



Organisation  
Mondiale  
de la Santé  
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## REPORT OF THE MEETING OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris (France), 30 September–4 October 2013

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at the OIE Headquarters from 30 September to 4 October 2013.

Details of participants and the adopted agenda are given at [Annexes 1 and 2](#).

The Aquatic Animals Commission reviewed the documents identified in the agenda, addressing comments that Member Countries had submitted by 30 August 2013 and amended texts in the *OIE Aquatic Animal Health Code* (the *Aquatic Code*) and the *OIE Manual of Diagnostic Tests for Aquatic Animals* (the *Aquatic Manual*) where appropriate. The amendments are shown in the usual manner by double underline and strikethrough and may be found in the Annexes to the report.

Member Countries should note that, unless stated otherwise, texts submitted for comment may be proposed for adoption at the 82nd OIE General Session in May 2014. Depending on the comments received on each text, the Commission will identify the texts proposed for adoption in May 2014 in the report of its February 2014 meeting.

The Aquatic Animals Commission strongly encourages Member Countries to participate in the development of the OIE's international standards by submitting comments on this report. It would be very helpful if comments were 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-change' function provided by word processing software as such changes are lost in the process of collating Member Countries' submissions into the Commission's working documents.

The table below summarises the texts presented in the Annexes. [Annexes 3 to 12](#) are presented for Member Country comment; [Annex 13](#) is presented for Member Countries' information.

Comments on this report must reach OIE Headquarters **prior to 24 January 2014** to be considered at the February 2014 meeting of the Commission. All comments should be sent to the OIE International Trade Department at: [trade.dept@oie.int](mailto:trade.dept@oie.int).

<b>Texts for Member Countries' comments</b>	<b>Annex number</b>
<b><i>Aquatic Code:</i></b>	
Glossary	Annex 3
Notification of diseases and epidemiological information (Chapter 1.1.)	Annex 4
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Necrotising hepatopancreatitis (Chapter 9.4.)	Annex 6
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<b><i>Aquatic Manual:</i></b>	
Infection with infectious salmon anaemia (Chapter 2.3.5.)	Annex 11
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Aquatic Animal Health Standards Commission Work Plan for 2012/2014	Annex 13

## 1. OIE Aquatic Animal Health Code

The Aquatic Animals Commission wished to emphasise that the issue of emerging diseases is important in light of the frequent emergence in aquaculture of new diseases some of which have significant impact on productivity, resource sustainability and trade. Diseases emerge regularly in aquatic animals due to newly domesticated species, the large number of species farmed and the rapid growth of aquaculture production.

During this meeting, the Aquatic Animals Commission touched upon several issues related to emerging diseases and how they are addressed in the *Aquatic Code*; this included the definition of emerging disease (Glossary), mechanisms for notification of an emerging disease (Chapter 1.1.) and the mechanism for listing emerging diseases (Chapter 1.2.). These sections of the *Aquatic Code* together assist Member Countries to recognise important emerging diseases and prevent their spread while continuing safe trade.

In this report, the relevant items are presented in Items 1.1, 1.2, and 1.3, (i.e. Glossary, Chapter 1.1. and Chapter 1.2., respectively). The Aquatic Animals Commission recommends that Member Countries comments are made taking into consideration the inter-relatedness of these items.

### 1.1. Glossary

#### *Veterinarian*

In light of the recently adopted amendment to the definition for 'veterinarian' in the *Terrestrial Code*, the Aquatic Animals Commission agreed to amend the definition in the *Aquatic Code* to align it with the *Terrestrial Code* definition.

### *Emerging disease*

The Aquatic Animals Commission considered the proposal for a revised definition of ‘emerging disease’ developed by the Terrestrial Code Commission which primarily aimed at improving clarity.

The Aquatic Animals Commission noted two different circumstances of disease emergence: new pathogens or an epidemiological change of known pathogens. This is reflected in the amended definition.

In aquaculture, emergence of diseases caused by unknown pathogens is not uncommon, and therefore the definition in the aquatic context needs to include the situation where an infectious agent is strongly associated with a disease although not proven to be the aetiological agent. The Aquatic Animals Commission noted that this situation is currently captured in the criteria listing an emerging disease (Chapter 1.2.). In light of this, the Commission further amended the definition to read: means a disease newly recognised or infection occurrence, which has a significant impact on animal or public health, of a disease, 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.~~

The Aquatic Animals Commission clarified that this definition excludes the diseases listed in the *Aquatic Code*.

The Aquatic Animals Commission wished to inform Member Countries that comments received on this amended definition will be submitted to the Terrestrial Code Commission at their next meeting (11-18 February, 2014) for their consideration to maintain alignment between the definitions used in the two *Codes*, where relevant. Subsequently, the Aquatic Animals Commission will consider Member Countries comments along with the Terrestrial Code Commission proposal at their next meeting.

Other relevant items on this topic are presented in Items 1.2. and 1.3. (i.e. Chapters 1.1. and 1.2.). The Aquatic Animals Commission recommends that Member Countries comments are made taking into consideration the inter-relatedness of these items.

### *Susceptible species*

The Aquatic Animals Commission considered that the second sentence of the current definition for ‘susceptible species’ does not contribute to defining this term. Consequently the Commission proposed to delete this sentence. In addition, the Commission proposed a change in the first sentence to improve clarity.

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

The revised Glossary is presented at [Annex 3](#) for Member Countries’ comments.

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

The Aquatic Animals Commission considered the amendments to this chapter being proposed by the Terrestrial Code Commission which are primarily aimed at improving clarity. The Aquatic Animals Commission incorporated the Terrestrial Code Commission’s proposed amendments into the revised chapter, with the exception in point e) of Article 1.1.3. For this point the Terrestrial Code Commission proposed the use of the words ‘unusual host species’. However, the Aquatic Animals Commission proposed to use the word ‘new host species’ instead of ‘unusual host species’ because they considered that the term ‘unusual’ is ambiguous and that ‘new host species’ are important for trade and should be reported.

The Aquatic Animals Commission wished to emphasise some significant changes with regards to emerging diseases have been made. These include:

- (i) the deletion of point f) in Article 1.1.3. (~~' an emerging disease with significant morbidity or mortality, or zoonotic potential;~~<sup>2</sup>) which clarifies that the definition for emerging disease excludes OIE listed diseases;
- (ii) the development of a new Article 1.1.3.bis which clearly separates the different notification obligations for an emerging disease, i.e. the obligation to notify an emerging disease, and the circumstances under which reporting should be terminated.

The Aquatic Animals Commission noted that the information provided to Member Countries on an emerging disease is currently made via the OIE Disease Information Alert System. The Commission had concerns that this mechanism may overemphasise the impact or importance of some emerging disease events and discourage reporting. The Commission agreed to raise this matter with the OIE Director General.

Other relevant items on this topic are presented in Items 1.1 and 1.3. (i.e. Glossary and Chapter 1.2). The Commission recommended that Member Countries' comments are made taking into consideration the inter-relatedness of these items.

The revised Chapter 1.1. is presented at [Annex 4](#) for Member Countries' comments.

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

The Aquatic Animals Commission considered the importance of recognising emerging diseases at an early stage and communicating information about these events among Member Countries. In particular, the Commission considered recent examples such as Acute hepatopancreatic necrosis syndrome (AHPNS) and Infection with ostreid herpesvirus 1 microvariant (OsHv-1).

Despite the current notification and reporting obligations for emerging diseases described in point 1.f) of Article 1.1.3., notification and reporting have been inconsistent. The Aquatic Animals Commission considered that the revised definition for an emerging disease and revised Chapter 1.1., including Article 1.3.bis, both provided clarity on notification and reporting obligations for emerging diseases.

The Aquatic Animals Commission reconsidered the utility of the mechanism for listing emerging diseases (which utilises the criteria described in Article 1.2.3.). The Commission considered that this mechanism is not sufficiently responsive for effective communication of the occurrence of an emerging disease. This article provides an additional mechanism that requires Member Countries to report on specific listed emerging diseases. However, this mechanism has established requirements for reporting only for a small number of listed emerging diseases.

The Aquatic Animals Commission also noted that the amended definition for emerging diseases now reflects the set of criteria in Article 1.2.3.

Considering the improved clarity on requirements for notification and reporting emerging diseases, clarified definition for emerging diseases, and the slow mechanism for listing an emerging disease, the Aquatic Animals Commission proposed that Article 1.2.3. be deleted.

Other relevant items on this topic are presented in Items 1.1. and 1.2. (i.e. Glossary and Chapter 1.1.). The Aquatic Animals Commission recommends that Member Countries comments are made taking into consideration the inter-relatedness of these items.

The revised Chapter 1.2. is presented at Annex 5 for Member Countries' comments.

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

##### **Acute hepatopancreatic necrosis syndrome**

Following on from previous discussions held by the Aquatic Animals Commission at their March 2013 meeting on Acute hepatopancreatic necrosis syndrome (AHPNS), the Commission reviewed current information on this syndrome.

The Aquatic Animals Commission considered that the available information may be insufficient to assess the disease against the criteria in Article 1.2.2. In particular, uncertainty on pathogen identity (among strains of *Vibrio parahaemolyticus*) and lack of availability of a specific diagnostic test (to differentiate the causative agent from other strains of *V. parahaemolyticus*) means that criteria for listing are unlikely to be met. In light of the proposal to delete Article 1.2.3. for listing of an emerging disease, the Aquatic Animals Commission does not consider it appropriate to list AHPNS as an emerging disease.

However, the Aquatic Animals Commission agreed that the disease meets the definition of an emerging disease and that Member Countries should notify the occurrence of this disease in accordance with Article 1.1.3.

The Commission drafted an OIE Technical Factsheet on Acute Hepatopancreatic Necrosis Syndrome (AHPNS) with input from experts. This Factsheet will be uploaded onto the OIE web page and will be regularly reviewed as new knowledge becomes available.

The Commission invites Member Countries to provide any relevant information that would contribute to improving the Technical Factsheet on Acute Hepatopancreatic Necrosis Syndrome (AHPNS), and more specifically information on how to diagnose the disease.

#### **1.5. Control of hazards in aquatic animal feeds (Chapter 6.1.)**

The Aquatic Animals Commission discussed how to review the purpose, scope and content of Chapter 6.1. The Commission agreed that the main objective of this chapter should be the prevention of spread of infectious diseases via aquatic animal feed. The Commission also agreed that this chapter should assist Member Countries in identifying risk pathways and assessing risk related to aquatic animal health hazards in aquatic animal feed.

The Commission will develop a revised chapter for consideration at their February 2014 meeting. The Commission invites Member Countries comments on this proposal.

#### **1.6. Necrotising hepatopancreatitis (Chapter 9.4.)**

In light of the outcomes of the International Committee for Taxonomy of Bacteria regarding the new name for necrotising hepatopancreatitis bacteria (NHP-B) to Candidatus *Hepatobacter penaei*, the Aquatic Animals Commission proposed to amend Article 9.4.1. to incorporate this change.

The revised Chapter 9.4. is presented at [Annex 6](#) for Member Countries' comments.

#### **1.7. Infection with infectious salmon anaemia virus (Chapter 10.5.)**

In response to a Member Country comment at the May 2013, OIE General Session requesting clarification of the text in Article 10.5.5. point 1) regarding the absence of susceptible species and the declaration of country freedom from HPR-deleted ISAV, the Aquatic Animals Commission proposed to delete this point and the equivalent point in Article 10.5.7. (zone or compartment) as they considered that these points are redundant and these pathways to claim freedom are covered in the Articles 10.5.4. and 10.5.6., respectively.

Whilst reviewing this chapter, the Aquatic Animals Commission also realised that a specific article on the 'Importation of aquatic animal products from a country, zone or compartment declared free from infection with HPR-deleted infectious salmon anaemia virus' was missing. The Commission introduced a new Article 10.5.15bis to address this circumstance.

The Aquatic Animals Commission wished to note that horizontal amendments proposed in Item 1.8. have also been incorporated into this amended chapter.

The revised Chapter 10.5. is presented at Annex 7 for Member Countries' comments.

### 1.8. Horizontal issues

The Aquatic Animals Commission noted a number of inconsistencies in the inclusion of some relevant text in some of the disease specific chapters and proposed the following amendments in all disease specific chapters.

#### All disease specific chapters, amend:

##### Point 2 of Articles 10.X.9. and 10.X.10. as indicated below:

These proposed amendments were adopted in May 2013, OIE General Session in Chapter 10.2. This amendment to all disease specific chapters would ensure alignment across all chapters.

Article 10.X.9.

- 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' or is disposed in a manner that prevents contact of waste with *susceptible species*.

Article 10.X.10.

- 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 10.X.3.

##### Point 3 of Articles 10.X.4. and 10.X.5. as indicated below:

The Aquatic Animals Commission proposed these amendments to improve clarity on the circumstances of claiming freedom for these points. The reference to ten years and absence of conditions conducive to clinical expression are unnecessary and create ambiguity.

- 3) A country where ~~the last observed occurrence of the disease was within the past ten years or where the disease~~ *infection* status prior to *targeted surveillance* 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 Disease X 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 last two years without detection of Disease X.

##### Point 4d) of Articles 10.X.4. and 10.X.5., as indicated below:

The Aquatic Animals Commission proposed these articles be amended as they considered that implementation of modified 'basic biosecurity conditions' is relevant immediately upon eradication of disease and the 2 year period is not relevant to this point.

- 4d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since ~~for at least the past two years~~ eradication of the disease.

Articles 10.X.7. (all disease specific chapters) and Article 10.X.11. (in fish and amphibian chapters) or Article 10.X.10 (in mollusc and crustacean chapters)

The Aquatic Animals Commission noted that some articles were missing the correct cross reference to other relevant articles. They proposed that the relevant text be amended to ensure the correct cross referencing to other articles regarding maintenance of free status.

‘Infection with pathogen X’ approach

In addition, the Aquatic Animals Commission noted that a number of existing disease specific chapters are titled ‘Infection with pathogen X’ but this approach has not been applied consistently throughout the chapter. The Commission will ensure that these inconsistencies will be corrected in all the relevant disease specific chapters in the 2014 edition of the *Aquatic Code*. The Commission agreed that the recently revised Chapters 10.2 and 10.5. should be used as the model for applying these changes to disease naming nomenclature.

Model articles are provided at [Annex 8](#) for Member Countries’ comments.

**1.9. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new chapter X.X.)**

Comments were received from: Australia, Canada, Chinese Taipei, European Union, Japan, Korea, New Zealand, Norway, Russia, Switzerland and the United States of America.

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

The Aquatic Animals Commission agreed with several Member Countries requesting that this chapter should focus on criteria for listing species as susceptible for their inclusion in Article X.X.2. of disease specific chapters in the *Aquatic Code*. In order to clarify this, the Commission proposed to amend the title of the chapter to ‘Criteria for listing species as susceptible to infection with specific pathogen’.

In response to Member Countries’ comments, the Aquatic Animals Commission clarified that provisions in the disease specific chapters of the *Aquatic Code* only apply to species that are susceptible to infection which does not include mechanical vectors. In addition, the Commission clarified that ‘disease’ is defined in the *Aquatic Code* as clinical and non-clinical infection and therefore susceptibility of a species means susceptibility to infection with the causative agent. The proposed criteria are designed to assess susceptibility to infection.

In response to Member Countries’ comments that only ‘definite susceptible species’ should be included in the *Aquatic Code*, the Aquatic Animals Commission recognised possible ambiguity in introducing ‘definite susceptible species’ and ‘possible susceptible species’. Consequently, the Commission amended the chapter to remove these terms.

In addition, Article X.X.7. was amended to clearly indicate that in the situation where evidence exists but is insufficient to demonstrate susceptibility of a species, this species would be included in section 2.2.1. of the relevant disease chapter in the *Aquatic Manual*. The Aquatic Animals Commission included some text emphasising that where a species can reasonably be expected to pose a risk of transmission for the pathogen under consideration, Competent Authorities should assess the risk of introduction of the causative agent through imports of a possible susceptible species, in accordance with the recommendations in the *Aquatic Code*. Consistent with the Aquatic Animals Commission’s intention to focus only on susceptible species, the Commission agreed that the proposed Article X.X.8. ‘Taxonomic relationship of susceptible species’ be deleted. The Aquatic Animals Commission clarified that once the new Chapter X.X. has been adopted, these criteria will be applied to determine susceptible species for each listed disease. The Commission proposed that an ad hoc Group would be convened to undertake assessments against the criteria which would then be circulated for Member Countries’ comments. Any subsequent changes to the list of susceptible species in Article X.X.2. of specific disease chapters in the *Aquatic Code*, would then be circulated for Member Countries’ comments prior to proposal for adoption.

If this draft chapter is adopted and once the species susceptibility determinations are completed, the Aquatic Animals Commission proposes that the following text in Article X.X.2 (Scope) of the disease specific chapters of the *Aquatic Code* 'These recommendations apply to any other susceptible species referred to in the *Aquatic Manual* when traded internationally' be deleted and be replaced with 'When the recommendations in this chapter are applied to any other species, *Competent Authorities* should conduct a risk analysis in accordance with the recommendations in the *Aquatic Code*'.

The revised Chapter X.X. is presented at [Annex 9](#) for Member Countries' comments.

#### **1.10. Infection with Salmonid alphavirus (New chapter 10.X.)**

Given the adoption of 'Infection with *Salmonid alphavirus*' as an OIE listed disease, at the May 2013 OIE General Session, the Aquatic Animals Commission developed a new draft chapter on Infection with *Salmonid alphavirus* for inclusion in the *Aquatic Code*.

The Aquatic Animals Commission noted that the list of commodities in Articles 10.X.3 and 10.X.12, and 10.X.13. have been placed 'under study'. The Commission requested that the *ad hoc* Group on Safe Commodities be reconvened to conduct assessments on a range of commodities commonly traded internationally against the criteria provided in Chapter 5.4. and on the safety of disinfected eggs. The Commission requested that they receive the *ad hoc* Group report prior to their February 2014 meeting so that relevant articles can be updated.

The Aquatic Animals Commission wished to note that horizontal amendments proposed in Item 1.8. have been incorporated into this draft chapter.

The new Chapter 10.X. is attached as [Annex 10](#) for Member Countries' comments.

#### **1.11. Future work on pathogen differentiation**

The Aquatic Animals Commission reviewed all listed diseases and considered possible issues related to the pathogen differentiation approach. The Commission recognised that the Yellow head disease chapter in the *Aquatic Code* requires clarification with regards to scope, and alignment of the *Aquatic Manual* with the *Code*. The Commission will discuss this issue further at their February 2014 meeting.

#### **1.12. Aquatic Code - Table of contents**

The Aquatic Animals Commission reviewed the Table of contents of the *Aquatic Code* and agreed that Chapter 6.1. would be better placed within Section 4. General recommendations: disease prevention and control. Subsequent to this proposed change, the Commission agreed that the title of Section 6 should be changed in order to better reflect its contents. The Commission proposed that Section 6 'Veterinary Public Health' be renamed 'Recommendations For Antimicrobial Use In Aquatic Animals'. The Commission requested that the OIE International Trade Department implement this proposal in the 2014 edition of the *Aquatic Code*.

## **2. OIE Manual of Diagnostic Tests for Aquatic Animals**

Ms Sara Linnane, Scientific Editor, from the Scientific and Technical Department, joined the meeting for this agenda item.

### **2.1. Review of the Aquatic Manual chapters**

Comments had been received from Australia and the European Union.

#### **2.1.1. Chapter 2.3.5 Infection with infectious salmon anaemia**

The Aquatic Animals Commission, in consultation with the OIE Designated Expert, reviewed Member Country comments and amended the text, as appropriate.

The revised Chapter 2.3.5. is presented at [Annex 11](#), in English, for Member Countries' comments. Note, the Spanish version will be made available following adoption of the chapter in 2014.

#### **2.1.2. Chapter 2.4.9 Infection with ostreid herpesvirus 1 microvariant**

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

The revised Chapter 2.4.9. is presented at [Annex 12](#), in English, for Member Countries' comments. Note, the Spanish version will be made available following adoption of the chapter in 2014.

#### **2.1.3. Chapter 2.1.4 Infection with salmonid alphavirus**

The Aquatic Animals Commission noted that the draft chapter for the *Aquatic Manual* is still under development but will be submitted to the OIE by mid-November 2013. Once received, the draft chapter will then be sent to all Member Countries for comment with the request to submit comments prior to the February 2014 meeting to enable prompt adoption of this chapter.

### **2.2. Disease specific guidance documents on surveillance for a fish, a mollusc and a crustacean disease**

The Aquatic Animals Commission considered the three stand-alone documents that provide examples of how to develop surveillance systems specific for a fish, a mollusc and a crustacean disease. Of these documents, the Commission agreed that the one for viral haemorrhagic septicaemia (VHS) be uploaded on the OIE website. The remaining examples for white spot disease and infection with *Bonamia ostreae* require further amendment prior to publication on the OIE website.

The Commission will undertake this work and will consider the revised documents at its next meeting in February 2014.

## **3. OIE Reference Centres**

### **3.1. Feedback on Reference Laboratory quality management systems**

Dr Min-Kyung Park, Scientific and Technical Department of the OIE, joined the meeting for this agenda item. At the previous meeting, Dr Park had presented an analysis of the activities of the OIE Reference Centres collected through the annual reports for 2012. The Aquatic Animals Commission noted that some laboratories are in the process of achieving an internationally recognised quality management system. The Commission noted that a letter of encouragement would be sent to these laboratories with a request that the OIE be provided with an update on any progress made during the year.

The Aquatic Animals Commission was concerned that some OIE Reference Laboratories do not have a quality management system nor plans to implement one, despite this being a requirement stated in the Terms of Reference for an OIE Reference Laboratory. The Commission noted that a letter would be sent to these laboratories indicating that failure to comply with Chapter 1.1.1 of the *Aquatic Manual* entitled: *Quality management in veterinary testing laboratories* could result in these laboratories being proposed for delisting.

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

The Third Global Conference of the OIE Reference Centres will be held in Seoul, Korea (Rep. of) from 14 to 16 October 2014. The main themes of the Conference will be: strengthening the OIE Reference Centre network, laboratory quality management systems, strengthening links between Reference Centres and Veterinary Services, and global databases of pathogen sequences.

The Commission agreed that the first three of these themes are of importance to Reference Centres for aquatic animals and welcomed the plan to conduct a breakout session specifically for Reference Centres for aquatic animals.

### 3.3. Withdrawal of OIE Reference Laboratories status

The Commission noted that the following OIE Reference Laboratories no longer wish to be designated as OIE Reference Laboratories and will be removed from the list:

- OIE Reference Laboratory for Spherical baculovirus (*Penaeus monodon*-type baculovirus) at the National Taiwan University in Chinese Taipei.
- OIE Reference Laboratory for Infection with *Batrachochytrium dendrobatidis* at the Australian Animal Health Laboratory in Australia.

The Aquatic Animals Commission noted that there is now no OIE Reference Laboratory for Infection with *Batrachochytrium dendrobatidis* and invites applications for designation as an OIE Reference Laboratory for this listed disease.

### 3.4. Review nominations for replacement experts

The Aquatic Animals Commission reviewed and accepted the following nomination for a replacement expert at the OIE Reference Laboratory for Epizootic haematopoietic necrosis and Infection with ranavirus:

Dr Nick Moody to replace Dr Alex Hyatt at the Australian Animal Health Laboratory in Geelong, Victoria, Australia.

## 4. OIE Twinning Projects

Dr Gounalan Pavade, Scientific and Technical Department of the OIE, updated the Commission regarding three twinning proposals: USA/People's Republic of China for infectious haematopoietic necrosis; USA/Indonesia for crustacean/shrimp diseases; and Japan/Indonesia for Koi herpes virus.

## 5. Aquatic Animals Commission Work Plan 2013

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

The detailed Commission's Work Plan is presented at [Annex 13](#) for Member Countries information.

## **6. Other business**

### **6.1. OIE Global Conference on Aquatic Animal Health**

Dr Gillian Mylrea, Deputy Head, International Trade Department, informed the Aquatic Animals Commission that the OIE proposes to hold the third Global Conference on Aquatic Animal Health in January 2015. The location of the conference is pending confirmation from a possible host country.

### **6.2. Online commenting**

The Aquatic Animals Commission discussed the use of on line commenting systems by the International Plant Protection Convention and CAC, and recommended the International Trade Department seek more information on how such a system could be used in the OIE, and report back to the Code Commission.

## **7. Next meeting**

The Aquatic Animals Commission proposed to hold their next meetings respectively from 24<sup>th</sup> February to 28<sup>th</sup> February 2014 and from 29<sup>th</sup> September to 3<sup>rd</sup> October 2014.

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.../Annexes



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

**Paris, 30 September–4 October 2013**

**List of participants**

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

**Paris, 30 September–4 October 2013**

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**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.)**
  - 1.5. Control of hazards in aquatic animal feeds (Chapter 6.1.)**
  - 1.6. Necrotising hepatopancreatitis (Chapter 9.4.)**
  - 1.7. Infection with infectious salmon anaemia virus (Chapter 10.5.)**
  - 1.8. Horizontal issues**
  - 1.9. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new chapter X.X.)**
  - 1.10. Infection with *Salmonid alphavirus* (new chapter 10.X.)**
  - 1.11. Future work on pathogen differentiation**
  - 1.12. *Aquatic Code* - Table of contents**
- 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**
    - 2.1.2. Chapter 2.4.9. Infection with ostreid herpesvirus 1 microvariant**
    - 2.1.3. Chapter 2.1.4. Infection with salmonid alphavirus**
  - 2.2. Disease specific guidance documents on surveillance for a fish, a mollusc and a crustacean disease**

Annex 2 (contd)

- 3. OIE Reference Centres**
  - 3.1. Feedback on Reference Laboratory quality management systems**
  - 3.2. Global Conference of the OIE Reference Centres, Seoul, Korea (Rep. of), 14–16 October 2014**
  - 3.3. Withdrawal of OIE Reference Laboratories status**
  - 3.4. Review nominations for replacement experts**
- 4. OIE Twinning Projects**
- 5. Aquatic Animals Commission Work Plan 2013**
- 6. Other business**
  - 6.1. OIE Global Conference on Aquatic Animal Health**
- 7. Next meeting**

## GLOSSARY

### **Emerging disease**

means a disease ~~newly recognised~~ infection ~~which has a significant impact on 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 natural cases or by experimental exposures to the *pathogenic agent* that mimics ~~the~~ natural pathways for *infection*. ~~Each disease chapter in the Aquatic Code and the Aquatic Manual contains a list of currently known susceptible species.'~~

### **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.

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## CHAPTER 1.1.

**NOTIFICATION OF DISEASES AND  
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.
- 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) 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., Member Countries shall also provide information on the measures taken to prevent the spread of *diseases*, 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;
  - c) first occurrence of a new strain of a pathogen of a *listed disease* new to a country, a *zone* or a *compartment*,

Annex 4 (contd)

- 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 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 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 for emerging disease, as described under point 1. These should continue until the disease has been eradicated or the situation becomes sufficiently stable or 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 animal health measures have been applied to prevent possible reappearance or spread of the *disease*. These measures will be found in detail in various chapters of the *Aquatic Code*.
- 3) A Member Country may be considered to regain freedom from a specific *disease* when all relevant conditions given in the *Aquatic Code* have been fulfilled.
- 4) The *Competent Authority* of a Member Country which sets up one or several *free zones* or *free compartments* shall inform the *Headquarters*, giving necessary details, including the criteria on which the free status is based, the requirements for maintaining the status and indicating clearly the location of the *zones* or *compartments* on a map of the territory of the Member Country.

## Article 1.1.5.

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

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## 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
<b>A. Consequences</b>		
1.	The disease has been shown to cause significant production losses at a national or multinational (zonal or regional) level.	There is a general pattern that the disease will lead to losses in susceptible species, and that morbidity or mortality are related primarily to the infectious agent and not management or environmental factors. (Morbidity includes, for example, loss of production due to spawning failure.) The direct economic impact of the disease is linked to its morbidity, mortality and effect on product quality.
2.	Or	The disease has been shown to or scientific evidence indicates that it is likely to cause significant morbidity or mortality in wild aquatic animal populations.
3.	Or	The agent is of public health concern.

Annex 5 (contd)

<b>And B. Spread</b>			
4.		Infectious aetiology of the disease is proven.	
5.	Or	An infectious agent is strongly associated with the disease, but the aetiology is not yet known.	Infectious diseases of unknown aetiology can have equally high-risk implications as those diseases where the infectious aetiology is proven. Whilst disease occurrence data are gathered, research should be conducted to elucidate the aetiology of the disease and the results be made available within a reasonable period of time.
6.	And	Likelihood of international spread, including via live animals, their products or fomites.	International trade in aquatic animal species susceptible to the disease exists or is likely to develop and, under international trading practices, the entry and establishment of the disease is likely.
7.	And	Several countries or countries with zones may be declared free of the disease based on the general surveillance principles outlined in Chapter 1.4.	Free countries/zones could still be protected. Listing of diseases that are ubiquitous or extremely widespread would render notification unfeasible. However, individual countries that run a control programme on such a disease can propose its listing provided they have undertaken a scientific evaluation to support their request. Examples may be the protection of broodstock from widespread diseases, or the protection of the last remaining free zones from a widespread disease.
<b>And C. Diagnosis</b>			
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.

## 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.

Annex 5 (contd)

And	
3.	<del>The agent is of public health concern.</del>
Or	
4.	<del>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.</del>

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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  $\alpha$ -Proteobacteria.

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

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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 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);

Annex 7 (contd)

- 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.
- 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 for ISAV, *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 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 are declared countries or *zones* free from infection with ISAV (see Article 10.5.6.).

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

OR

- 2) A country where the species referred to in Article 10.5.2. are present but there has been no detectable occurrence of infection with ISAV may make a *self-declaration of freedom* from infection with ISAV 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 last two years without detection of infection with ISAV.

OR

- 3) A country that has made a *self-declaration of freedom* from infection with ISAV but in which infection with ISAV is subsequently detected may make a *self-declaration of freedom* from infection with ISAV again when the following conditions have been met:

Annex 7 (contd)

- a) on detection of 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 ISAV, 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 last 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~~ since eradication of the *disease*.

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 10.5.6.

## 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 are declared countries or *zones* free from infection with HPR-deleted ISAV (see Article 10.5.7.).

- ~~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 the species referred to in Article 10.5.2. are present but 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 *basic biosecurity conditions* have been continuously met in the country for at least the past ten years.

OR

- 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* 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:
  - 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 last two years without detection of infection with HPR-deleted ISAV.

Annex 7 (contd)

OR

- 34) A country that has made a *self-declaration of freedom* from infection with HPR-deleted ISAV 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 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 (see *Aquatic Manual*) have been completed; and
  - c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last 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 since eradication of the *disease*.

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 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.

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* is present may be declared free from infection with ISAV 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 species referred to in Article 10.5.2. are present but there has been no detectable occurrence of infection with ISAV may be declared free from infection with ISAV 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 last two years without detection of infection with ISAV.

OR

- 3) A *zone* previously declared free from infection with ISAV but in which any ISAV is detected may be declared free from infection with ISAV again when the following conditions have been met:

Annex 7 (contd)

- 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 ISAV, 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 last 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~~ since 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.

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 species referred to in Article 10.5.2. are present but 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*, 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* 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:

- 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 last two years without detection of infection with HPR-deleted ISAV.

OR

- 34) A *zone* previously declared free from infection with HPR-deleted ISAV but in which infection with HPR-deleted ISAV is detected may be declared free from infection with HPR-deleted ISAV again when the following conditions have been met:

Annex 7 (contd)

- 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 (see *Aquatic Manual*) have been completed; and
- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last 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~~ since 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.

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) the treatment of all effluent and waste materials in a manner that ensures inactivation of ISAV.

Annex 7 (contd)

- 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 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*.

Annex 7 (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 10.5.14.

**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 to products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 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.

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 infection with HPR-deleted ISAV.

Annex 7 (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 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 the following *commodities* which have been prepared and packaged for retail trade and complying 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.

- 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:

Annex 7 (contd)

- 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.

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**MODEL DISEASE CHAPTER X.X.  
SHOWING HORIZONTAL AMENDMENTS**

CHAPTER X.X.

**DISEASE X**

[...]

Article X.X.4.

**Disease X**

A country may make a *self-declaration of freedom* from 'Disease X' 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 'Disease X' if all the areas covered by the shared water are declared 'Disease X' free countries or *zones* (see Article X.X.5.).

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

OR

- 2) A country where the species referred to in Article X.X.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 'Disease X' when *basic biosecurity conditions* have been continuously met in the country for at least the past ten years.

OR

- 3) A country ~~where the last observed occurrence of the disease was within the past ten years or where the disease infection status prior to *targeted surveillance* 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 'Disease X' 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 last two years without detection of 'Disease X'.

OR

- 4) A country that has made a *self-declaration of freedom* from 'Disease X' but in which the *disease* is subsequently detected may make a *self-declaration of freedom* from 'Disease X' again when the following conditions have been met:

Annex 8 (contd)

- 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 last two years without detection of 'Disease X'; and
- d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place ~~since for at least the past two years~~ eradication of the *disease*.

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 X.X.5.

## Article X.X.5.

**'Disease X' free zone or free compartment**

A *zone* or *compartment* within the *territory* of one or more countries not declared free from '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 *Competent Authorities* confirm that the conditions have been met.

- 1) A *zone* or *compartment* where none of the *susceptible species* is present may be declared free from 'Disease X' 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 species referred to in Article X.X.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 be declared free from 'Disease X' 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 last observed occurrence of the *disease* was within the past ten years or where the *disease infection* status prior to *targeted surveillance* 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 'Disease X' 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 last two years without detection of 'Disease X'.

OR

- 4) A zone previously declared free from 'Disease X' but in which the *disease* is detected may be declared free from 'Disease X' 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
  - c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of 'Disease X'; and
  - d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place ~~since for at least the past two years~~ eradication of the *disease*

[...]

Article X.X.7.

#### **Importation of live aquatic animals from a country, zone or compartment declared free from '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 '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.4. or X.X.5. (as applicable) and X.X.6., the place of production of the *aquatic animal* is a country, *zone* or *compartment* declared free from 'Disease X'.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11. This Article does not apply to *commodities* referred to in point 1 of Article X.X.3.

[...]

Annex 8 (contd)

## 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 '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 '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 'Disease 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.

**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 '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 '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 to products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of '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 chapters) and X.X.10. (in mollusc and crustacean chapters)

**Importation of aquatic animal products from a country, zone or compartment declared free from '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 '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.4. or X.X.5. (as applicable) and X.X.6., the place of production of the *commodity* is a country, *zone* or *compartment* declared free from 'Disease X'.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.11. This Article does not apply to *commodities* referred to in point 1 of Article X.X.3.

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## 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 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 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**

There are three stages approach is outlined in this chapter to assessing susceptibility of a species to *infection* with a specified aetiological agent:

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

- a) natural occurrence; includes all situations where *infection* has arisen without direct experimental intervention e.g. *infection* arising in wild or farmed populations;

Annex 9 (contd)

- b) non-invasive experimental procedure; 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.

Article X.X.5.

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

The aetiological 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 agent constituted 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 agent is multiplying in the host, or ~~that~~ developing ~~or latent~~ stages of the aetiological agent are present in or on the host;
- B. viable aetiological 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 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 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 *infection* in accordance with Article X.X.4.;

AND

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

AND

- 3e) there is evidence of *infection* with the aetiological 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*.

**Species for which there is incomplete evidence for susceptibility**

Where evidence exists but is insufficient to demonstrate susceptibility of a species because either transmission does not mimic natural pathways of infection, or the identity of the aetiological agent has not been confirmed, or infection is only partially supported, the information will be included in the relevant disease 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*.

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:~~

- ~~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.~~

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## 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*.

Annex 10 (contd)

- 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 transmission for SAV, *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 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 are declared countries or *zones* free from infection SAV (see Article 10.X.5.).

- 1) A country where none of the *susceptible species* is present 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 two years.

OR

- 2) A country where the species referred to in Article 10.X.2. are present but there has been no observed occurrence of infection SAV 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 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 unknown may make a *self-declaration of freedom* from infection with SAV 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 last two years without detection of infection with SAV.

OR

- 4) A country that has made a *self-declaration of freedom* from infection with SAV but in which the *disease* is subsequently detected may make a *self-declaration of freedom* from infection with SAV again when the following conditions have been met:
- a) on detection of 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 (see *Aquatic Manual*) have been completed; and

Annex 10 (contd)

- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of SAV; and
- 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 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.

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* is present 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 two years.

OR

- 2) A *zone* or *compartment* where the species referred to in Article 10.X.2. are present but there has been no observed occurrence of infection with SAV 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 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 unknown may be declared free from infection with SAV 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 last two years without detection of infection with SAV.

OR

- 4) A *zone* previously declared free from infection with SAV but in which infection with SAV is detected may be declared free from infection with SAV again when 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
  - b) infected populations have been destroyed or removed from the *infected zone* by means that minimise the *risk* of further spread of SAV, and the appropriate *disinfection* procedures (see *Aquatic Manual*) have been completed; and

Annex 10 (contd)

- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of infection with SAV; and
- 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:
  - 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.

Annex 10 (contd)

- 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 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*.

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 10 (contd)

## 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 to products authorised by the *Competent Authority*; and
- 2) water used in transport and all effluent and waste materials from the processing are treated in a manner that ensures inactivation of 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 the following *commodities* which have been prepared and packaged for retail trade and complying 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 10 (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 infectious 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.



## CHAPTER 2.3.5.

# INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

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### 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 is known to cause transient subclinical infection and has mainly been detected localised to the gills. There is evidence of 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 ISAV from HPR0 ISAV.

### 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 (Cottet *et al.*, 2011; 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.

## Annex 11 (contd)

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 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 (Kibenge *et al.*, 2009). Recent studies show that HPR0 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<sup>-2</sup> and 50 Jm<sup>-2</sup>, respectively, while the corresponding value for ISAV in wastewater from a fish-processing plant was approximately 72 Jm<sup>-2</sup>. Ozonated seawater (4 minutes with 8 mg ml<sup>-1</sup>, 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<sup>-1</sup>) 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  $\alpha$ -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 (reviewed in Kibenge *et al.*, 2004). Following experimental infection by bath immersion, ISAV has been detected by RT-PCR in herring (*Clupea harengus*) 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 (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).

## Annex 11 (contd)

### 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, 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 (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. 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- $\beta$ -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.

#### 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.

Annex 11 (contd)**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.

**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 with HPR-deleted ISAV appear lethargic and may keep close to the wall of the net pen.

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.

Annex 11 (contd)**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.

**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.

Annex 11 (contd)

- 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<sup>-1</sup>) and Naphthol AS-MX phosphate (0.2 mg ml<sup>-1</sup>) 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.

**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<sup>-1</sup>) and 2-mercaptoethanol (40 µM) (this latter may be omitted).

For virus isolation, cells grown in 25 cm<sup>2</sup> 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 cm<sup>2</sup> tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers (25 cm<sup>2</sup> 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.

Annex 11 (contd)

## 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.

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.

Annex 11 (contd)

- 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 iodid (100 µg ml<sup>-1</sup> 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

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
	Larvae	PLs	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.

Annex 11 (contd)**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 uneven spread of infection within a farm, 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, large sample sizes need to be tested at 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 or pathological changes consistent with ISA (Section 4.2) whether or not the pathological changes are associated with clinical signs of disease;
- 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 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 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

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### 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, variants of OsHV-1 (ostreid herpesvirus 1) 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.  $\mu$ Var is a microvariant ~~Ostreid herpesvirus 1  $\mu$ Var is strictly~~ defined by a 12 bp deletion in a microsatellite locus upstream of the ORF4 and additional mutations in ORF4 and ORF2/43; however, the scope of this chapter includes related variants with a deletion of around 12 base pairs in ~~this the microsatellite locus~~ region. The term OsHV-1 microvariants is used in this chapter to refer to the OsHV-1  $\mu$ Var microvariant and these related variants. 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), 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.

## Annex 12 (contd)

More recently, polymerase chain reactions (PCRs) using different primer sets and PCR product sequencing enabled the detection of a variant called  $\mu$ Var and other related variants in association with high mortality events reported in Europe, Australia and New Zealand (Lynch *et al.*, 2012; Martenot *et al.*, 2011; Peeler *et al.*, 2012; Renault *et al.*, 2012; Segarra *et al.*, 2010). 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  $\mu$ 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  $1 \times 10^5$  ml<sup>-1</sup>, and reached a maximum of  $1 \times 10^6$  ml<sup>-1</sup> 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 pg  $\mu$ l<sup>-1</sup> was detected for 16, 9 and 1 day at 4, 11 and 20°C respectively, and in a second experiment, 100 pg  $\mu$ l<sup>-1</sup> 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.

### 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).

## 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*, suminee oyster, *C. ariakensis*, European flat oyster, *O. edulis*, Manila clam, *R. philippinarum*, carpet shell clam, *R. decussates*, and scallops, *P. maximus* (Arzul *et al.*, 2001a; 2001b; Renault *et al.*, 2000). Until now, mortality attributable to OsHV-1 microvariants Ostroid herpesvirus 1  $\mu$ Var (Segarra *et al.*, 2010; Renault *et al.*, 2012) has been mainly reported as affecting in the Pacific oyster, *Crassostrea gigas* and in the Portuguese cupped oyster *Crassostrea angulata* (Batista *et al.*, pers. comm.).~~

### 2.2.2. Susceptible stages of the host

Although OsHV-1 microvariants can be detected in all oyster stages, mortality due to OsHV-1 microvariants only 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. decussatus* and *P. maximus*-*C. angulata* are naturally infected by OsHV-1 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 healthy ones.

### 2.2.4. Target organs and infected tissue

The infection-associated lesions in juveniles are mainly observed in connective tissues of all organs in which fibroblastic-like cells 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  $\text{mg}^{-1}$  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. Determining the levels of viral DNA in oysters by quantitative PCR (qPCR) might be a summation means 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 (Schikorski *et al.*, 2011a; 2011b).

### 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 can be experimentally transmitted horizontally via the water (Schikorski *et al.*, 2011a), which presumably is the main natural mode of OsHV-1 transmission.~~

Annex 12 (contd)

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  $\mu$ Var has been described by Schikorski *et al.* (2011a; 2011b). The disease can be transmitted to spat at 22°C following intramuscular injection of an extract of naturally infected oysters, and also by cohabiting injected oysters with healthy oysters. Based on 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 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, Australia, New Zealand, and Korea, but is known to occur elsewhere in the absence of oyster mortalities.

### 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 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; Seletchnik *et al.*, 1999). High seawater temperatures appear to be one of the potential factors influencing OsHV-1 infection.

Moreover, stressful conditions particularly rearing techniques seem to favour OsHV-1 infection. In France, during summer, many oyster transfers occur 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 OsHV-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

### 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 less susceptible to OsHV-1 including the variant OsHV-1 microvariants μVar 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.

Annex 12 (contd)**2.4.6. Blocking agents**

None

**2.4.7. Disinfection of eggs and larvae**

None

**2.4.8. General husbandry practices**

Biosecurity 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<sub>2</sub>SO<sub>4</sub> present in seawater may stabilise herpesviruses (Wallis & Melnick, 1965).

In controlled rearing conditions (mollusc hatchery/nursery), OsHV-1 outbreaks may therefore be controlled through quarantine and hygienic measures including virus inactivation through adapted treatments such as ultraviolet irradiation 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 or other standard histology fixatives are also acceptable. For PCR assays, samples must be preserved in 95–100% ethanol 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, 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.

Annex 12 (contd)

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

###### 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 ×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 any *OsHV-1* infected mollusc.

*Levels of validation:*

- *Specificity and sensitivity:* 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*, a positive result is presumptive evidence of *OsHV-1* 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 × 10 minutes), dehydrated in a graded series of ethanol (70%, 1 × 10 minutes; 95%, 2 × 15 minutes; 100%, 3 × 20 minutes), washed in propylene oxide (2 × 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 identification

##### 4.3.1.2.1. 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<sup>®</sup> Green real-time PCR was first developed (Pépin *et al.*, 2008). Martenot *et al.* (2010) developed an alternative protocol based on TaqMan<sup>®</sup> chemistry. The quantitation limits were 1000 and 18 UG mg<sup>-1</sup> of tissues for the Sybr<sup>®</sup> Green-based method and the TaqMan<sup>®</sup> method, respectively, and the latter protocol has a detection limit of 6 UG mg<sup>-1</sup> 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<sup>®</sup> 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.

Annex 12 (contd)

*Samples to be taken:* Live or moribund molluscs. Larvae (100–200 mg), spat (100–200 mg) or 2–3 mm<sup>2</sup> 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).

#### 4.3.1.2.3.2. OshV-1 specific Sybr<sup>®</sup> 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<sup>®</sup>) 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.*, 2001b; 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'

Annex 12 (contd)

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 ( $\geq 50$  copies  $\mu\text{l}^{-1}$ ). 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 OsHV-1 DNA is important in order to define more precisely viral strains and isolates. 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  $\mu\text{l}$  with 96-microwell plates. Each well (25  $\mu\text{l}$ ) contains 5  $\mu\text{l}$  extracted DNA dilution (sample) or OsHV-1 genomic DNA (positive control), 12.5  $\mu\text{l}$  Brilliant® SYBR® Green I PCR Master Mix or FullVelocity® Master Mix (Stratagene), 2.5  $\mu\text{l}$  each diluted primer (final concentration 200 nM) and 2.5  $\mu\text{l}$  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  $\mu\text{l}$  sample dilutions as DNA template or a viral DNA control.

Absolute quantitation of copies of OsHV-1 DNA (copies  $\mu\text{l}^{-1}$ ) 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*<sup>2</sup>) 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.

Regarding the test's sensitivity, it is considered that it can detect systematically 4 DNA copies  $\mu\text{l}^{-1}$ . 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  $5 \times 10^6$  copies  $\mu\text{l}^{-1}$ .

#### 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).

Annex 12 (contd)

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.

#### 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.

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 µg ml<sup>-1</sup> 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).

A prehybridisation step is carried out with pre-hybridisation buffer (50% formamide, 10% dextran sulfate, 4 × SSC [0.06 M Na<sub>3</sub>citrate, 0.6 NaCl, pH 7], 250 µg ml<sup>-1</sup> yeast tRNA and 10% Denhart) for 30 minutes at 42°C in a humid chamber. The prehybridisation buffer solution is replaced with 100 µl hybridisation buffer solution containing 50 µl digoxigenin-labelled probe (5 ng µl<sup>-1</sup>) and 50 µl hybridisation buffer (50% formamide, 10% dextran sulfate, 4× SSC, 250 µg ml<sup>-1</sup> 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 × 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 × PBS (1 hour at room temperature). Unbound peroxidase-conjugated antibody was removed by six washes in 1 × PBS (5 minutes). Diaminobenzidine (DAB) tetrahydrochloride was diluted in 1 × PBS (0.7 mg ml<sup>-1</sup>). The colour solution was added to tissue sections (500 µl) and incubated at room temperature in the dark for 20 minutes. The reaction was stopped with two 1 × 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 presence of intracellular viral proteins, specific OsHV-1 messenger RNA, non-structural proteins and TEM demonstrating virions within cells constitute evidence for replication, but detection of viral 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 those 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 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.

Annex 12 (contd)**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 OsHV-1 microvariants is a case of mortality of susceptible species associated with detection of OsHV-1 by PCR, qPCR, or *in-situ* hybridisation.

**7.2. Definition of confirmed case**

A confirmed case is defined as a suspect case followed by sequencing of the microsatellite locus upstream the ORF4 (Segarra *et al.*, 2010) leading to sequences consistent with the definition of microvariants.

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Annex 12 (contd)

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## AQUATIC ANIMALS COMMISSION WORK PLAN 2013–2014

### Aquatic Code:

Task	Oct 2013	February 2014	May GS 2014	Sept 2014
<b>OsHV-1 <math>\mu</math>var</b> - listed as an emerging disease		Review status as a listed emerging disease		
<b>Chapter 10.X. – Infection with salmonid alphavirus</b>	AAC developed new <i>Code</i> chapter and circulated for Member comments	Review Member comments	Propose for adoption	
<b>Chapter 6.1. – Chapter on control of hazards in feed</b>	AAC agreed on the structure of a revised chapter. AAC to develop revised chapter	Review draft chapter (developed by AAC) and circulate for Member comments		Review Member comments
<b>Chapter 6.X. – Risk analysis for antimicrobial resistance in aquaculture (new)</b>	Request report from AHG re progress	Review AHG report		
<b>Chapter X.X. – Criteria for listing susceptible species (new)</b>	Review Member comments and circulate revised text for Member comments	Review Member comments	Propose for adoption	
<b>Chapter on Evaluation of AAHS (new)</b>				Consider the development of a new chapter
<b>Revision of Section 4 to improve guidance on the control of disease</b>	Develop Concept Note for revision of this section	Review new introductory chapter for this section (to be developed by AAC)		
<b>Article 1.2.3. –Criteria for listing a disease and an emerging disease</b>	AAC proposed deletion and circulated for Member comments	Review Member comments	Propose for adoption	
<b>Chapter 1.1.</b>	AAC revised text and circulated for Member comments	Review Member comments	Propose for adoption	
<b>Glossary</b>	AAC revised definitions for emerging disease, susceptible species and veterinarian. Circulated for Member comments	Review Member comments	Propose for adoption	

Annex 13 (contd)**Aquatic Manual:**

Manual Tasks	Oct. 2013	Feb 2014	May GS 2014	Sept 2014
<b>Chapter 2.3.5. – Infection with ISAV</b>	AAC revised chapter and circulated for comments	Review Member comments	Propose for adoption	
<b>Chapter 2.4.9. – OsHV-1 <math>\mu</math>var</b>	AAC revised chapter and circulated for comments	Review Member comments	Propose for adoption	
<b>Chapter 1.1.3. – Disinfection</b>	<b>November 2013:</b> Circulate revised text for Member comments	Review Member comments	Propose for adoption	
<b>Chapter X.X.X. – Infection with salmonid alphavirus</b>	<b>November 2013:</b> Circulate new chapter for Member comments.	Review Member comments	Propose for adoption	

**Other items:**

Tasks	Oct. 2013	Feb. 2014	late 2014	early 2015
<b>OIE Global Aquatic Animal Health Conference (January 2015, TBC)</b>	Establish Scientific Committee. Develop the programme.	Finalise programme		Conference
<b>OIE Ref. Lab. Conference (7–9 October 2014)</b>	AAC to provide input into the programme and Scientific Committee.		Conference (7-9 October 2014)	

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