *et al.*, 2000).

~~A protocol for quantifying OsHV-1 in Pacific oysters based on a Sybr[®] Green real-time PCR was first developed (Pépin *et al.*, 2008). Martenot *et al.* (2010) developed an alternative protocol based on TaqMan[®] chemistry. The quantitation limits were 1000 and 18 UG mg⁻¹ of tissues for the Sybr[®] Green-based method and the TaqMan[®] method, respectively, and the latter protocol has a detection limit of 6 UG mg⁻¹ of tissues. Comparing the two protocols using DNA samples obtained from 210 spat, the kappa index (0.41) indicated a moderate concordance between the protocols, according to the measures of Landis and Koch. All samples that were positive by the reference protocol were also positive by the alternative protocol. Of the 76 samples that were negative by the reference protocol, 49 were positives by the alternative protocol. Although these results may suggest that the alternative protocol can be more sensitive than the reference protocol, formal validation is needed. A protocol based on TaqMan[®] chemistry is under development and validation for the detection of virus specimens or variants presenting the deletion reported in the microsatellite upstream from the ORF4 area (microsatellite) for OsHV-1 the variant microvariants (Pépin *et al.*, pers. comm.).~~

~~A loop-mediated isothermal amplification (LAMP) assay was also developed for OsHV-1 DNA detection (Ren *et al.*, 2010). A set of four primers was designed, based on the sequence of the ATPase subunit of the OsHV-1 DNA packaging terminase gene. This LAMP technique can be used both in the laboratory and on farms.~~

Samples to be taken: Live or moribund molluscs. Larvae (100–200 mg), spat (100–200 mg) or 2–3 mm² tissue pieces are excised aseptically from mantle, placed into 1.5 ml tubes, preserved in 95° alcohol or kept frozen (–80°C). Dissecting utensils should be flamed between samples to prevent cross-contamination.

4.3.1.2.3.1. Conventional PCR assays

Conventional PCR assays have been used successfully to detect OsHV-1 DNA in bivalves and different primer pairs have been designed (see Batista *et al.*, 2007 for a review).

Two pairs of primers (A3/A4 and A5/A6) were designed and used to detect virus DNA in Pacific oyster larvae and spat via nested PCR (Renault *et al.*, 2000). The specificity of these primer pairs was evaluated using DNA from *C. gigas* as well as DNA from vertebrate herpesviruses; 500 fg of virus DNA extracted from purified particles was routinely detected. The one-step PCR assay with the A3/A4 primer pair not only allowed amplification of OsHV-1 DNA but also the detection of a variant of this virus in *C. gigas* and *R. philippinarum* larvae (Arzul *et al.*, 2002).

Other primers were then designed including C2/C6. The combination of primer pairs A3/A4 and A5/A6 allowed less PCR amplification than C2/C6 (21.4% vs 32.4%) when the same larval samples were analysed (Renault & Arzul, 2001). C2/C6 primer pair systematically allowed the detection of 1 fg of purified viral DNA (Renault *et al.*, 2004). A detection limit of 10 fg of purified viral DNA for both primer pairs C13/C5 and Gp3/Gp4 has been reported (Vigneron *et al.*, 2004). As little as 1 pg and 10 pg allowed the C9/C10 and the OsHVDPFor/OsHVDPRev primer pairs, respectively, to detectably amplify a specific product (Webb *et al.*, 2007).

Although PCR specificity has been assessed for some of the primer pairs used to detect virus DNA (see above), this has not been done for all designed primer pairs. Moreover, the amplification conditions that have been used in PCR assays using different primer pairs were based on the conditions optimised for A3/A4 and A5/A6 (Renault *et al.*, 2000). An experimental procedure scheme used for the detection of OsHV-1 DNA by conventional PCR has been proposed by Bastista *et al.* (2007).

Annex 18 (contd)*4.3.1.2.3.2. OsHV-1 specific Sybr® Green PCR assay (Pepin et al., 2008)*

Fifty mg of larvae/spat/mantle tissue are ground in 50 µl double-distilled water using a disposable piston. The crushed tissues are diluted six-fold and clarified at 10,000 *g* for 5 minutes. One hundred µl recovered supernatant are treated using a commercial DNA tissue kit (QIAGEN – QIamp tissue mini kit®) according to the manufacturer's protocol. Final elution of the DNA is performed with 100 µl TE buffer. The DNA is stored at –20°C. Prior to PCR, DNA concentrations can be measured by absorbance at 260 nm. According to total DNA concentration measured in samples, they are diluted in order to obtain 20 ng total DNA per PCR reaction.

Three sets of primers can be used targeting three regions of viral DNA: (ORF4, ORF88 and ORF99). Primer pairs B4/B3 (Arzul *et al.*, 2001⁵; ORF99 encoding a BIR protein) and C9/C10 (Barbosa-Solomieu *et al.*, 2004; ORF4) were previously designed for single PCR, whereas the Gp4/Gp7 primer pair (ORF88 encoding a class I membrane protein) was assessed for qPCR. The primer pairs B4/B3, C9/C10 and Gp4/Gp7 yield PCR products of 207, 197 and 85 bp, respectively.

B4: 5'-ACT-GGG-ATC-CGA-CTG-ACA-AC-3'

B3: 5'-GTG-GAG-GTG-GCT-GTT-GAA-AT-3'

C9: 5'-GAG-GGA-AAT-TTG-CGA-GAG-AA-3'

C10: 5'-ATC-ACC-GGC-AGA-CGT-AGG-3'

Gp4: 5'-GGC-GTC-CAA-ACT-CGA-TTA-AA-3'

Gp7: 5'-TTA-CAC-CTT-TGC-CGG-TGA-AT-3'

The C9/C10 primer pair yield reliable parameters for qPCR with OsHV-1 DNA, as well as the B3/B4 primer pair, which show closely similar parameters with a slightly lower *E* value (96.3%). The Gp4/Gp7 primer pair is less efficient (*E* = 91.3%) and less sensitive (≥ 50 copies μl^{-1}). The primer pair C9/C10 appears to be the most sensitive and efficient.

An additional primer pair DPFor/DPRev can be also used producing a 197 bp product (ORF100, DNA polymerase).

DPFor: 5'-ATT-GAT-GAT-GTG-GAT-AAT-CTG-TG-3'

DPRev: 5'-GGT-AAA-TAC-CAT-TGG-TCT-TGT-TCC-3'

Targeting different **segments of OsHV-1 DNA** is important in order to define more precisely viral **strains and isolates genotypes**. Although ORF4 is an interesting candidate to describe diversity because virus polymorphism has been already reported in this area, ORF100 (DNA polymerase) appears to be less polymorphic.

All amplification reactions are performed in a total volume of 25 µl with 96-microwell plates. Each well (25 µl) contains 5 µl extracted DNA dilution (sample) or OsHV-1 genomic DNA (positive control), 12.5 µl Brilliant® SYBR® Green I PCR Master Mix or FullVelocity® Master Mix (Stratagene), 2.5 µl each diluted primer (final concentration 200 nM) and 2.5 µl distilled water. Thermal cycle conditions are: 1 cycle of pre-incubation at 95°C for 10 minutes; 40 cycles of amplification at 95°C for 30 seconds (15 seconds with FullVelocity® Master Mix), 60°C for 45 seconds (30 seconds with FullVelocity® Master Mix) and 72°C for 45 seconds with Brilliant® Master Mix; and melting temperature curve analysis at 95°C for 60 seconds, 60°C for 30 seconds and 95°C for 30 seconds. Real time PCR analysis should be performed in triplicate with 5 µl sample dilutions as DNA template or a viral DNA control.

Absolute quantitation of copies of OsHV-1 DNA (copies μl^{-1}) is carried out by comparing CT (threshold cycle) values obtained with the standard curve, using the Thermocycler software. Each experiment includes a positive DNA control (OsHV-1 genomic DNA for absolute quantitation) and blank controls (NTC, no template control consisting of deionised sterile water). PCR efficiency (*E*) is calculated from standard curves as the percentage of template molecules that is doubled during each cycle ($[10^{(-1/\text{slope})} - 1] \times 100$), with requirements that it fell into the range 95–105% and that the coefficient of determination (*R*²) is > 0.98 . In order to allow detection of non-specific products, a dissociation protocol (melt curve) takes place after the amplification cycles. The temperature at which SYBR®Green fluorescence is generated by the double-stranded amplicon dissociation is recorded.

Annex 18 (contd)

Regarding the test's sensitivity, it is considered that it can detect systematically 4 DNA copies μl^{-1} . The dynamic range for the qPCR was estimated from several standard curve assays, and a linear relationship was obtained between input copy number of the viral DNA template and CT value for over 5 log 10 dilutions. It was possible to quantitate OsHV-1 DNA copy numbers at least from 10 to 5×10^6 copies μl^{-1} .

4.3.1.2.3.3. OsHV-1 specific TaqMan® PCR assay (Martenot *et al.*, 2010)

The target was the B region of the OsHV-1 genome, which encodes a putative apoptosis inhibitor (Arzul *et al.*, 2001b). Primer pairs and two TaqMan® probes were designed to detect simultaneously the target gene and an internal control (IC). The IC was a synthesised sequence containing at each end the forward OsHV1BF (5'-GTC-GCA-TCT-TTG-GAT-TTA-ACA-A-3') and reverse B4 (5'-ACT-GGG-ATC-CGA-CTG-ACA-AC-3') primers. The B4 primer used for the TaqMan PCR was the same as that published by Pepin *et al.* (2008).

The amplification of the targeted region and IC was performed by using the OsHV1BF and B4 primers. The B (5'-TGC-CCC-TGT-CAT-CTT-GAG-GTA-TAG-ACA-ATC-3') and the IC (5'-ATC-GGG-GGG-GGG-GGT-TTT-TTT-TTT-ATC-G-3') probes were labelled at the 5' end with the fluorescent reporter dyes TxR and FAM, respectively, and at the 3' end with an appropriate quencher (BHQI or BHQII).

The reaction mixture contained 12.5 μl premix ExTaq® 2× Takara® (Lonza, Verviers, Belgium), 0.5 μl each primer (20 μM), 0.5 μl TaqMan® probes (10 μM) and 9 μl water. Two μl DNA sample was added to 23 μl reaction mixture. The amplification was performed in two stages under the following conditions: 1 cycle of 95°C for 10 seconds, followed by 40 cycles of amplification at 95°C for 5 seconds, 60°C for 20 seconds. The virus quantitation was carried out by comparison with standard curve values.

The protocol for quantifying OsHV-1 in Pacific oysters based on a Sybr® Green real-time PCR was first developed (Pepin *et al.*, 2008). Martenot *et al.* (2010) developed an alternative protocol based on TaqMan® chemistry. The quantitation limits were 1000 and 18 UG mg^{-1} of tissues for the Sybr® Green-based method and the TaqMan® method, respectively, and the latter protocol has a detection limit of 6 UG mg^{-1} of tissues. Comparing the two protocols using DNA samples obtained from 210 spat, the kappa index (0.41) indicated a moderate concordance between the protocols, according to the measures of Landis and Koch. All samples that were positive by the reference protocol were also positive by the alternative protocol. Of the 76 samples that were negative by the reference protocol, 49 were positives by the alternative protocol. Although these results may suggest that the alternative protocol can be more sensitive than the reference protocol, formal validation is needed. A protocol based on TaqMan® chemistry is under development and validation for the detection of virus specimens or variants presenting the deletion reported in the microsatellite upstream from the ORF4 area for microvariants (Pepin *et al.*, pers. comm.).

4.3.1.2.3.4. OsHV-1 specific in-situ hybridisation

The *in-situ* hybridisation (ISH) procedure described here uses a digoxigenin (DIG)-labelled DNA probe to detect OsHV-1 in formalin-fixed, paraffin-embedded tissue (Arzul *et al.*, 2002; Lipart & Renault, 2002). This assay can detect the generic and emergent strains microvariants but cannot distinguish between genotypes.

Sections of tissue that include mantle, digestive gland, gills and adductor muscle should be fixed for 24 hours in Davidson's AFA or other suitable fixative and processed using standard procedures for histological examination.

~~Seven μm thick~~ Tissue sections on silane-prep™ slides are dewaxed in xylene (2 × 5 minutes), treated in absolute ethanol (2 × 5 minutes) and air dried at room temperature (15 minutes). Sections are then permeabilised with proteinase K (100 $\mu\text{g ml}^{-1}$ in distilled water) for 30 minutes at 37°C in a humid chamber. Proteolysis is stopped by one 3-minute wash in 0.1 M Tris, 0.1 M NaCl buffer (pH 7.5) at room temperature. Sections are dehydrated in 95° ethanol for 1 minute, absolute ethanol for 1 minute and air dried (15 minutes).

Annex 18 (contd)

A prehybridisation step is carried out with pre-hybridisation buffer (50% formamide, 10% dextran sulfate, $4 \times \text{SSC}$ [0.06 M $\text{Na}_3\text{citrate}$, 0.6 NaCl, pH 7], $250 \mu\text{g ml}^{-1}$ yeast tRNA and 10% Denhart) for 30 minutes at 42°C in a humid chamber. The prehybridisation buffer solution is replaced with $100 \mu\text{l}$ hybridisation buffer solution containing $50 \mu\text{l}$ digoxigenin-labelled probe ($5 \text{ ng } \mu\text{l}^{-1}$) and $50 \mu\text{l}$ hybridisation buffer (50% formamide, 10% dextran sulfate, $4 \times \text{SSC}$, $250 \mu\text{g ml}^{-1}$ yeast tRNA and 10% Denhart). Slides are covered with plastic coverslips (Polylabo, France). DIG-labelled probes are synthesised from OsHV-1 genomic DNA (100 pg per reaction) by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim, Germany) during conventional PCR. The primer pair C1/C6 is used:

C1: 5'- TTC-CCC-TCG-AGG-TAG-CTT-TT -3'

C6: 5'- GTG-CAC-GGC-TTA-CCA-TTT-TT -3'

Target DNA and digoxigenin-labelled probe are denatured at 95°C for 5 minutes and the hybridisation is carried out overnight at 42°C in a humid chamber.

After hybridisation, coverslips were removed carefully and slides were washed for 10 minutes in $1 \times \text{SSC}$ (0.2% BSA) at 42°C . Specifically bound probe was detected using a peroxidase-conjugated mouse IgG antibody against digoxigenin (Boehringer Mannheim, Germany) diluted 1:250 in $1 \times \text{PBS}$ (1 hour at room temperature). Unbound peroxidase-conjugated antibody was removed by six washes in $1 \times \text{PBS}$ (5 minutes). Diaminobenzidine (DAB) tetrahydrochloride was diluted in $1 \times \text{PBS}$ (0.7 mg ml^{-1}). The colour solution was added to tissue sections ($500 \mu\text{l}$) and incubated at room temperature in the dark for 20 minutes. The reaction was stopped with two $1 \times \text{PBS}$ washes. Slides were stained for 20 seconds in Unna Blue (RAL, France) followed by ethanol dehydration and mounted in Eukitt via xylene.

Specific dark brown intra-cellular staining is indicative of the presence of viral DNA.

Thirty Pacific oyster adults have been analysed using three different techniques: PCR, ISH and immunochemistry, in order to detect OsHV-1 in subclinical individuals (Arzul *et al.*, 2002). PCR and ISH allowed detection of oyster herpes virus DNA in 93.3% and 86.6%, respectively, of analysed oysters while polyclonal antibodies allowed detection of viral proteins in 76.6% of analysed adult oysters.

4.3.1.2.4. Agent purification

OsHV-1 can be purified from infected animals using a previously developed technique (Le Deuff & Renault, 1999)

4.3.2. Serological methods

None applicable.

5. Rating of tests against purpose of use

Should perinuclear chromatin be observed by histology, electron microscopy at least should be undertaken to identify any virus-like particles present and demonstrate their location within cells. Viruses observed by EM should be described as e.g. herpesvirus-like until further investigations are done to provide further evidence of the identity of the virus. As different herpesviruses are morphologically similar, a virus should only be described as OsHV-1 if it had been shown to have identity with the latter virus using OsHV-1 specific primers or probes.

For OsHV-1, the demonstration of the presence of intracellular viral structural and non-structural proteins, specific OsHV-1 specific messenger RNA, non-structural proteins and TEM demonstrating and virions within cells constitute evidence for replication, but detection of viral DNA presence by PCR alone does not. As many moribund/dead oysters from populations with abnormal mortalities had high copy numbers of viral DNA, it may be possible in some cases to extrapolate those data to infer that OsHV-1 has replicated in animals (from known or new host species) with such high levels of viral DNA. However, rigorous evaluation and validation is required before these data could be used in that way.

It may be possible to demonstrate viral infectivity by passage to a susceptible host with appropriate control animals (bioassay). Detection of mortality or characteristic changes associated with detection of the virus is an important consideration in the assessment but not conclusive evidence of host susceptibility. The anatomical location of the pathogen is important also to exclude potential passive contamination of the host. This information can be obtained by techniques such as TEM, immunohistochemistry or ISH.

As an example, the methods currently available for targeted surveillance and diagnosis 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.

Table 5.1. Methods for **targeted** surveillance and diagnosis

Method	Targeted surveillance			Presumptive diagnosis surveillance			Confirmatory diagnosis		
	Larvae	Juveniles	Adults	Larvae	Juveniles	Adults	Larvae	Juveniles	Adults
Gross signs	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>
Bioassay	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>c</u>	<u>c</u>	<u>d</u>
Histopathology	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>
Transmission EM	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>b</u>	<u>b</u>	<u>b</u>
Antibody-based assays	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>	<u>d</u>
DNA probes – <i>in situ</i>	c	c	c	<u>c</u>	<u>c</u>	<u>c</u>	<u>b</u>	<u>b</u>	<u>b</u>
PCR	a	a	a	<u>a</u>	<u>a</u>	<u>a</u>	<u>b</u>	<u>b</u>	<u>b</u>
qPCR	a	a	a	<u>a</u>	<u>a</u>	<u>a</u>	<u>b</u>	<u>b</u>	<u>b</u>
Sequence	d	d	d	<u>d</u>	<u>d</u>	<u>d</u>	<u>a</u>	<u>a</u>	<u>a</u>

EM = electron microscopy; PCR = polymerase chain reaction; qPCR = real-time PCR.

6. Test(s) recommended for targeted surveillance to declare freedom from OsHV-1 infection

Not applicable. PCR and real-time PCR are recommended.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case of infection with microvariants is a case of mortality of susceptible species associated with detection of OsHV-1 by PCR or qPCR, or *in situ* hybridisation. Infection can exist without evidence of mortality.

7.2. Definition of confirmed case

A confirmed case is defined as a suspect case of infection with ostreid herpesvirus 1 microvariants is confirmed when detection by histology, transmission electron microscopy, or PCR is followed by sequencing of the microsatellite locus upstream of the ORF4 (Segarra *et al.*, 2010) leading to sequences consistent with the definition of microvariants.

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CHAPTER 2.3.X.

INFECTION WITH SALMONID ALPHAVIRUS

1. Scope

For the purpose of this chapter, infection with salmonid alphavirus (SAV) means infection with any subtype of SAV of the genus *Alphavirus* of the family *Togaviridae*.

Infection with SAV may cause pancreas disease (PD) or sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* [Walbaum]) and brown trout (*Salmo trutta* L.) (Boucher *et al.*, 1995; McLoughlin & Graham, 2007). The virus is horizontally transmitted, and the main reservoirs of SAV are clinically diseased or covertly infected fish (Viljugrein *et al.*, 2009). The disease is a generalised systemic disease and lethal condition characterised microscopically by necrosis and loss of exocrine pancreatic tissue, and severe heart and skeletal muscle changes. The mortality varies significantly, from negligible to over 50% in severe cases, of PD and up to 15% of surviving fish will develop into long, slender fish ('runts') (McLoughlin & Graham, 2007).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

SAV is an enveloped, spherical, single-stranded, positive-sense RNA virus, approximately 55–65 60–70 nm in diameter, with a genome of ~12 kb. The genome codes for eight proteins: four capsid glycoproteins (E1, E2, E3 and 6K) and four nonstructural proteins (nsP1–4). Glycoprotein E2 is considered to be the site of most neutralising epitopes, while E1 contains more conserved, cross-reactive epitopes (McLoughlin & Graham, 2007). SAV is considered to belong to the genus *Alphavirus* of the family *Togaviridae*. This is based on nucleotide sequence studies of SAV isolates, and is also supported by biological properties of the virus, including cross-infection and neutralisation trials. In addition, four conserved nucleotide sequence elements (CSEs) and a conserved motif (GDD), characteristic of alphaviruses, are present in the SAV genome (McLoughlin & Graham, 2007).

SAV has been divided into six subtypes (SAV1–SAV6) based solely on nucleic acid sequence differences for the proteins E2 and nsP3 (Fringuelli *et al.*, 2008). The level of antigenic variation among subtypes is considered low as monoclonal antibodies (MAbs) raised against a specific SAV subtype are likely to cross react with other SAV isolates (Graham *et al.*, 2013a; Jewhurst *et al.*, 2004). The genotype groups and their geographical distributions are presented in the table below (abbreviations: SW= sea water, FW = fresh water):

SAV subtype	Host and environment	Country
SAV 1 (PD)	Atlantic salmon (SW) Rainbow trout (FW)	Ireland, UK (Northern Ireland, Scotland)
SAV 2 FW (SD)	Rainbow trout (FW) Atlantic salmon (SW)	France, Germany, Italy, Spain, Switzerland, Poland, UK (England, Scotland)
SAV 2 Marine (PD)	Atlantic salmon (SW)	Norway, UK (Scotland)
SAV 3 (PD)	Rainbow trout (SW) Atlantic salmon (SW)	Norway only
SAV 4 (PD)	Atlantic salmon (SW)	Ireland, UK (Northern Ireland, Scotland)
SAV 5 (PD)	Atlantic salmon (SW)	UK (Scotland)
SAV 6 (PD)	Atlantic salmon (SW)	Ireland only

Annex 19 (contd)

2.1.2. Survival outside the host

Laboratory tests suggest that SAV would survive for extended periods in the aquatic environment. Laboratory tests have shown that in these tests, virus survival was inversely related to temperature and is reduced by the presence of organic matter. In the presence of organic matter, markedly longer survival times are observed in sea water compared with fresh water. In the presence of organic matter, marked longer survival times were observed in sea water compared with fresh water (Graham *et al.*, 2007c). SAV has been detected in fat leaking from dead PD diseased fish, indicating that this may be a route for transmission. Fat droplets may accumulate at the sea water surface, contributing to long distance spread (Stene *et al.*, submitted).

The half-life of SAV in serum has been found to be inversely related to temperature, emphasising the need for rapid shipment of samples at 4°C to laboratories for virus isolation. For long-term conservation of SAV-positive samples and cultured virus, storage at –80°C is recommended (Graham *et al.*, 2007c).

2.1.3. Stability of the agent

SAV is rapidly inactivated in the presence of high levels of organic matter at 60°C, at pH 7.2, and at pH 4 and pH 12 at 4°C, suggesting that composting, ensiling and alkaline hydrolysis would all be effective at inactivating virus in fish waste (Graham *et al.*, 2007a).

2.1.4. Life cycle

Probable infection routes are through the gills or via the intestine. In the acute stages of the disease, large amounts of SAV can be detected and live virus can be isolated from the heart, kidney, blood and several other organs, but the actual target cells for the virus has not yet been identified.

Viraemia precedes both the onset of histological changes and clinical signs (McLoughlin & Graham, 2007). The route of shedding may be through natural excretions/secretions, supported by the detection of SAV by reverse-transcriptase polymerase chain reaction (RT-PCR) in the faeces and mucus of experimentally infected Atlantic salmon. These matrices may therefore play a role in the horizontal transmission of SAV through water (Graham *et al.*, 2012). Virus has been detected in water 4–13 days after infection, indicating that virus shedding coincides with the viraemic stage (Andersen *et al.*, 2010). An incubation period of 7–10 days at sea water temperatures of 12–15°C has been estimated based on analysis of antibody production in intraperitoneally infected fish and cohabitants in an experimental trial (McLoughlin & Graham, 2007). Several studies have shown that SAV RNA can be detected in fish for an extended period post-infection (Jansen *et al.*, 2010a; McLoughlin & Graham, 2007). Subclinical infection has been reported, suggesting that the severity of an outbreak may be influenced by several environmental factors (McLoughlin & Graham, 2007), and recent data show that seasonal increase in water temperature may trigger PD disease outbreaks in SAV-infected farms (Stene *et al.*, 2013).

2.2. Host factors

2.2.1. Susceptible host species

Disease outbreaks and infection experiments have shown that Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* [Walbaum]) and brown trout (*Salmo trutta* L.) are susceptible. (Boucher *et al.*, 1995; McLoughlin & Graham, 2007).

2.2.2. Susceptible stages of the host

All life stages should be considered as susceptible to SAV.

Farmed rainbow trout in fresh water are affected at all stages of production (Kerbarth Boscher *et al.*, 2006). Experience from Norway show that farmed rainbow trout and Atlantic salmon are susceptible at all stages in sea water, probably reflecting a sea water reservoir of SAV. Experimental infection by injection indicates has also shown susceptibility of Atlantic salmon parr in fresh water (McVicar, 1990).

2.2.3. Species or subpopulation predilection (probability of detection)

There is no known species or subpopulation predilection.

2.2.4. Target organs and infected tissue

Infection with SAV is a systemic disease with an early viraemic phase. After infection, SAV has been detected in all organs that have been examined: brain, gill, pseudobranch, heart, pancreas, kidney and skeletal muscle (Andersen *et al.*, 2007; McLoughlin & Graham, 2007) as well as in mucus and faeces (Graham *et al.*, 2012).

2.2.5. Persistent infection with lifelong carriers

SAV has been detected in surviving fish 6 months after experimental infection (Andersen *et al.*, 2007). At the farm level, an infected population will harbour SAV until slaughter (Jansen *et al.*, 2010a; 2010b). On an individual level, however, lifelong persistent infection has not been documented.

2.2.6. Vectors

SAV has been detected in salmon lice (*Lepeophtheirus salmonis*) collected during acute PD-disease outbreaks in Atlantic salmon, but transfer to susceptible fish species has not been studied (Pettersen *et al.*, 2009). Vectors are not needed for transmission of SAV.

2.2.7. Suspected aquatic animal carriers

In surveys of wild marine fish, SAV RNA has been detected in the flatfish species common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*) (McCleary *et al.*, submitted; Snow *et al.*, 2010). The importance of wild marine and/or anadromous fish, as well as fresh water salmonids species as virus carriers needs to be clarified.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of SAV occurs horizontally. This is supported by phylogenetic studies, successful transmission among fish in cohabitant studies, proven transmission between farming sites, and studies on survival of SAV in sea water and the spread via water currents (Graham *et al.*, 2000c; 2011; Jansen *et al.*, 2010a; Kristoffersen *et al.*, 2009; Viljugrein *et al.*, 2009).

Long-distance transmission and thus introduction of SAV in a previously uninfected area is most likely assigned to movement of infected live fish (Kristoffersen *et al.*, 2009; Rodger & Mitchell, 2007). Once SAV has been introduced into an area, shared ownership, close proximity and water currents are factors involved in local transmission (Aldrin *et al.*, 2010; Kristoffersen *et al.*, 2009; Viljugrein *et al.*, 2009). Risk factors for outbreaks of PD on a farming site include a previous history of infection with SAV-PD, high feeding rate, high sea lice burden, the use of autumn smolts and previous outbreak of infectious pancreas necrosis (IPN) (Bang Jensen *et al.*, 2012; Kristoffersen *et al.*, 2009; Rodger & Mitchell, 2007).

Vertical transmission of SAV has been suggested (Bratland & Nylund, 2009), but the evidence is not convincing (Kongtorp *et al.*, 2010; McLoughlin & Graham, 2007). The Norwegian Scientific Committee for Food Safety has recently carried out a risk assessment on brood fish surveillance and vertical transmission of infection, concluding that the risk of vertical transmission of SAV is insignificant/negligible.

2.3.2. Prevalence

The prevalence of infected fish within an SAV-infected fish farm may vary. During disease outbreaks, the prevalence is usually high; prevalences of 70–100% have been reported in Atlantic salmon farming sites (Graham *et al.*, 2010). If moribund or thin fish or runts are sampled, the probability of detecting SAV-infected fish is higher than if randomly selected, apparently healthy fish are sampled (Jansen *et al.*, 2010b). Prevalence estimates will also vary with the diagnostic method used.

The prevalence of infected fish within an SAV-infected fish farm is high. Exact estimates vary with the diagnostic method used, but prevalence of 70–100% has been reported from field samples of Atlantic salmon farming sites (Graham *et al.*, 2005; Stene *et al.*, 2013; Taksdal *et al.*, 2007). A single study indicates that the prevalence amongst farmed rainbow trout in sea water may be less, but this needs to be confirmed by other studies (Taksdal *et al.*, 2007).

Annex 19 (contd)

Prevalence in wild fish is largely unknown. SAV RNA has been detected in some flatfish species in sea water in Scotland (Snow *et al.*, 2010). A serological survey of wild salmonids in fresh water river systems in Northern Ireland did not detect virus neutralisation antibodies against SAV in any of 188 sera tested, whereas the majority of sera from farmed salmon in sea water in the same area tested positive (Graham *et al.*, 2003).

2.3.3. Geographical distribution

Infection with SAV is known to be present in farmed salmonid fish in Croatia, France, Germany, Ireland, Italy, Norway, Poland, Spain, Switzerland and the United Kingdom (England, Scotland and Northern Ireland).

2.3.4. Mortality and morbidity

Mortality rates due to infection with SAV may vary with subtype, season, year, use of biosecurity measures and species of fish (Bang Jensen *et al.*, 2012; Graham *et al.*, 2011; Rodger & Mitchell, 2007; Stormoen *et al.*, 2013). The cumulative mortality at the farm level ranges from negligible to over 50% in severe cases for PD and from negligible to over 22% for SD (Bang Jensen *et al.*, 2012; Graham *et al.*, 2003, Rodger & Mitchell, 2007; Ruane *et al.*, 2008; Stene *et al.*, 2013).

Duration of PD-disease outbreaks, defined as the period with increased mortality, varies from 1 to 32 weeks (Jansen *et al.* 2010a; 2014; Ruane *et al.*, 2008).

2.3.5. Environmental factors

Clinical outbreaks and mortality of PD are influenced by water temperature and season more often occur in the spring and summer months when temperatures are high (McLoughlin & Graham, 2007; Rodger & Mitchell, 2007; Stene *et al.*, 2013; Stormoen *et al.*, 2013) indicating an influence of temperature on disease development. Stressing the fish by movement, crowding or treatment may initiate disease outbreaks on infected farms.

2.4. Control and prevention**2.4.1. Vaccination**

At present, one vaccine is commercially available. This vaccine was introduced in 2007 and is widely used in Atlantic salmon farms in PD-endemic areas in Norway, Ireland and Scotland. This vaccine is based on inactivated PD-virus-SAV subtype 1, and claims a reduction in mortality of at least 50% in comparisons of vaccinated fish against unvaccinated fish at the same farm. The vaccine does not seem to offer complete protection, but a field evaluation carried out in Norway demonstrated that the mortality in farms with vaccinated fish is comparable with mortality in farms without infection with SAV PD. Furthermore, a small reduction in the number of outbreaks was seen (Bang Jensen *et al.*, 2012).

A vaccine based on inactivated PD-virus-SAV of another subtype is under development. Furthermore, a DNA-based vaccine is showing promising results. To date, only North America-Canada has allowed the use of DNA-based vaccines for control of fish diseases; it is not certain whether this vaccine will be licensed for use in other markets.

2.4.2. Chemotherapy

No chemotherapy is available.

2.4.3. Immunostimulation

No immunostimulation is available.

2.4.4. Resistance breeding

Differences in susceptibility among different family groups of Atlantic salmon have been observed in challenge experiments and in the field, indicating the potential for resistance breeding. Both in Ireland and Norway, efforts are being made to breed fish that are more resistant to PD-infection with SAV (McLoughlin & Graham, 2007). Selection of brood fish by using gene markers for resistance is in an early phase.

2.4.5. Restocking with resistant species

Not relevant.

2.4.6. Blocking agents

Not relevant.

2.4.7. Disinfection of eggs and larvae

The OIE methods for disinfection of eggs and larvae given in Chapter 1.1.3 *Methods for disinfection of aquaculture establishments* Traditional disinfection methods are considered efficient effective against SAV (Graham *et al.*, 2007b; Kongtorp *et al.*, 2010).

2.4.8. General husbandry practices

To avoid infection with SAV, general good hygiene practices should be applied: use of appropriate sites for farming, segregation of generations, stocking with good quality fish, removal of dead fish, regular cleaning of tanks and pens, controlling parasites and other pathogens as well as careful handling of fish. Once a site has been infected, mortality may be reduced by imposing a general stop on handling of the fish as well as a general stop on feeding the fish.

3. Sampling

3.1. Selection of individual specimens

All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Extremely weak ('sleeping') fish may be found at the bottom of a tank or in the net-cages. If the number of clinically diseased fish is low, samples from long, thin fish ('runts') may be added (Jansen *et al.*, 2010b).

3.2. Preservation of samples for submission

Histology and immunohistochemistry:	Fixation in neutral phosphate-buffered 10% formalin
Molecular biology (RT-PCR and sequencing):	Appropriate medium for preservation of RNA
Cell culture:	Virus transport medium
Serology:	Blood plasma or serum

3.3. Pooling of samples

For diagnostic purposes, pooling of samples from different individuals is not considered necessary or recommended as detection of SAV and characteristic histopathological changes in the same individual will strengthen the connection between the virus and the observed disease for confirmation of SAV as it is usually of interest to compare results from the various examinations of individual fish. For surveillance purposes, pooling of samples for virological examination (PCR or cell culture) may be accepted, but will may decrease the sensitivity of the tests.

3.4. Best organs or tissues

Heart and mid-kidney are the recommended organs for detection of SAV either by molecular biological methods or by cell culture. During the course of the disease, the heart usually contains more SAV than other tissues and should always be sampled. After disease outbreaks, gills and heart (Graham *et al.*, 2010) and pools of heart and mid-kidney (Jansen *et al.*, 2010a; 2010b) remained PCR positive for months after initial detection as these organs contain SAV both during the initial viraemic phase and later.

During the initial viraemic phase, serum samples are also suitable for detection of SAV either by molecular biological methods or by cell culture. Serum sampling may therefore be used for early warning screening tests (Graham *et al.*, 2010). From approximately 3 weeks after SAV infection, blood serum or plasma is suitable for a virus neutralisation test that identifies neutralising antibodies against SAV in fish exposed to SAV (Graham *et al.*, 2003).

Annex 19 (contd)

Tissues for histological examinations should include gill, heart, pyloric caeca with attached pancreatic tissue, liver, kidney, spleen and skeletal muscle containing both red (aerobe) and white (anaerobe) muscle. Skin with associated skeletal muscle sample should be taken at the lateral line level and deep enough to include both red and white muscle. The latter is most easily found close to the side line of the fish.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

A sudden drop in appetite may be observed 1–2 weeks before the detection of enhanced mortality. Clinically diseased fish may be observed swimming slowly at the water surface. In some cases, extremely weak (“sleeping”) fish can be found at the bottom of tanks or in net-cages. An increased number of faecal casts may also be observed in the water. However, it is important to notice that clinical signs are not pathognomonic and that careful observation and examinations of any dead, weak or abnormally behaving fish is necessary.

Initially, nutritional status is usually normal, but in the months after an outbreak or in the later stages of disease, long slender fish (‘runts’) with low condition are typically observed. The development of long, slender fish can be caused by factors other than SAV.

4.2. Clinical methods

4.2.1. Gross pathology

Yellow mucoid gut contents are a usual post-mortem finding, as is typically seen in fish that are not eating. Occasionally signs of circulatory disturbances, such as petechial haemorrhages, small ascites or reddening of the pancreatic region between the pyloric caeca, may be seen. Some diseased fish may show pale hearts or heart ruptures. It is important to note that post-mortem findings are not pathognomonic.

4.2.2. Clinical chemistry

Not documented for diagnostic use.

4.2.3. Microscopic pathology

The changes most commonly found in clinically diseased fish are severe loss of exocrine pancreatic tissue, cardiomyocytic necrosis and inflammation, red (aerobe) skeletal muscle inflammation and white (anaerobe) skeletal muscle degeneration or inflammation. A less frequent but supporting finding is the detection of cells with many cytoplasmic eosinophilic granules along kidney sinusoids.

As the disease progresses, the development of these changes is not simultaneous in all organs: In a very short, early phase, the only lesion present can be necrosis of exocrine pancreatic tissue and a variable inflammatory reaction in the peripancreatic fat. Shortly thereafter, heart muscle cell degeneration and necrosis develops before the inflammation response in the heart becomes more pronounced. The pancreatic necrotic debris will seemingly disappear and the typical picture of severe loss of exocrine pancreatic tissue will soon appear simultaneously with the increasing inflammation in the heart. Somewhat later, skeletal muscle degeneration, inflammation and fibrosis develop. In a proportion of fish, severe fibrosis of the peri-acinar tissue may occur, and in this case the pancreas does not recover (runts) (Christie *et al.*, 2007; Kerbart Boscher *et al.*, 2006; McLoughlin & Graham, 2007; Taksdal *et al.*, 2007).

4.2.4. Wet mounts

Not relevant.

4.2.5. Smears

Not relevant.

4.2.6. Fixed sections, immunohistochemistry

The single immunohistochemical method published (Taksdal *et al.*, 2007) is only recommended for samples from fish with acute necrosis of exocrine pancreatic tissue.

4.2.6.1. Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for immunohistochemistry sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for histopathology and immunohistochemistry as described below.

4.2.6.2. Staining procedure for immunohistochemistry

All incubations are carried out at room temperature and all washing steps are done with Tris-buffered saline (TBS).

- i) Nonspecific antibody binding sites are first blocked in 5% bovine serum albumin (BSA) in TBS for 20 minutes. The solution is then poured off without washing.
- ii) Sections are incubated with primary antibody (monoclonal mouse antibody 4H1 against E1 SAV glycoprotein [Todd *et al.*, 2001]), diluted 1/3000 in 2.5% BSA in TBS and then incubated overnight, followed by two wash out baths lasting a minimum of 5 minutes.
- iii) Sections are incubated with secondary antibody (biotinylated rabbit anti-mouse Ig) diluted 1/300 for 30 minutes, followed by wash out baths as in step ii above.
- iv) Sections are incubated with streptavidin with alkaline phosphatase 1/500 for 30 minutes followed by wash out baths as in step ii above.
- v) For detection of bound antibodies, sections are incubated with Fast Red¹ (1 mg ml⁻¹) and Naphthol AS-MX phosphate (0.2 mg ml⁻¹) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) and allowed to develop for 20 minutes followed by one wash in tap water before counterstaining with Mayer's haematoxylin and mounting in aqueous mounting medium.

SAV-positive and SAV-negative tissue sections are included as controls in every setup (Taksdal *et al.*, 2007)

4.2.7. Electron microscopy/cytopathology

Not relevant for diagnostic use.

4.2.8. Differential diagnoses

4.2.8.1. Differential diagnoses relevant for microscopic pathology (Section 4.2.3)

Tissues that are changed by **infection with SAV-SD and PD** are also changed by heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and IPN. However, if all the main organs are examined by histopathology, the pattern of affected organs will usually appear different:

	PD Infection with SAV	HSMI	CMS	IPN
Heart*	+	+	+	–
Pancreas	+	–	–	+
Skeletal muscle	+	+	–	–

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

Annex 19 (contd)

*Heart changes in CMS affects mainly the inner spongy layer of the ventricle and the atrium, whereas in PD Infection with SAV and HSMI, the compact layer of the ventricle is more severely affected.

Although these three diseases induce epicarditis, HSMI causes the most severely inflamed epicardium. In a very short, early acute stage of infection PD and SD, when only necrosis of exocrine pancreas has developed, infection with SAV might can, when examined by histopathology only, be mistaken for IPN caused by IPN virus (IPNV). In such cases, virological examination will clarify the causal agent.

Virological and serological examinations combined with histopathological examination of 5–10 clinically diseased fish will usually clarify the situation. HSMI and CMS have only been detected in Atlantic salmon.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture

Isolation of field isolates of SAV in cell culture may be challenging (Christie, 1998; Graham, 2007c; Petterson et al., 2013). CHSE-214 are commonly used for primary SAV isolation, but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used. Variation in cell line susceptibility among different SAV field isolates has been reported (Graham *et al.*, 2008; Herath *et al.*, 2009), and it is therefore recommended that several cell lines are tested for initial cell culture isolation of SAV in a new laboratory or for a new virus strain.

The CHSE-214 cells are grown at 20°C in Eagle's minimal essential medium (EMEM) with non-essential amino acids and 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) buffer, or Leibovitz's L-15 cell culture medium, both supplemented with fetal bovine serum (FBS) (5% or 10%) and L-glutamine (4 mM).

For virus isolation, cells are grown in tissue culture flasks or multi-well cell culture plates. SAV-positive controls may be inoculated in parallel with the tissue samples as a test for cell susceptibility to SAV. When positive controls are included, measures must be taken to avoid contamination (this should be performed in a separate location from that of the test samples).

i) Inoculation of cell monolayers

Prepare a 2% suspension of tissue homogenate or a 10% suspension of serum using L-15 medium or EMEM without serum or other medium with documented suitability. Remove growth medium from actively growing monolayers (1- to 2-day-old cultures or cultures of 70–80% confluency) grown in tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers with a low volume of the 2% tissue homogenate or 10% serum dilution (for 25 cm² flasks: 1.5 ml). Adjust volume to the respective surface area in use. Allow 2–3 hours' incubation at 15°C followed by removal of the inoculum, and addition of fresh L-15 or EMEM medium supplemented with 2–5% fetal calf bovine serum (for 25 cm² flasks: 5 ml).

When fish samples come from production sites where IPNV is regarded as endemic, the tissue homogenate supernatant should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

ii) Monitoring incubation

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of cytopathic effect (CPE). Typical CPE due to SAV appears as plaques of pyknotic, vacuolated cells. However, Norwegian SAV field isolates (both SAV3 and marine SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV subtypes (Graham et al., 2008; Petterson et al., 2013). If no CPE has developed after 14 days, subculture to fresh cell cultures.

iii) Subcultivation procedure

14 days (or earlier when obvious CPE appears) after inoculation, the cultures are freeze–thawed at –80°C (the procedure can be repeated 1–2 times) to release virus from the infected cells.

Annex 19 (contd)

Following centrifugation at 3000 **g** for 5 minutes, the supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 2–3 hours before addition of fresh medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]), as virus replication may occur without development of apparent CPE.

iv) Antibody-based verification of SAV growth in cell culture

All incubations below are carried out at room temperature unless otherwise stated.

- a) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well plates), or on cover-slips, depending on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). The necessary monolayers for negative and positive controls must be included.
- b) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 9–11 days.
- c) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. If necessary, the fixed cell cultures may be stored dry for 14 days at 4°C until staining.
- d) Incubate the cell monolayers with anti-SAV MAb in an appropriate dilution in phosphate-buffered saline (PBS) for 1 hour and rinse three times with PBS with 0.05% Tween 20.
- e) Incubate with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin for 1 hour (or if the primary Ab is polyclonal from rabbits, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity of the test, FITC-conjugated anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with rinsing as in step d in between the steps. The nuclei can be stained with propidium iodide (100 µg ml⁻¹ in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in the dark until examination. For long periods of storage (more than 2–3 weeks) a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.3.1.2.3. Reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and genotyping by sequencing

The primers described below for real-time RT-PCR and RT-PCR with sequencing will detect all known subtypes of SAV.

RT-PCR may be used for detection of SAV from total RNA (or total nucleic acids) extracted from recommended organs or tissues (see Section 3.4). Real-time RT-PCR for the detection of SAV is recommended as it increases the specificity and also the sensitivity of the test.

For genotyping, RT-PCR with subsequent sequencing of fragments from the E2 and nsP3 genes is recommended.

The primers and probe for real-time RT-PCR from the nsP1 gene, as well as primers for genotyping, are listed below. The E2-primers may also be used for conventional RT-PCR detection of SAV, if necessary.

Annex 19 (contd)

RT-PCR: Primer and probe sequences	Named	Genomic segment	Product size	Reference
QnsP1F: 5'-CCG-GCC-CTG-AAC-CAG-TT-3' QnsP1R: 5'-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3' QnsP1probe: 5'FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3'	forward primer reverse primer Taqman@probe	QnsP1	107 nt	Hodneland <i>et al.</i> , 2006
E2F: 5'-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3' E2R: 5'-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3'	forward primer reverse primer	E2	516 nt	Fringuelli <i>et al.</i> , 2008
nsP3F: 5'-CGC-AGT-CCA-GCG-TCA-CCT-CAT-C-3' nsP3R: 5'-TCA-CGT-TGC-CCT-CTG-CGC-CG-3'	forward primer reverse primer	nsP3	490 nt	Fringuelli <i>et al.</i> , 2008

4.3.2. Serological methods

4.3.2.1 Immunoperoxidase-based **virus serum** neutralisation assay (Graham *et al.*, 2003)

Experimental studies have shown that neutralising antibodies can first be detected 10–16 days post-infection (Graham *et al.*, 2003), and virus neutralisation (VN) assays can be used as a diagnostic tool for the detection of SAV antibodies. VN assays are based on the presence or absence of detectable virus growth in cultured cells following incubation with serum that may contain neutralising antibodies. In addition, the assay allows detection of virus in serum or plasma, if present.

CHSE-214 cells are grown as described in Section 4.3.1.2.1 Cell culture. A suspension of trypsinised cells, diluted 1/3 in growth medium (10% FBS) is prepared for the VN assay.

- i) 1/20 and 1/40 dilutions of each test serum are prepared in maintenance medium (2% FBS), and transferred to two duplicate wells (15 µl per well) on a flat-bottomed tissue culture grade microtitre plate. An equal volume of virus (100 TCID₅₀ [median tissue culture infective dose]) is added and the plate is incubated for 2 hours at room temperature.
- ii) 70 µl of maintenance medium, and 50 µl of the CHSE-214 cell suspension is added to each well, and the plates are incubated for 3 days at 15°C.
- iii) The cell monolayer is then fixed and stained as described in Section 4.3.1.2.1, step iv Antibody-based verification of SAV growth in cell culture, or using the following procedure: monolayers of CHSE-214 cells are fixed for 30 minutes at room temperature in 10% neutral buffered formalin. Following two washes with 0.01 M PBS, a MAb against SAV is added to the monolayers in an appropriate dilution. Bound MAb is visualised using a labelled streptavidin–biotin system according to the manufacturer's instructions.
- iv) VN titres (ND₅₀) are then calculated according to the method of Karber (1931), with titres ≥ 1:20 being considered positive. Both serum controls (without virus added) and a virus control (without serum added) must always be included in the assay, to ensure valid results.

5. Rating of tests against purpose of use

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

Table 5.1. Methods for targeted surveillance and diagnosis

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Fry	Juveniles	Adults		
Gross signs	d	d	d	c	d
Histopathology	c	c	c	a	a
Immunohistochemistry	d	d	d	b	b
Isolation in cell culture	d	d	d	c	c
Virus-Serum neutralisation assay	d	c	e-b	a	b
Real-time RT*-PCR	b	a	a	a	a
RT*-PCR with sequencing	d	d-b	d-b	d-b	a

* RT-PCR = Reverse transcriptase polymerase chain reaction.

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

The recommended test to be used in surveillance of susceptible fish populations for declaration of freedom from SAV is ~~real-time~~ RT-PCR as described in Section 4.3.1.2.3 in this chapter.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspected case of infection with SAV is defined as:

i) ~~Detection of SAV~~

~~i) Clinical signs consistent with infection with SAV (Section 4.1.1)~~

~~or~~

~~ii) Gross and microscopically pathology consistent with the disease (Sections 4.2.1 and 4.2.3)~~

~~or~~

iii) Detection of antibodies against SAV ~~(Section 4.3.2.1)~~ or ~~detection of SAV (Section 4.3.1.2.)~~

or

~~iii) Detection of histopathological changes consistent with the disease~~

iv) If epidemiological information of infectious contact with suspected or confirmed case(s) appears.

Annex 19 (contd)**7.2. Definition of confirmed case**

~~Detection of SAV or detection of antibodies against SAV and detection of histopathological changes consistent with the disease.~~

Evidence for the presence of SAV from two independent laboratory tests as microscopic pathology (Section 4.2.3), cell culture (Section 4.3.1.2), RT-PCR (Section 4.3.1.2.3) or serology (Section 4.3.2)

8. References

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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 the Taxonomy has assigned IHHNV (a parvovirus) as a tentative species in the genus *Brevidensovirus*, family *Parvoviridae* with the species name of PstDENV (for *Penaeus stylirostris* densovirus) (Fauquet *et al.*, 2005). For the purpose of this *Aquatic Manual*, most references to the viral agent of IHHN will be as IHHNV.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

IHHNV is the smallest of the known penaeid shrimp viruses. The IHHN virion is a 20–22 nm, non-enveloped icosahedron, with a density of 1.40 g ml⁻¹ in CsCl, contains linear single-stranded DNA with an estimated size of 3.9 kb, and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (Bonami *et al.*, 1990; Nunan *et al.*, 2000; GenBank AF218266).

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

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective inactivation methods)

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

2.1.4. Life cycle

Not applicable.

Annex 20 (contd)**2.2. Host factors****2.2.1. Susceptible host species**

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

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

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

2.2.2. Susceptible stages of the host

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

2.2.3. Species or subpopulation predilection (probability of detection)

See Sections 2.2.1 and 2.2.2.

2.2.4. Target organs and infected tissue

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

2.2.5. Persistent infection with lifelong carriers

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infections and/or epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (Bell & Lightner 1984; Lightner, 1996a; 1996b; Morales-Covarrubias & Chavez-Sanchez, 1999; Motte *et al.*, 2003).

2.2.6. Vectors

No vectors are known in natural infections.

2.2.7. Known or suspected wild aquatic animal carriers

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

2.3. Disease pattern

2.3.1. Transmission mechanisms

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

2.3.2. Prevalence

In regions where the virus is enzootic in wild stocks, the prevalence of IHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHNV 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 IHNV positive included the brown shrimp, *P. californiensis* and the Western white shrimp *P. occidentalis*. In farms where IHNV 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.*, 1992; 1983a; Martinez-Cordova 1992).

2.3.3. Geographical distribution

IHNV 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, IHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although IHNV 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). IHNV has also been reported in cultured penaeid shrimp from Pacific islands including the Hawaiian Islands, French Polynesia, Guam, and New Caledonia. In the Indo-Pacific region, the virus has been reported from cultured and wild penaeid shrimp in East Asia, South-East Asia, and the Middle East (Bondad-Reantaso *et al.*, 2001; Lightner, 1996a). An IHNV-like virus has been reported from Australia (Krabetsve *et al.*, 2004; Owens *et al.*, 1992), and the presence of IHNV in farmed prawns in Australia was reported to the OIE in 2008. As discussed in Section 2.1.1, IHNV-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, IHNV may take three distinct forms: in unselected *P. stylirostris*, infection by IHNV 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 IHNV 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 IHNV genome has been found to be inserted in the genome of some genetic lines of *P. monodon*. There is no evidence that this variant of IHNV is infectious (Tang & Lightner, 2002; 2006).

2.3.5. Environmental factors

The replication rate of IHNV at high water temperatures was significantly reduced in a study in which viral replication was compared in *P. vannamei* experimentally infected and held at 24°C and 32°C. After a suitable incubation period, shrimp held at 32°C had approximately 10² lower viral load than shrimp held at 24°C. However, even at the higher temperature, significant (up to 10⁵ virus copies 50 ng⁻¹ of shrimp DNA) IHNV replication still occurred in shrimp held at 32°C (Montgomery-Brock *et al.*, 2007).

Annex 20 (contd)**2.4. Control and prevention****2.4.1. Vaccination**

No effective vaccination methods for IHHNV have been developed.

2.4.2. Chemotherapy

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Resistance breeding

Selected stocks of *P. stylirostris* that are resistant to IHHN disease have been developed and these have had some successful application in shrimp farms (Clifford, 1998; Lightner, 1996a; 1996b; Weppe 1992; Zarian-Herzberg & Ascencio-Valle, 2001). Some selected lines of *P. stylirostris* that were bred for IHHN disease resistance, were found to be refractory to infection (Tang *et al.*, 2000). However, such stocks have no increased resistance to diseases such as white spot syndrome virus (WSSV), and, hence, their use has been limited, although with some stocks a genetic basis for IHHN susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.*, 1997).

2.4.5. Restocking with resistant species

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

2.4.6. Blocking agents

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

2.4.7. Disinfection of eggs and larvae

IHHNV has been demonstrated to be transmitted vertically by the transovarian route (Motte *et al.*, 2003). Hence, while disinfection of eggs and larvae is good management practice (Chen *et al.*, 1992) and is recommended for its potential to reduce IHHNV contamination of spawned eggs and larvae produced from them (and contamination by other disease agents), the method is not effective for preventing transmission of IHHNV (Motte *et al.*, 2003).

2.4.8. General husbandry practices

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

3. Sampling

3.1. Selection of individual specimens

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

IHNV infects tissues of ectodermal and mesodermal origin. The principal target tissues for IHNV 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

IHNV 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 IHNV (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 IHNV (see Section 4.2.1.2). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHNV. As *P. vannamei*, *P. stylirostris*, and *P. monodon* can be infected by IHNV 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 IHNV disease is required.

4.1.2. Behavioural changes

In acute IHNV 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 & Quintio, 2000; Pruder *et al.*, 1995).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Acute IHHNV infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained histological methods (see Section 4.2.6). Chronic IHHNV infections and RDS are much more difficult to diagnose using routine H&E histological methods. For diagnosis of chronic infections, the use of molecular methods are recommended for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or ISH of histological sections).

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of IHHNV infection. These characteristic IHHN inclusion bodies are eosinophilic and often haloed (with H&E stains of tissues preserved with fixatives that contain acetic acid, such as Davidson's AFA and Bouin's solution) (Bell & Lightner, 1988; Lightner, 1996a), intranuclear inclusion bodies within chromatin-margined, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies caused by IHHNV may be easily confused with developing intranuclear inclusion bodies caused by WSSV infection. ISH assay (see Section 4.3.1.2.3 of this chapter) of such sections with a specific DNA probe to IHHNV provides a definitive diagnosis of IHHNV infection (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

4.2.4. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

4.2.5. Smears

Not applicable.

4.2.6. Fixed sections

Histopathology: histology may be used to provide a definitive diagnosis of IHNV 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 IHNV.

4.3. Agent detection and identification methods**4.3.1. Direct detection methods****4.3.1.1. Microscopic methods***4.3.1.1.1. Wet mounts*

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See section 4.2.6.

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

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

4.3.1.2.2. Antibody-based antigen detection methods

None has been successfully developed.

4.3.1.2.3. Molecular techniques

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

Annex 20 (contd)

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*² (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 equivalent): cut pieces to fit samples and controls and mark with soft-lead pencil making 1 cm squares for each sample. Include a positive and a negative control on each filter. Lay out on to a piece of filter paper (Whatman 3MM).
- ii) If necessary, dilute samples to be assayed in TE (Tris/EDTA [ethylene diamine tetra-acetic acid]) buffer plus 50 µg ml⁻¹ salmon sperm DNA, using 1 µl sample in 9 µl buffer in 1.5 ml microcentrifuge tubes. Samples for dot-blot can be haemolymph, tissues homogenised in TN (Tris/NaCl: 0.4 M NaCl and 20 mM Tris-HCl, pH 7.4) buffer, or extracted DNA in 10 mM Tris/HCl.
- iii) Boil samples for 10 minutes and quench on ice for 5 minutes. Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein. Keep on ice until samples are dotted on to the membrane.
- iv) Dot 1–3 µl of each sample on to an appropriate place on the filters. Allow to air-dry and then fix samples on to the membrane by baking at 80°C for 30 minutes or by UV cross-linking using a DNA transilluminator for 3 minutes.
- v) Adjust a water bath to 68°C and prepare the prehybridisation solution. For a 10 × 15 cm membrane, prepare 8 ml per membrane. Set a stirring hot plate to 'low' and stir while warming the solution for 30 minutes until the blocking agent has dissolved and the solution is cloudy. Also, prepare some heat-seal bags that are slightly larger in size than the membrane: five to six bags will be needed per membrane.
- vi) Remove membranes from the oven or transilluminator and put into a heat-seal bag with 4 ml per membrane of prehybridisation solution. Seal the bags and put into a 68°C water bath for 0.5–1 hour.
- vii) Boil the DIG-labelled probe for 10 minutes, quench on ice and then microfuge in the cold to bring all the liquid down in the microcentrifuge tube. Keep on ice. Remove the prehybridisation solution from the bags. Add 2 ml of fresh prehybridisation solution to each bag and then add the correct, predetermined amount of DIG-labelled probe to each, mixing well as it is being added. Seal the bags, place back in the 68°C water bath and incubate for 8–12 hours.

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

Annex 20 (contd)

- viii) Wash membranes well with:
- | | | |
|--|-----|--------------------------------|
| 2 × standard saline citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) | 2 × | 5 minutes at room temperature |
| 0.1 × SSC/0.1% SDS
(use 4 ml/filter and seal in bags) | 3 × | 15 minutes at 68°C |
| Buffer I | 1 × | 5 minutes at room temperature |
| Buffer II | 1 × | 30 minutes at room temperature |
| Buffer I
(Buffers are prepared ahead of time). | 1 × | 5 minutes at room temperature |
- 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 IHNV and generally follows the methods outlined in Mari *et al.* (1993) and Lightner (1996a). Formulas for the required reagents are given after the protocols.

- i) Embed tissue in paraffin and cut sections at 4–6 µm thickness. Place sections on to positively charged microscope slides (do not put gelatine in water to float sections; just use water).
- ii) Put slides in a slide rack, such as a Tissue-Tek rack. Heat the slides in an oven for 45 minutes at 60°C. In the staining centre, rehydrate the tissue as follows:
- | | | |
|---------------------------------|-----|--|
| Xylene (or suitable substitute) | 3 × | 5 minutes each |
| Absolute alcohol | 2 × | 1 minute each |
| 95% alcohol | 2 × | 10 dips each |
| 80% alcohol | 2 × | 10 dips each |
| 50% alcohol | 1 × | 10 dips |
| Distilled water | | six rinses (do not let slides dry out) |
- iii) Wash the slides for 5 minutes in phosphate buffered saline (PBS or Tris/NaCl/EDTA [TNE] buffer). Prepare fresh proteinase K at 100 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 20 (contd)

- ix) Wash the slides for 5 minutes in Buffer I at room temperature. Put the slides flat in a humid chamber and block with 0.5 ml per slide of Buffer II. Incubate for 15 minutes at 37°C. Drain the fluid on to blotting paper.
- x) Dilute the anti-DIG AP conjugate (Roche Applied Science cat. 10686322) 1/1000 in Buffer II (1 µl anti-DIG AP per 1 ml buffer). Cover tissue with 500 µl of diluted conjugate and incubate in a humid chamber for 30 minutes at 37°C.
- xi) Place the slides in a slide rack. Wash in Buffer I twice for 5–10 minutes each time at room temperature. Wash once with Buffer III for 5–10 minutes.
- xii) Prepare the development solution by first adding 4.5 µl NBT per 1 ml buffer III. Mix well. Then add 3.5 µl X-phosphate per ml of solution and mix well. Pipette on 500 µl per slide and incubate in a humid chamber in the dark for 2–3 hours at room temperature.
- xiii) Stop the reaction by returning the slides to a slide rack and washing in Buffer IV for 15 minutes at room temperature.
- xiv) Counterstain the slides by dipping for 5 minutes in 0.5% aqueous Bismarck brown Y.
- xv) Dehydrate the slides in the staining centre as follows:
- | | | |
|---------------------------------|-----|--------------|
| 95% alcohol | 3 × | 10 dips each |
| Absolute alcohol | 3 × | 10 dips each |
| Xylene (or suitable substitute) | 4 × | 10 dips each |
- Do not allow the slides to dry out – leave them in the last xylene (or xylene substitute) container until ready for cover-slips.
- xvi) Mount with cover-slips and mounting medium (Permount).
- xvii) Examine the slides under bright-field for a dark-blue or black precipitate that marks sites where IHNV DNA is present. Pathodiagnostic intranuclear Cowdry type A inclusions are well marked with the probe. Also often marked are host cell nuclei without obvious inclusions, cytoplasmic inclusions, and accumulation of free virus in the tissue spaces and haemolymph.

NOTE: Always run a known positive and negative control.

Reagent formulas for ISH method:

- i) 10 × phosphate buffered saline

NaCl	160 g
KH ₂ PO ₄	4 g
Na ₂ HPO ₄	23 g
KCl	4 g
DD H ₂ O	1950 ml (qs to 2 litres)

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

- ii) 10 × Tris/NaCl/EDTA (TNE) buffer

Tris base	60.57 g
NaCl	5.84 g
EDTA	3.72 g
DD H ₂ O	900 ml (qs to 1 litre)

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

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

PBS	10 ml 1 × PBS
Proteinase K	1 mg

- iv) 0.4% formaldehyde

37% formaldehyde	5.4 ml
DD H ₂ O	500 ml

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

Annex 20 (contd)

- v) Prehybridisation buffer (50 ml final volume)
- | | |
|---------------------|----------------------------|
| 4 × SSC | 10 ml 20 × SSC |
| 50% formamide | 25 ml 100% formamide |
| 1 × Denhardt's | 2.5 ml 20 × Denhardt's |
| 5% dextran sulphate | 10 ml 25% dextran sulphate |
- Warm to 60°C
Boil 2.5 ml of 10 mg ml⁻¹ salmon sperm DNA and add to buffer for final concentration of 0.5 mg ml⁻¹ salmon sperm DNA; store at 4°C.
- vi) 20 × SSC buffer
- | | |
|---|--------------------------------------|
| 3M NaCl | 175.32 g NaCl |
| 0.3 M Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O | 88.23 g Na citrate·2H ₂ O |
| DD H ₂ O | 1000 ml (qs) |
- pH to 7.0; autoclave; store at 4°C.
To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H₂O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H₂O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H₂O.
Filter solutions through a 0.45 µm filter; store at 4°C.
- vii) 20 × Denhardt's solution
- | | |
|---------------------|----------------------------|
| BSA (Fraction V) | 0.4 g bovine serum albumin |
| Ficoll 400 | 0.4 g Ficoll |
| PVP 360 | 0.4 g polyvinylpyrrolidone |
| DD H ₂ O | 100 ml |
- Filter solutions through a 0.45 µm filter; store at 4°C. Aliquot 2.5 ml into small tubes and store frozen.
- viii) 25% dextran sulphate
- | | |
|---------------------|--------|
| Dextran sulphate | 25 g |
| DD H ₂ O | 100 ml |
- Mix to dissolve; store frozen in 10 ml aliquots.
- ix) Salmon sperm DNA (10 mg ml⁻¹)
- | | |
|---------------------|--------|
| Salmon sperm DNA | 0.25 g |
| DD H ₂ O | 25 ml |
- To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18-gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.
- x) 10 × Buffer I
- | | |
|---------------------|-------------------|
| 1 M Tris/HCl | 121.1 g Tris base |
| 1.5 M NaCl | 87.7 g NaCl |
| DD H ₂ O | 1000 ml (qs) |
- pH to 7.5 with HCl. Autoclave; store at 4°C.
To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD 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 20 (contd)

xii) Buffer III

100 mM Tris/HCl	1.21 g Tris base
100 mM NaCl	0.58 g NaCl
DD H ₂ O	100 ml (qs)
pH to 9.5 with HCl	
Then add:	
50 mM MgCl ₂	1.02 g MgCl ₂ ·6H ₂ O
Filter through a 0.45 µm filter; store at 4°C.	

xiii) 10% polyvinyl alcohol (PVA)

Polyvinyl alcohol	10 g
DD H ₂ O	100 ml

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

xiv) Development solution

Mix 90 ml Buffer III with 10 ml of 10% PVA. Store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

4.5 µl NBT	75 mg NBT ml ⁻¹ in 70% dimethylformamide (Roche Diagnostics 1-383-213)
3.5 µl X-phosphate	5-bromo-4-chloro-3-indoyl phosphate, toluidine salt (50 mg ml ⁻¹ in dimethylformamide) (Roche Diagnostics 1-383-221)

xv) Buffer IV

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

xvi) 0.5% Bismarck Brown Y

Bismarck Brown Y	2.5 g
DD H ₂ O	500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

Polymerase chain reaction for IHNV: 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 IHNV detection. Nested methods are also available from commercial sources.

There are multiple geographical variants of IHNV, some of which are not detected by all of the available methods for IHNV. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHNV (Krabsetsve *et al.*, 2004; Tang & Lightner, 2002), 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). Primer set 309F/R amplifies only a segment from IHNV types 1 and 2 (the infectious forms of IHNV), but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.*, 2007). Primer set MG831F/R reacts only with types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang *et al.*, 2007). Hence, confirmation of unexpected positive and/or negative PCR results for IHNV with a second primer set, or use of another diagnostic method (i.e. PCR using primers from another region of the genome, real-time PCR, bioassay, ISH) is highly recommended.

Table 4.1. Recommended primer sets for one-step PCR detection of IHNV

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

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region (ORF 1) of the IHNV genome. Primers 77353/77012 are from a region in between the nonstructural and the structural (coat protein) protein-coding regions of the genome. In the event that results are ambiguous using the 389F/R 'universal' primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012/77353 or the 392F/R primer sets for confirmation.

General PCR method for IHNV: the PCR method described below for IHNV 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), or reagents from Gibco Life Sciences (DNazol Cat. No. 10503-027). 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 IHNV: a) DNA from a known negative tissue sample; b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and c) a 'no template' control.
- iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHNV-infected material. Prepare primers at 100 ng µl⁻¹ in distilled water. Keep frozen at -70°C.
- iv) Use a 'hot start' method for the polymerase: if Applied Biosystem's AmpliTaq Gold is used, this involves a 5-minute step at 95°C to denature DNA prior to the primers binding and activation of the enzyme. This programme is then linked to the cycling programme (35 cycles) and an extension programme. The programme is set as follows:

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

Annex 20 (contd)

- 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 H ₂ O	32.5 µl × number of samples
10 × PCR buffer	5 µl × number of samples
10 mM dTTP	1 µl × number of samples
10 mM dATP	1 µl × number of samples
10 mM dCTP	1 µl × number of samples
10 mM dGTP	1 µl × number of samples
25 mM MgCl ₂	4 µl × number of samples
Forward primer (100 ng µl ⁻¹)	1.5 µl × number of samples
Reverse primer (100 ng µl ⁻¹)	1.5 µl × number of samples
AmpliTaq Gold	0.5 µl × number of samples

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

NOTE: The volume of the PCR reaction may be modified. Previously, the PCR reactions for IHNV 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 IHNV: qPCR methods have been developed for the detection of IHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHNV genome (Dhar *et al.*, 2001; Tang & Lightner, 2001).

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

- i) The PCR primers and TaqMan probe are selected from a region of the IHNV genomic sequence (GenBank AF218266) that encodes for non-structural protein. The primers and TaqMan probe are designed by the Primer Express software (Applied Biosystems). The upstream (IHNV1608F) and downstream (IHNV1688R) primer sequences are: 5'-TAC-TCC-GGA-CAC-CCA-ACC-A-3' and 5'-GGC-TCT-GGC-AGC-AAA-GGT-AA-3', respectively. The TaqMan probe (5'-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3'), which corresponds to the region from nucleotide 1632 to 1644, is synthesised and labelled with fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (Applied Biosystems, part no. 450025).
- ii) Preparation of DNA template: the extraction and purification of DNA template is the same as that described in the section of traditional PCR.

Annex 20 (contd)

- iii) The qPCR reaction mixture contains: TaqMan Universal PCR Master Mix (Applied Biosystems, part no. 4324018), 0.3 μ M of each primers, 0.15 μ M of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 μ l. For optimal results, the reaction mixture should be vortexed and mixed well.
- iv) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 or equivalent can also be used). The cycling profile is: activation of AmpliTaq Gold for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.
- v) At the end of the reaction, real-time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. Samples will be defined as negative if the Ct (threshold cycle) values exceed 40 cycles. Samples with a Ct value lower than 40 cycles are considered to be positive. To confirm the real-time PCR results, an aliquot of PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. An 81-bp DNA fragment can be visualised in the samples that are positive for IHHNV.
- vi) It is necessary to include a 'no template' control in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of the thermal cycler. A positive control should also be included, and it can be a plasmid containing the target sequence, or purified virions, or DNA from IHHNV-infected tissue.

Sequencing: PCR products may be cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified product from the infectious form of the virus and demonstrate the presence of the insertion of non-infectious IHHNV genome in host DNA (Tang & Lightner, 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 (Saksmerprome *et al.*, 2011; Tang & Lightner, 2006). 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.

4.3.2. Serological methods

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

5. Rating of tests against purpose of use

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

Table 5.1. IHHNV surveillance, detection and diagnostic methods

Method	Surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	d	d
Bioassay	d	d	d	d	c	c
Direct LM	d	d	d	d	d	d
Histopathology	d	d	c	c	a	b
Transmission EM	d	d	d	d	c	c
Antibody-based assays	d	d	d	c	d	d
DNA probes – <i>in situ</i>	d	d	b	b	a	a
PCR, qPCR	a	a	a	a	a	a
Sequence	d	d	d	d	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infectious hypodermal and haematopoietic necrosis

As indicated in Table 5.1, PCR is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, demonstration of pathognomonic IHHNV-induced lesions in the cuticular epithelium by histology (with or without confirmation by ISH with IHHNV-specific DNA probes) is a suitable method (Table 5.1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

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

In farmed stocks of *P. stylirostris*, juveniles, subadults and adults may show persistently high mortality rates. In *P. vannamei*, *P. stylirostris*, and possibly *P. monodon*, IHHNV-infected stocks may show poor and highly disparate growth, poor overall culture performance, and cuticular deformities, including especially bent rostrums and deformed sixth abdominal segments.

Demonstration of eosinophilic to pale basophilic intranuclear inclusion bodies in the typical target tissues for IHHNV. As IHHNV intranuclear inclusion bodies are nearly identical in appearance to those occurring in the early stages of WSSV infections, their presence in tissue sections should be considered as a presumptive diagnosis of IHHNV until confirmed with a second test method, such as dot-blot or ISH with IHHNV-specific DNA probes or positive PCR test results for IHHNV.

7.2. Definition of confirmed case

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

- Positive dot-blot hybridisation test results for IHNV.
- ISH positive histological signal to IHNV-type lesions.
- PCR positive results for IHNV.
- Sequencing of PCR specific products may be required when the purpose is to determine the genotype of IHNV.

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

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

GUIDE TO THE USE OF THE AQUATIC ANIMAL HEALTH CODE

A. Introduction

- 1) The OIE *Aquatic Animal Health Code* (hereafter referred to as the *Aquatic Code*) sets out standards for the improvement of aquatic animal health worldwide. More recently, the *Aquatic Code* has also included standards for the welfare of farmed fish. 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 measures providing for early detection, 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.
- 3) The OIE standards are based on the most recent scientific and technical information. Correctly applied, they protect aquatic animal health and welfare of farmed fish during production and trade in aquatic animals and aquatic animal products.
- 4) The absence of chapters, articles or recommendations on particular pathogenic agents or commodities does not mean that Veterinary Authorities and other Competent Authorities may not apply appropriate animal health and welfare measures. However, such measures should be based on sound scientific justification according to the principles of the WTO SPS Agreement.
- 5) The complete text of the *Aquatic Code* is available on the OIE website and may be downloaded from: <http://www.oie.int>.

B. *Aquatic Code* content

- 1) Key terms and expressions used in more than one chapter in the *Aquatic Code* with a contextual meaning are defined in the Glossary. The reader should be aware of the contextual definitions given in the Glossary when reading and using the *Aquatic Code*. Defined terms appear in italics. In the on-line version of the *Aquatic Code*, a hyperlink leads to the relevant definition.
- 2) The term '(under study)' is found in some rare instances, with reference to an article or part of an article. This means that this part of the text has not been adopted by the World Assembly of OIE Delegates and the particular provisions are thus not part of the *Aquatic Code*.
- 3) The standards in the chapters of Section 1 are designed for the implementation of measures for the diagnosis, surveillance and notification of pathogenic agents. The standards include the criteria for listing aquatic animal diseases, diseases listed by the OIE and procedures for notification to the OIE.
- 4) The standards in the chapters of Section 2 are designed to guide the importing country in conducting import risk analysis in the absence of OIE trade standards. The importing country may also use these standards to justify import measures which are more trade restrictive than existing OIE trade standards.
- 5) The standards in the chapters of Section 3 are designed for the establishment, maintenance and evaluation of Aquatic Animal Health Services, including communication. These standards are intended to assist the Veterinary Services and Aquatic Animal Health Services of Member Countries to meet their objectives of improving aquatic animal health and welfare of farmed fish, as well as to establish and maintain confidence in their international aquatic animal health certificates.

Annex 21 (contd)

- 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 and disposal of aquatic animal waste.
- 7) The standards in the chapters of Section 5 are designed for the implementation of general sanitary measures for trade. In particular, chapters address certification and the measures applicable by the exporting, transit and importing countries. Section 5 also includes a range of model health certificates to be used as a harmonised basis for international trade.
- 8) The standards in the chapters of Section 6 are designed to ensure the responsible and prudent use of antimicrobial agents in aquatic animals.
- 9) The standards in the chapters of Section 7 are designed for the implementation of welfare measures for farmed fish. The standards cover the general principles for welfare of fish, their welfare during their transport, at the time of stunning and killing for human consumption, as well as in the situation of killing for disease control purpose.
- 10) The standards in each of the chapters of Sections 8 to 11 are designed to prevent the aetiological agents of OIE listed diseases from being introduced into an importing country. 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, are compulsorily notifiable, Member Countries are encouraged also to provide information to the OIE on other animal health events of epidemiological significance.

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

Chapter 1.3. provides diseases 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, standards should be proportionate to the risk posed by different strains of the pathogenic agent. The first listed disease offering risk management options based on strain differentiation 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 numbers of species, and the number of new species existing in aquaculture.

4) Trade requirements

International aquatic animal health trade measures should be based on OIE standards. A Member Country may authorise the importation of aquatic animals or aquatic animal products into its territory under conditions more or less restrictive than those recommended by the *Aquatic Code*. To scientifically justify more trade restrictive measures the importing country should conduct 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 in international trade. Veterinary Authorities and other Competent Authorities and all veterinarians or certifying officials directly involved in international trade should be familiar with these chapters. These chapters provide guidance for informal mediation by the OIE.

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 that can be traded for any purpose from a country, zone or compartment not declared free from disease X. The criteria for inclusion of aquatic animal products in point 1 of Article X.X.3. are based on the absence of the pathogenic agent in the traded aquatic animals and aquatic animal products or inactivation of the pathogenic agent by treatment or processing.

Based on assessments using criteria in Article 5.4.2, in all disease chapters, point 1 of Article X.X.12. (amphibian and fish disease chapters) and Article X.X.11. (crustacean and mollusc disease chapters) lists aquatic animals or aquatic animal products for retail trade for human consumption. The criteria for inclusion of aquatic animals or aquatic animal products in point 1 of Article X.X.12. (amphibian and fish disease 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.

5) International health certificates

An international aquatic animal health certificate is an official document the Veterinary Authority or other Competent Authority draws up in accordance with Chapter 5.1. and Chapter 5.2. Certificates list 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 and aquatic animal products. This includes the Veterinary Services' or Aquatic Animal Health Services' ethical approach to the provision of 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 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*.

Annex 21 (contd)

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

- a) List the diseases, for which the importing country is justified in seeking protection in regards to its own 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 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.
- d) As further guidance to Member Countries, Chapter 5.10. provides model certificates that should be used as a baseline.

6) Guidance notes for importers and exporters

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, during transport and unloading, 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 animals and animal products.

— Text deleted.

CHAPTER X.X

DISEASE X

[...]

Article X.X.2.

Scope

The recommendations in this chapter apply to the following susceptible species which meet the criteria for determining susceptibility as described in Chapter X.X: [species X, Y, Z].

~~These recommendations also apply to any other susceptible species referred to in the Aquatic Manual when traded internationally.~~

[...]

— Text deleted.



Original: English
February 2014

REPORT OF THE ELECTRONIC OIE AD HOC GROUP ON SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS

January–February 2014

The *ad hoc* Group on Safety of Products Derived from Aquatic Animals (the *ad hoc* Group) worked remotely as an electronic *ad hoc* group during January and February 2014.

Details of participants and the adopted agenda are presented in [Annexes 1 and 2](#).

The *ad hoc* Group was convened at the recommendation of the Aquatic Animal Health Standards Commission (the Aquatic Animals Commission) to conduct assessments on a range of commodities commonly traded internationally against the criteria provided in Chapter 5.4. of the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) and to evaluate whether *S. alphavirus* is vertically transmitted in disinfected eggs.

The *ad hoc* Group conducted assessments for a range of aquatic animal products against the ‘Criteria to assess the safety of aquatic animal commodities for any purpose from a country, zone or compartment not declared free from disease X’ (Article 5.4.1.) and against the ‘Criteria to assess the safety of aquatic animal commodities for retail trade for human consumption from a country, zone or compartment not declared free from disease X’ (Article 5.4.2.) for inclusion in the new draft chapter on Infection with *S. alphavirus* for inclusion in the *Aquatic Code*.

The following aquatic animal products were assessed and did meet the criteria in Article 5.4.1.:

- i) heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
- ii) pasteurised fish products that have been subjected to a heat treatment at 90°C for 10 minutes (or any time/temperature equivalent that has been demonstrated to inactivate SAV);
- iii) mechanically dried, eviscerated fish (i.e. a heat treatment of 100°C for 30 minutes or any time/temperature equivalent that has been demonstrated to inactivate SAV);
- iv) fish oil and fish meal;
- v) fish skin leather.

Annex 23 (contd)

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.1.:

- i) naturally dried, eviscerated fish (i.e. sun-dried or wind-dried);
- ii) frozen, eviscerated fish;
- iii) frozen fish fillets or steaks;
- iv) chilled, eviscerated fish;
- v) chilled fish fillets or steaks.

The following aquatic animal products did meet the criteria in Article 5.4.2.:

- i) frozen fish fillets and steaks;
- ii) chilled fish fillets and steaks.

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.2.:

- iii) frozen, eviscerated fish;
- iv) chilled, eviscerated fish.

The individual product assessments are presented in Annex 3.

The *ad hoc* Group also evaluated whether salmon alphavirus is vertically transmitted in disinfected eggs and whether an article (10.X.13.) on importation of disinfected eggs for aquaculture is appropriate for the disease-specific chapter. The *ad hoc* Group considered that available epidemiological evidence suggests that vertical transmission or egg associated transmission via gonadal fluids is unlikely if eggs are disinfected using an appropriate rinsing step to eliminate extraneous organic material. The *ad hoc* Group therefore recommended that an article on the importation of disinfected eggs for aquaculture should be included in the draft chapter for infection with *Salmonid alphavirus*, although Members should consider clearly specifying disinfection procedures in any health certification required.

References:

D. A. Graham, K. Cherry, C. J. Wilson and H. M. Rowley (2007). Susceptibility of salmonid alphavirus to a range of chemical disinfectants. *Journal of Fish Diseases*, 30, 269–277.

.../Annexes

Annex 23 (contd)

Annex 1

**REPORT OF THE ELECTRONIC OIE AD HOC GROUP ON
SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS**

February 2014

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Annex 23 (contd)

Annex 2

**REPORT OF THE ELECTRONIC OIE AD HOC GROUP ON
SAFETY OF PRODUCTS DERIVED FROM AQUATIC ANIMALS**

February 2014

Adopted agenda

1. Conduct assessments for the following aquatic animal products against the ‘Criteria to assess the safety of aquatic animal commodities for any purpose from a country, zone or compartment not declared free from disease X’ (Article 5.4.1.) and the ‘Criteria to assess the safety of aquatic animal commodities for retail trade for human consumption from a country, zone or compartment not declared free from disease X’ (Article 5.4.2.):
 - heat sterilised, hermetically sealed fish products;
 - pasteurised fish products;
 - mechanically dried, eviscerated fish;
 - fish oil and fish meal;
 - fish skin leather;
 - naturally dried, eviscerated fish (i.e. sun-dried or wind-dried);
 - chilled, eviscerated fish;
 - frozen, eviscerated fish;
 - frozen fish fillets or steaks;
 - chilled fish fillets or steaks.
2. Evaluate whether salmon alphavirus is vertically transmitted in disinfected eggs and whether an article on importation of disinfected eggs for aquaculture is appropriate for the disease-specific chapter.
3. Submit a report to the OIE Aquatic Animal Health Standards Commission for review at their February 2014 meeting.

Annex 23 (contd)

Annex 3

I. Aquatic Animal Product Assessments for infection with *Salmonid alphavirus* (SAV)

1. Assessments using criteria in Article 5.4.1. (for Article XX.9.3. point 1)

The following aquatic animal products were assessed and did meet the criteria in Article 5.4.1.:

- i) heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
- ii) pasteurised fish products that have been subjected to a heat treatment at 90°C for 10 minutes (or any time/temperature equivalent that has been demonstrated to inactivate SAV);
- iii) mechanically dried, eviscerated fish (i.e. a heat treatment of 100°C for 30 minutes or any time/temperature equivalent that has been demonstrated to inactivate SAV);
- iv) fish oil and fish meal;
- v) fish skin leather.

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.1.:

- i) naturally dried, eviscerated fish (i.e. sun-dried or wind-dried);
- ii) frozen, eviscerated fish;
- iii) frozen fish fillets or steaks;
- iv) chilled, eviscerated fish;
- v) chilled fish fillets or steaks.

Annex 23 (contd)Annex 3 (contd)

Commodity under consideration		Heat sterilised, hermetically sealed fish products	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Muscle, skin and fins may be present in the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Heat treatment is 121°C for 3.6 minutes or equivalent (e.g. 111°C for 36 minutes) (Ababouch, 1999; Ababouch, 2002). SAV is heat-sensitive under experimental conditions. SAV is inactivated within one hour at 60°C. (Graham <i>et al.</i> , 2007).	Yes
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV will be inactivated by this process. Therefore, heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) are eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Pasteurised fish products	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Pasteurised fish products include edible portions of the fish. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Pasteurisation involves heating at 90°C for 10 minutes (or equivalent) (FDA, 2001; Gould, 1999). SAV is heat sensitive under experimental conditions. SAV is inactivated within one hour at 60°C in liquid media in the presence of organic matter (Graham <i>et al.</i> , 2007). A 60 minute temperature combination that is equivalent to 90°C for 10 minutes is reached at 83°C i.e. 90°C for 10 minutes delivers that same thermal treatment as 83°C for 60 minutes. This exceeds 60°C for 60 minutes. Therefore, SAV would be expected to be inactivated (UK Food Safety Agency, 2014).	Yes
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV will be inactivated by this process. Therefore, pasteurised fish products that have been subjected to heat treatment at 90°C for 10 minutes, or to any time/temperature equivalent that has been demonstrated to inactivate SAV, are eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)Annex 3 (contd)

Commodity under consideration		Mechanically dried, eviscerated fish	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Muscle, bones, head, gills, skin, and fins may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Mechanical drying involves heating at 100°C for 30 minutes (or equivalent) (OECD, 2008). SAV is heat sensitive under experimental conditions. SAV is inactivated within one hour at 60°C (Graham <i>et al.</i> , 2007).	Yes
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV will be inactivated by this process. Therefore, mechanically dried, eviscerated fish (i.e. a heat treatment at 100°C for at least 30 minutes or any time/temperature equivalent that has been demonstrated to inactivate SAV) are eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Naturally dried, eviscerated fish	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Muscle, bones, head, gills, skin, and fins may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Natural drying involves exposure to the sun and/or wind (OECD, 2008). Typical air temperatures are 25–30°C for 1–3 days.	No
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	There is insufficient evidence to assess this commodity. Therefore, naturally dried, eviscerated fish are not eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Fish oil and fish meal	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Fish oil is derived from whole fish or by-products of processing. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	During production, fish oil and fish meal undergo multiple heat treatments and the final water content of the product is extremely low. Raw material is cooked (may be pre-heated to 50–60°C before cooking at temperatures of 95–100°C for 15–20 minutes). For reasons of energy cost and nutritional content, some processors use 80–85°C for 20 minutes). Cooked material is pressed to produce press liquor and presscake that can be dried (75–80°C, ≥30 minutes) and milled to presscake meal. Press liquor is heated to 90–95°C, which produces oil and stick-water. Oil is purified with hot water (at 90°C). Stick-water is evaporated at ≥100°C (<130°C) and the resulting fish solubles are added to the presscake. Presscake and fish soluble mix is dried at 75–80°C for ≥30 minutes to reduce water content to ≤12%. This is then milled to whole fishmeal. SAV is heat sensitive under experimental conditions. SAV is inactivated within one hour at 60°C in liquid media in the presence of organic matter (Graham <i>et al.</i> , 2007).	Yes
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV will be inactivated by this process. Therefore, fish oil and fishmeal are eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Fish skin leather	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	This commodity is composed of skin with fins removed. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Skin tissue has not been tested.	N o
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>		
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>	The fish skin is exposed to alkaline metal sulphide, solvents, proteases, acid pH 1.5–4, chromium or other tanning solutions and dyes. The final leather product is usually pH <5. Each step takes between 15 minutes and 24 hours in a commercial setting (<i>Pocket Book for the Leather Technologist</i> 4 th edition). SAV is acid labile. In experimental <i>in vitro</i> studies virus titre dropped to undetectable levels within 5 minutes at pH4, when HCl is used. When HCl is used to induce the pH drop, but also formic acid, SAV titres drop by 4 log ₁₀ if exposed to pH4 for 5 minutes, and is not detectable after one day (Graham <i>et al.</i> , 2007).	Ye s
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV will be inactivated by this process. Therefore, fish skin leather is eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)Annex 3 (contd)

Commodity under consideration		Frozen, eviscerated fish	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Head, gills, muscle, skin, and fins may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Fish are frozen and maintained at a temperature of -18°C or lower (WHO/FAO, 2009). SAV will survive freezing at -20°C for at least 48 weeks (Graham <i>et al.</i> , 2007).	No
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV is unlikely to be inactivated by this process. Therefore, frozen, eviscerated fish are not eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Frozen fish fillets and steaks	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Muscle and skin may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Fish are frozen and maintained at a temperature of minus 18°C or lower (WHO/FAO, 2009). SAV will survive freezing at minus 20°C for at least 48 weeks (Graham <i>et al.</i> , 2007).	No
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	SAV is unlikely to be inactivated by this process. Therefore, frozen fish fillets and steaks are not eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)Annex 3 (contd)

Commodity under consideration		Chilled, eviscerated fish	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Head, gills, muscle, skin, and fins may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Fish are chilled and maintained at a temperature approaching that of melting ice (WHO/FAO, 2009). No data are available on the survival of SAV in fish tissues at this temperature.	No
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	There is insufficient evidence to assess chilled, eviscerated fish. Therefore, chilled, eviscerated fish is not eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Chilled fish fillets and steaks	
Criteria 5.4.1.		Assessment	
1.	Absence of pathogenic agent in the traded commodity:		
1a.	<i>There is strong evidence that the pathogenic agent is not present in the tissues from which the commodity is derived.</i>	Muscle and skin may be part of the commodity. Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV.	No
AND			
1b.	<i>The water (including ice) used to process or transport the commodity is not contaminated with the pathogenic agent and the processing prevents cross contamination of the commodity to be traded.</i>		
OR			
2.	Even if the pathogenic agent is present in, or contaminates, the tissues from which the commodity is derived, the treatment or processing to produce the commodity to be traded inactivates the pathogenic agent:		
2a.	<i>Physical (e.g. temperature, drying, smoking)</i>	Fish are chilled and maintained at a temperature approaching that of melting ice (WHO/FAO, 2009). No data are available on the survival of SAV in fish tissues at this temperature.	No
AND/OR			
2b.	<i>Chemical (e.g. iodine, pH, salt, smoke)</i>		
AND/OR			
2c.	<i>Biological (e.g. fermentation).</i>		
Conclusion	There is insufficient evidence to assess criterion 2a. Therefore, chilled fish fillets and steaks are not eligible for inclusion in point 1 of Article 10.X.3.		

Annex 23 (contd)

Annex 3 (contd)

2. Assessments using Criteria in Article 5.4.2. (for Article XX.9.12. point 1)

The following aquatic animal products were assessed and did meet the criteria in Article 5.4.2.:

- i) frozen fish fillets and steaks;
- ii) chilled fish fillets and steaks.

The following aquatic animal products were assessed and did not meet the criteria in Article 5.4.2.:

- iii) frozen, eviscerated fish;
- iv) chilled, eviscerated fish.

Annex 23 (contd)Annex 3 (contd)

Commodity under consideration		Frozen fish fillets and steaks	
Criteria 5.4.2.		Assessment	
1.	<i>The aquatic animal product is prepared and packaged for retail trade for human consumption.</i>	It is part of the definition.	Yes
AND EITHER			
2.	<i>It includes only a small amount of waste tissues generated by the consumer.</i>	Waste may include skin and bones.	Yes
OR			
3.	<i>The pathogenic agent is not normally found in the waste tissues generated by the consumer.</i>		No
Conclusion	Frozen fish fillets and steaks that are prepared and packaged for retail trade for human consumption produce small amounts of waste. Therefore, this product is eligible for inclusion in Article 10.X.12.		

Commodity under consideration		Chilled fish fillets and steaks	
Criteria 5.4.2.		Assessment	
1.	<i>The aquatic animal product is prepared and packaged for retail trade for human consumption.</i>	It is part of the definition.	Yes
AND EITHER			
2.	<i>It includes only a small amount of waste tissues generated by the consumer.</i>	Waste may include skin and bones.	Yes
OR			
3.	<i>The pathogenic agent is not normally found in the waste tissues generated by the consumer.</i>		No
Conclusion	Chilled fish fillets and steaks that are prepared and packaged for retail trade for human consumption produce small amounts of waste. Therefore, this product is eligible for inclusion in Article 10.X.12.		

Annex 23 (contd)

Annex 3 (contd)

Commodity under consideration		Frozen, eviscerated fish	
Criteria 5.4.2.		Assessment	
1.	<i>The aquatic animal product is prepared and packaged for retail trade for human consumption.</i>	It is part of the definition.	Yes
AND EITHER			
2.	<i>It includes only a small amount of waste tissues generated by the consumer.</i>	Waste may include head, skin, fins and bones.	No
OR			
3.	<i>The pathogenic agent is not normally found in the waste tissues generated by the consumer.</i>	Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV. Fish are frozen and maintained at a temperature of minus 18°C or lower (WHO/FAO, 2009). SAV will survive freezing at minus 20°C for at least 48 weeks (Graham <i>et al.</i> , 2007).	No
Conclusion	Frozen, eviscerated fish that are prepared and packaged for retail trade for human consumption produce amounts of waste that cannot be considered small and SAV may not be inactivated by the process of freezing. Therefore, frozen, eviscerated fish is not considered to be eligible for inclusion in Article 10.X.12.		

Commodity under consideration		Chilled, eviscerated fish	
Criteria 5.4.2.		Assessment	
1.	<i>The aquatic animal product is prepared and packaged for retail trade for human consumption.</i>	It is part of the definition.	Yes
AND EITHER			
2.	<i>It includes only a small amount of waste tissues generated by the consumer.</i>	Waste may include head, skin, fins and bones.	No
OR			
3.	<i>The pathogenic agent is not normally found in the waste tissues generated by the consumer.</i>	Infection with SAV may induce a viraemia during the acute phase. SAV has been detected by RT-PCR or virus isolation in the following tissues: blood, brain, gill, and heart (Graham <i>et al.</i> , 2006; Graham <i>et al.</i> , 2007; Jansen, <i>et al.</i> , 2010; Graham <i>et al.</i> , 2011) as well as in mucus and faeces (Graham <i>et al.</i> , 2012). Demonstration of presence or absence of viable virus in skin, fins, skeletal muscle and other tissues has not been systematically studied in fish undergoing slaughter/destruction although all ages of finfish can experience infection with SAV. Fish are chilled and maintained at a temperature approaching that of melting ice (WHO/FAO, 2009). No data are available on the survival of SAV in fish tissues at this temperature.	No
Conclusion	Chilled, eviscerated fish that are prepared and packaged for retail trade for human consumption produce amounts of waste that cannot be considered small, and there is insufficient evidence to assess this commodity against some of the criteria. Therefore, chilled, eviscerated fish is not considered to be eligible for inclusion in Article 10.X.12.		

[Annex 23](#) (contd)[Annex 3](#) (contd)**REFERENCES**SAV specific

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AQUATIC ANIMALS COMMISSION WORK PLAN 2014–2015

Aquatic Code

Task	Oct 2013	February 2014	May GS 2014	Sept 2014
Glossary	AAC revised definitions for emerging disease, susceptible species and veterinarian. Circulated for Member comments	Reviewed Member comments	Propose for adoption	
Chapter 1.1.	AAC revised text and circulated for Member comments	Reviewed Member comments	Propose for adoption	
Article 1.2.3. – Criteria for listing a disease and an emerging disease	AAC proposed deletion and circulated for Member comments	Reviewed Member comments	Propose for adoption	
OsHV-1 μvar – Listed as an emerging disease		Proposed deletion in line with proposed deletion of Article 1.2.3.	Propose for adoption	
Chapter 1.3. – Listing of IHNN				Review delisting process
Revision of Section 4 to improve guidance on the control of disease	Develop Concept Note for revision of this section	Reviewed section 4 and prioritised chapter revisions		
Chapter 4.3. – Disinfection		Recommended that an AHG be convened to develop a revised chapter		Review draft and circulate for Member comments
Chapter 6.1. – Chapter on control of hazards in feed	AAC agreed on the structure of a revised chapter. AAC to develop revised chapter	Reviewed draft chapter (developed by AAC)		Review draft and circulate for Member comments
Chapter 6.X. – Risk analysis for antimicrobial resistance in aquaculture (new)	Request report from AHG regarding progress	Reviewed draft chapter and requested further work by AHG		Review draft and circulate for Member comments
Chapter 10.X. – Infection with salmonid alphavirus	AAC developed new Code chapter and circulated for Member comments	Reviewed Member comments	Propose for adoption	
Chapter X.X. – Criteria for listing susceptible species (new)	Review Member comments and circulate revised text for Member comments	Reviewed Member comments	Propose for adoption	
Chapter on Evaluation of AAHS (new)				Consider the development of a new chapter
User's Guide		AAC developed revised draft and circulated for comment		Review Member comments

Annex 24 (contd)*Aquatic Manual*

Manual Tasks	Oct. 2013	Feb 2014	May GS 2014	Sept 2014
Chapter 2.3.5. – Infection with ISAV	AAC revised chapter and circulated for comments	Reviewed Member comments	Propose for adoption	
Chapter 2.4.9. – OsHV-1 μvar	AAC revised chapter and circulated for comments	Reviewed Member comments	Propose for adoption	
Chapter 1.1.3. – Disinfection		Decided to relocate guidance on disinfection to the Code		
Chapter X.X.X. – Infection with salmonid alphavirus	November 2013: Circulate new chapter for Member comments.	Reviewed Member comments	Propose for adoption	

Other items

Tasks	Oct. 2013	Feb. 2014	late 2014	early 2015
OIE Global Aquatic Animal Health Conference (January 2015, TBC)	Establish Scientific Committee. Develop the programme.	Finalised programme		Conference (20-22 January, 2015, Vietnam)
OIE Ref. Lab. Conference (7–9 October 2014)	AAC to provide input into the programme and Scientific Committee.	Provided input into the programme	Conference (7-9 October, 2014, Seoul)	

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