



Organisation  
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## ANNEX 2

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March 2013

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

Paris (France), 11–15 March 2013

#### EU comments

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

**The intended positions of the EU on the draft Aquatic Code and Manual chapters proposed for adoption at the 81<sup>st</sup> OIE General Session are inserted in the text of the report of the March meeting below, while some specific comments are inserted in the text of the respective annexes to the report.**

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

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at the OIE Headquarters in Paris from 11 to 15 March 2013.

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

The Aquatic Animals Commission thanked the following Member Countries for providing written comments: Argentina, Australia, Canada, Chile, China (People's Rep. of), Chinese Taipei, the European Union (EU), Japan, New Zealand, Norway, Russia, Switzerland, Thailand and the United States of America (USA).

The Aquatic Animals Commission reviewed comments that Member Countries had submitted by 8 February 2013 and amended texts in the OIE *Aquatic Animal Health Code* (the *Aquatic Code*) where appropriate. The amendments are shown in the usual manner by 'double underline' and '~~strikethrough~~' and may be found in the Annexes to the report. The amendments made at the March 2013 meeting are highlighted with a coloured background in order to distinguish them from those made at the September 2012 meeting.

All Member Countries' comments were considered by the Aquatic Animals Commission. However, the Commission was not able to prepare a detailed explanation of the reasons for accepting or not accepting every proposal received. However, all Member Countries' comments were considered by the Aquatic Animals Commission. Member Countries are reminded that if comments are resubmitted without modification or new justification, the Commission will not, as a rule, repeat previous explanations for decisions. The Commission encourages Member Countries to refer to previous reports when preparing comments on longstanding issues. The Commission also draws the attention of Member Countries to the reports of *ad hoc* Groups, which include important information and encourages Member Countries to review these reports together with the report of the Commission, where relevant.

The table below summarises the texts as presented in the Annexes. Member Countries should note that texts in Annexes 3 to 14 are proposed for adoption at the 81st General Session in May 2013; Annex 15 is presented for Member Countries' comments; Annexes 16 and 17 for information.

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

Comments on this report must reach OIE Headquarters by **30<sup>th</sup> August 2013** to be considered at the October 2013 meeting of the Aquatic Animals Commission.

All comments should be sent to the OIE International Trade Department at: [trade.dept@oie.int](mailto:trade.dept@oie.int).

<b>Texts proposed for adoption:</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
Criteria for listing aquatic animal diseases (Chapter 1.2.)	Annex 5
Diseases listed by the OIE (Chapter 1.3.)	Annex 6
Risk analysis (Chapters 2.1., 2.2. and 5.X.)	Annex 7
Killing for disease control purposes (Articles 7.4.2, 7.4.3. and 7.4.3.)	Annex 8
Epizootic ulcerative syndrome (Chapter 10.2)	Annex 9
Infectious salmon anaemia (Chapter 10.5.)	Annex 10
<b><i>Aquatic Manual:</i></b>	
Epizootic ulcerative syndrome (Chapter 2.3.2.)	Annex 11
Infectious salmon anaemia (Chapter 2.3.5.)	Annex 12
Viral encephalopathy and retinopathy (Chapter 2.3.11.)	Annex 13
Infection with ostreid herpesvirus-1 (Chapter 2.4.9.)	Annex 14
<b>Texts for Member Countries' comment:</b>	<b>Annex number</b>

Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new chapter X.X.)	Annex 15
<b>Annexes for Member Countries' information:</b>	
Joint FAO/OIE Aquatic Animal Health Coordination Group Meeting Report, January 2013	Annex 16
Aquatic Animal Health Standards Commission Work Plan for 2013/2014	Annex 17

## Meeting with the OIE Director General

Dr Bernard Vallat, OIE Director General, welcomed members of the Aquatic Animals Commission and thanked them for their commitment to the work of the OIE. Dr Vallat noted that along with the rapid growth in aquaculture there are also many challenges facing aquatic animal health. The work of this Commission is to ensure that the *Aquatic Code* and the *Manual of Diagnostic Tests for Aquatic Animals (Aquatic Manual)* are up to date and provide assistance to OIE Member Countries for improving their ability to respond to these challenges. He also indicated that relevant *ad hoc* Groups could be convened, on request, to support the work of the Aquatic Animals Commission.

Dr Franck Berthe, the President of the Aquatic Animals Commission, highlighted some topics of importance to the Aquatic Animals Commission. Dr Berthe noted that the issue of pathogen differentiation has been discussed for three years, initially by the *ad hoc* Group on pathogen differentiation and now in association with the amended chapter on infectious salmon anaemia (ISA), which has been circulated three times for Member Country comments. He stated that the General Session in May 2013 was of importance as the revised chapter would be proposed for adoption; he was hopeful that Member Countries' concerns had been addressed in this revised version that will be proposed. Dr Berthe noted that the criteria for listing diseases in the *Aquatic Code* differ from those in the *Terrestrial Animal Health Code (Terrestrial Code)*, with special criteria for the listing of an emerging disease. Although some Member Countries have requested the criteria for listing be aligned with those in the *Terrestrial Code*, Dr Berthe proposed that this section of the *Aquatic Code* be reviewed once the OIE has completed its current review of the issue of an emerging disease. Dr Vallat stated that it is essential that emerging diseases be reported and that the Terrestrial Animal Health Standards Commission (Code Commission) and the Scientific Commission for Animal Diseases (Scientific Commission) are undertaking a review of the definition of emerging disease. Dr Vallat agreed it is important that the Aquatic Animals Commission follow the outcome of this work and review the criteria used in the *Aquatic Code*.

Dr Berthe noted the upcoming International Conference of the OIE Reference Centres to be held in Seoul (Republic of Korea) in October 2014 and requested that at least one session focused on aquatic animals be included in the programme, as this conference provides an excellent opportunity to raise important issues with Reference Centres for aquatic animal diseases. Dr Vallat indicated his support for this request.

Dr Berthe reiterated the Aquatic Animals Commission support for a third OIE global conference on aquatic animal health. The Aquatic Animals Commission suggested that the conference include a focus on compartmentalisation and the importance of public–private partnerships. Dr Vallat supported the proposal for a conference and indicated he would seek a possible host country for this event. He encouraged the Aquatic Animals Commission to continue to develop this proposal.

## 1. OIE Aquatic Animal Health Code

The Aquatic Animals Commission emphasised that all relevant comments on a specific disease have been considered to ensure alignment between the relevant *Code* and *Manual* chapters.

### 1.1. Glossary

Comments were received from: Australia, Canada, the EU, Norway, Chinese Taipei and Thailand.

'Aquatic animal health professional'

Several Member Countries asked if it is necessary to limit the definition of Aquatic animal health professional (AAHP) to those persons carrying out tasks under the responsibility and direction of the Competent Authority (CA) as this may exclude persons working in the private sector. The Aquatic Animals Commission clarified that based on the definition of CA and Aquatic Animal Health Services (AAHS), the tasks under the responsibilities and direction of the CA include the work provided by the private sector.

The Aquatic Animals Commission proposed to delete the words ~~'is under the responsibility and direction of the Competent Authority'~~ from the proposed definition for AAHP.

'Aquatic Animal Health Services'

The Aquatic Animals Commission did not agree with a Member Country comment to maintain the term veterinary paraprofessional in the proposed amendment to the definition for AAHS as the term 'veterinary paraprofessionals' is neither used in nor a defined term in the *Aquatic Code*.

The Aquatic Animals Commission noted that should this definition be adopted, the words 'other' should be deleted from the phrase 'veterinarian or other aquatic animal health professional' in Chapters 6.3. and 6.4.

**Other definitions**

A Member Country requested that the Aquatic Animals Commission consider amending the definition of 'disease' in the *Aquatic Code*, which is defined as clinical or non-clinical infection, to the definition used in the *Terrestrial Code*, which is more consistent with common use of the term.

The Aquatic Animals Commission noted that the definition in the *Aquatic Code* had been developed to ensure that measures in the *Aquatic Code* apply to infection with the aetiological agents of listed diseases, regardless of whether there is clinical manifestation. If the definition of disease were changed to include clinical manifestation of infection only, each use of the term 'disease' would need to be checked carefully to ensure that a change to alternative terminology would not result in any consequential changes in meaning. The current definition, which has been in place since 2002, remains functional.

The Aquatic Animals Commission agreed that it would be appropriate to reconsider a change to the definition at a time when the transition of disease names in the *Aquatic Code* to 'infection with [aetiological agent]' has been completed.

The Commission wished to remind Member Countries that defined terms are always italicised when used in the *Aquatic Code*. It is necessary to check the Glossary to ensure correct understanding of the text in the *Aquatic Code*.

The revised Glossary is attached as Annex 3 for adoption at the 81<sup>st</sup> General Session in May 2013.

**EU position**

**The EU thanks the OIE for having taken some of its comments into account and in general supports the adoption of the modified glossary. A comment is inserted in the text of Annex 3.**

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

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

The Aquatic Animals Commission considered Member Country comments and also amendments proposed by the Code Commission to the equivalent Chapter 1.1. in the *Terrestrial Code*. To ensure alignment of the two *Codes*, the Aquatic Animals Commission only considered comments specific to the aquatic situation and referred comments relevant to both *Codes* to the Code Commission for

consideration at its September meeting. The Aquatic Animals Commission will review these amendments at its next meeting in October 2013.

The Aquatic Animals Commission noted the proposal of the Code Commission to amend the equivalent Article 1.1.4. in the *Terrestrial Code* by deleting the word 'compartment' in points 1, 2 and 4. The Aquatic Animals Commission proposed not to make this amendment in the *Aquatic Code* article due to differences in practical application of compartmentalisation for aquatic animals.

The revised Chapter 1.1. is attached as Annex 4 for adoption at the 81<sup>st</sup> General Session in May 2013.

### **EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter. The EU notes that several of the amendments proposed are in line with the proposed changes to the corresponding Terrestrial Code chapter, which the EU supports. Furthermore, the EU acknowledges that the changes proposed to this chapter aim at harmonising the text as far as possible with that of the relevant chapter in the Terrestrial Code, which the EU strongly supports. Specific EU comments made on the Terrestrial Code Chapter 1.1. equally apply to the Aquatic Code and are therefore repeated in the text of Annex 4 for ease of reference.**

#### **1.3. Criteria for listing aquatic animal diseases (Chapter 1.2.)**

Comments were received from Australia, Canada, Chinese Taipei, the EU, New Zealand, Norway and USA.

In response to several Member Country comments, the Aquatic Animals Commission wishes to remind Member Countries that the mechanism for listing emerging diseases has been in place in the *Aquatic Code* since 2005. The first diseases were listed under this article in 2007 and, since this time, abalone viral mortality, white tail disease and milky haemolymph of spiny lobsters have been listed under this article. Two of these diseases were listed subsequently under existing Article 1.2.1. of the *Aquatic Code* (white tail disease and infection with abalone herpes virus). Following new information about the disease, milky haemolymph disease of spiny lobsters was found not to meet the criteria for listing under the existing Article 1.2.1. and was delisted as an emerging disease.

The mechanism for listing emerging diseases was developed in response to the need to respond to the rapid emergence of new and damaging diseases of aquatic animals. This need remains. New and emerging diseases may meet several listing criteria (e.g. some elements of part A. *Consequences* and part B. *Spread*) but due to a lack of knowledge a thorough assessment cannot be made against the listing criteria under existing Article 1.2.1. By listing diseases as 'emerging', these diseases can be recognised to assist the collection of epidemiological information and improve understanding of the disease to allow a later assessment against the listing criteria under existing Article 1.2.1.

The Aquatic Animals Commission agreed that this mechanism is functional and does not propose to make an amendment to this approach at this time. The Commission reiterated that the text proposed in the new Article 1.2.1. is proposed in response to Member Country requests for the *Aquatic Code* to better describe the obligations for diseases listed under the existing Article 1.2.2.

The revised Chapter 1.2. is attached as Annex 5 for adoption at the 81<sup>st</sup> General Session in May 2013.

### **EU position**

**The EU can in principle support the adoption of this modified chapter. While the new text added at the end of the proposed new Article 1.2.1. takes into account some of the EU concerns expressed in previous EU comments, the EU's general position as to the listing of emerging diseases remains unchanged. For instance, the EU is of the opinion that article 1.2.3. is not necessary. Indeed, member countries are already obliged to notify an emerging disease as per Article 1.1.3. point 1 f), thus making the listing of**

**emerging diseases complying only with the criteria in Article 1.2.3. superfluous. Only diseases meeting the criteria of Article 1.2.2 should be listed on the OIE list. However, the EU is aware that the OIE is currently undertaking an important review of the entire concept of "emerging disease" within the context of the Terrestrial Code, a process which is very much welcomed and supported by the EU. The EU therefore suggests revisiting the concept of "emerging diseases", including the need to list them, in the Aquatic Code once that process has been completed in the Terrestrial Code.**

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

Comments were received from: Australia, Canada, Chile, China (People's Rep. of), Chinese Taipei, EU, Japan, New Zealand, Norway, Russia and USA.

##### **1.4.1. Infection with ostreid herpesvirus-1 $\mu$ var as an emerging disease**

All Member Countries commenting on this item supported the listing of this disease. The Aquatic Animals Commission agreed with a Member Country proposal not to use abbreviations in the name of the disease in this chapter and instead to use the full name, i.e. Infection with ostreid herpesvirus-1 microvariant.

The Aquatic Animals Commission clarified that as this disease is proposed for listing as an emerging disease (as per criteria for listing an emerging aquatic animal disease in the existing Article 1.2.2.), no disease-specific *Aquatic Code* chapter would be developed. However, given that an *Aquatic Manual* chapter for Infection with ostreid herpesvirus 1 (Chapter 2.4.9.) had been adopted in 2012, the Commission proposed amendments to this chapter to ensure alignment with this proposal (see also Item 3.1.1.).

Listing of infection with ostreid herpesvirus-1 microvariant, as an emerging disease, will be proposed for adoption in May 2013.

##### **1.4.2. Infectious salmon anaemia**

Several Member Countries requested clarification on the issue of reporting and notification with regard to the proposed listing of ISA virus (ISAV) (HPR0 or HPR-deleted; HPR = highly polymorphic region). The Aquatic Animals Commission clarified that, if the proposed amendment is adopted, both variants (HPR0 and HPR-deleted) would be notifiable. It also clarified that if adopted, Member Countries would be able to report on the status of both HPR0 and HPR-deleted genotypes in the World Animal Health Information System (WAHIS).

The Commission clarified that notification obligations for each of HPR0 and HPR-deleted ISAV genotypes would be in accordance with Chapter 1.1. of the *Aquatic Code*. In practice this would mean routine reporting on status in 6 monthly reports and in other circumstances meeting requirements in Chapter 1.1. for immediate notification.

In order to further clarify this obligation the Aquatic Animals Commission amended the proposed name in Article 1.3.1. to 'Infection with HPR-deleted or HPR0 infectious salmon anaemia virus'. This is also in line with the current OIE approach to name diseases on the basis of the causative agent.

Several Member Countries commented that differentiation of ISAV variants based solely on the highly polymorphic region of segment 6, is too narrow. The Aquatic Animals Commission noted that all virulent variants of ISAV have deletions in the HPR region of the HE gene and they have either an insertion or the Q266L mutation in the F gene. The Commission clarified that HPR0 and 'non-HPR0' (i.e. HPR-deleted) variants included all possible genotypes of the virus. In addition, a strong and consistent relationship between deletions in this part of the genome and virulence has been reported (*EFSA Journal*, 2012; **10** [11], 2971).

Some Member Countries commented on the risk of mutation of HPR0 ISAV. The Commission highlighted the conclusions of the Report of the *ad hoc* Group on Pathogen Differentiation for Aquatic Animal Diseases (refer to Annex XVII in the October 2011 Report of the Aquatic Animals

Commission: [http://www.oie.int/fileadmin/Home/eng/Internationa\\_Standard\\_Setting/docs/pdf/A\\_AAC\\_October\\_2011.pdf](http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/A_AAC_October_2011.pdf)), further supported by the 2012 EFSA opinion (*EFSA Journal*, 2012; **10** [11], 2971): epidemiological and historical data from isolated ISA outbreaks indicate that the risk of emergence of virulent ISAV from HPR0 is low but not negligible. The revised name 'Infection with HPR-deleted or HPR0 infectious salmon anaemia virus', in Article 1.3.1., will be proposed for adoption in May 2013.

The Aquatic Animals Commission made amendments to both the *Code* and *Manual* chapters on ISA to ensure alignment with this proposed amendment. See Items 1.9. and 3.1.2.

#### **1.4.3. Epizootic ulcerative syndrome**

The Aquatic Animals Commission noted that no additional technical information had been provided by Member Countries on the EUS assessment (against the criteria in Article 1.2.1.), that concluded that EUS should remain as an OIE listed disease (note, this assessment was provided in Annex 10 of the September 2012 Report of the Aquatic Animals Commission). The Commission noted that the proposed name change was supported by Member Countries, i.e. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome).

Canada requested that its assessment of EUS for delisting be provided to Member Countries. This assessment was provided in the September 2012 Report of the Aquatic Animals Commission (refer to Annex 3 of Annex 10 of the September 2012 report, [http://www.oie.int/fileadmin/Home/eng/Internationa\\_Standard\\_Setting/docs/pdf/Aquatic Commission/A\\_AAC\\_September\\_2012.pdf](http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Aquatic_Commission/A_AAC_September_2012.pdf))

The revised name 'Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), in Article 1.3.1., will be proposed for adoption in May 2013.

The Aquatic Animals Commission made amendments to both the *Code* and *Manual* chapters on EUS to ensure alignment with this proposed amendment. See Items 1.8. and 3.1.1.

#### **1.4.4. Infection with salmonid alphavirus (previously referred to as pancreas disease)**

Canada, New Zealand and the USA questioned the process of considering infection with salmon pancreas disease virus for listing in Chapter 1.3. The Aquatic Animals Commission noted that the proposed listing of infection with salmon pancreas disease virus was informed by an assessment carried out by an *ad hoc* Group on the OIE List of Aquatic Animal Diseases (Finfish Team) and provided to Member Countries in the March 2012 Report of the Aquatic Animals Commission (see Annex 21 [http://www.oie.int/fileadmin/Home/eng/Internationa\\_Standard\\_Setting/docs/pdf/Aquatic Commission/A\\_AAC\\_March\\_2012.pdf](http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Aquatic_Commission/A_AAC_March_2012.pdf)). This assessment supported all criteria for listing except criterion 7. In the September 2012 Report of the Aquatic Animals Commission, the Commission indicated that it had re-assessed the interpretation of the *ad hoc* Group report on this criterion and had noted that the purpose of listing diseases in the *Aquatic Code* is to assist Member Countries to prevent the transboundary spread of important diseases of aquatic animals through consistent and transparent reporting. The Commission indicated that the application of the listing criteria should be considered in this context. Therefore, criterion 7 should not be interpreted as a requirement for countries to make a self-declaration of freedom for a disease as a prerequisite to a disease being listed. Rather, there should be evidence to indicate that 'several countries may be declared free' of the disease if the surveillance principles outlined in Chapter 1.4. of the *Aquatic Code* were to be applied. The Commission considered it essential that this criterion be interpreted consistently for all diseases considered for listing. At the September 2012 meeting, the Commission concluded that information provided in the *ad hoc* Group assessment indicated that some countries may be declared free and therefore criterion 7 was satisfied. Consequently the Commission proposed infection with salmon pancreas disease virus for listing in Chapter 1.3.

The Commission also noted that no technical comments were provided by Member Countries on the proposal to list infection with salmon pancreas disease virus.

Several Member Countries suggested that the name be amended to infection with salmonid alphavirus. The Commission agreed with this proposal because it is in line with the commonly used

scientific name. The Commission noted that if adopted, it will develop chapters for the *Aquatic Code* and *Aquatic Manual* for this disease. In the interim, a Disease Card for infection with salmonid alphavirus will be uploaded onto the OIE website.

The Aquatic Animals Commission will propose the listing of infection with salmonid alphavirus for adoption in May 2013.

The revised Chapter 1.3. is attached as Annex 6 for adoption at the 81<sup>st</sup> General Session in May 2013.

#### **EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter. As regards infection with salmonid alphavirus, the EU encourages the OIE to draft chapters for the Aquatic Code and Manual with the relevant recommendations for trade, including on safe commodities, to be presented for adoption by the World Assembly as soon as possible.**

#### **1.5. Import risk analysis (Chapters 2.1. and 2.2.)**

In response to Member Country comments at the 2012 General Session to harmonise the import risk analysis chapters between the *Aquatic Code* and *Terrestrial Code*, the Aquatic Animals Commission had requested that the OIE International Trade Department review the relevant chapters in the two *Codes* and provide an amended text for its consideration. Dr Gillian Mylrea (Deputy Head, OIE International Trade Department) informed the Commission that the OIE International Trade Department had merged Chapters 2.1. (General considerations) and 2.2. (Import risk analysis) into a revised Chapter 2.1. Import risk analysis to align with the equivalent Chapter 2.1 Import risk analysis in the *Terrestrial Code*. She also presented a new Chapter 5.X. OIE Procedures relevant to the Agreement on the Application of Sanitary and Phytosanitary Measures of the World Trade Organization (SPS Agreement) to align with the equivalent horizontal chapter in the *Terrestrial Code*.

The Commission reviewed the draft chapters and agreed to propose them for adoption. It noted that the proposed text is identical to that used in the relevant chapters of the *Terrestrial Code* except for the addition of the word 'aquatic' in front of animals or animal products.

Revised Chapters 2.1., 2.2. and 5.X. are attached as Annex 7 for adoption at the 81<sup>st</sup> General Session in May 2013.

#### **EU position**

**The EU in general supports the adoption of these modified chapters as well as the adoption of this new chapter. A comment is inserted in the text of Annex 7.**

#### **1.6. Control of hazards in aquatic animal feed (Chapter 6.1.)**

The Aquatic Animals Commission considered the structure and scope of Chapter 6.1. including consideration of comments previously received from Member Countries. The Commission agreed that the text should address general principles for the control of hazards in aquatic animal feed and that biological hazards of aquatic animal health relevance in aquatic animal feed should be a strong focus of any revised chapter. Alignment with any relevant Codex Alimentarius standards on food safety should also be ensured.

The Aquatic Animals Commission will consider the structure of a revised chapter at its next meeting.

#### **EU position**

**The EU thanks the OIE and supports this approach and would support references to Codex standards in this OIE Aquatic Code chapter, if and where relevant, in line with the approach already taken in the Terrestrial Code.**



### 1.7. Killing for disease control purposes (Chapter 7.4.)

Comments were received from the USA.

In response to a Member Country comment raised at the 80<sup>th</sup> General Session in May 2012 at the time of adoption of this chapter suggesting that an alternative to the word 'pain' be used, the Aquatic Animals Commission proposed to replace the words 'pain or distress' with the word 'stress'. The Commission noted that the concept of pain in fish is subject to scientific debate; however, 'stress' is measurable and its use does not change the intent in this chapter.

The revised articles in Chapter 7.4. are attached as [Annex 8](#) for adoption at the 81<sup>st</sup> General Session in May 2013.

#### EU position

**The EU cannot support the adoption of this revised chapter at the General Session in May as current scientific knowledge on pain in fish justifies the wording of the existing chapter. Scientific findings are summarised in the text of Annex 8 together with the references.**

### 1.8. Epizootic ulcerative syndrome (Chapter 10.2.)

For consistency with the proposed name change in Chapter 1.3. (see item 1.4.3.), the Aquatic Animals Commission amended 'epizootic ulcerative syndrome' to 'infection with *Aphanomyces invadans*' throughout the chapter, where relevant.

The Aquatic Animals Commission considered a Member Country comment regarding the difficulty of achieving self-declaration of freedom under the provisions of point 2 of Article 10.2.4. where there is an absence of conditions conducive to clinical expression of infection with *A. invadans*. The Commission noted that this circumstance is only one of many that would lead to the application of this pathway for self-declaration of freedom. The Commission proposed to delete the example in this point, because it may lead to misinterpretation of this pathway.

In response to a Member Country comment, the Aquatic Animals Commission acknowledged that demonstrating freedom from a disease with a high level of confidence may be difficult in the absence of conditions conducive to its clinical expression. In conditions not conducive to clinical expression, the risks associated with the spread of a pathogen may be lower compared with epizootic conditions. The level of risk could be determined through a specific risk analysis.

Given that many species are susceptible to infection with *A. invadans* and the known host range is increasing as the disease spreads into new geographical areas, the Aquatic Animals Commission proposed to delete the words 'the species referred to in Article 10.2.2. are present but' in point 1 of Article 10.2.4. The Commission considered the text to be too restrictive for infection with *A. invadans*, because the disease is regularly reported in species that have not previously been identified as susceptible. The revised Chapter 10.2. is attached as [Annex 9](#) for adoption at the 81<sup>st</sup> General Session in May 2013.

#### EU position

**The EU supports the adoption of this modified chapter.**

### 1.9. Infectious salmon anaemia (Chapter 10.5.)

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

A Member Country commented that the three possible options for disease status proposed are only based on a narrow view of the genetic diversity and thus limit the vision of scientific research. The Aquatic Animals Commission clarified that HPR-deleted ISAV variants cover a broad genetic diversity including, for example, mutations in the F gene. The Commission reiterated that HPR0 and

'non-HPRO' (i.e. HPR-deleted) variants includes all possible genotypes of the virus. The three possible options for disease status are based on combinations of presence or absence of all genetic variants classified under HPRO and HPR-deleted forms.

Several Member Countries requested clarification on the trade implications of the proposed chapter and the ability of Member Countries to prevent the introduction of ISAV taking into consideration the three possible levels of disease status. The Commission clarified that the proposed chapter provides specific trade measures for each of HPRO ISAV and HPR-deleted ISAV. There are no measures proposed for demonstrating freedom from HPRO ISAV exclusively. This is because there is unlikely to be any practical circumstance where a country would want to declare freedom from HPRO ISAV without simultaneously declaring freedom from HPR-deleted ISAV. A Member Country questioned the need to revise the listing of fish oil and fish meal as safe commodities in Article 10.5.3. The Aquatic Animals Commission considered that there was no new information that would warrant a reassessment of fish oil and fish meal as safe commodities. The Commission reiterated that the original assessment remains valid and noted that this can be found in the October 2010 Report of the Aquatic Animals Commission (in Annex IV of Annex XVIII; <http://web.oie.int/aac/eng/FDC%20reports/Oct%202010%20%28English%29.pdf>).

Several Member Countries questioned the rationale for point 1 in Article 10.5.4.: 'A country where none of the susceptible species is present may make a self-declaration of freedom from ISA when basic biosecurity conditions have been continuously met in the country for at least the past two years.' The Aquatic Animals Commission wished to clarify that aquaculture is a very dynamic sector and that there are situations in which a country may wish to apply this pathway, for example, when starting a new aquaculture production industry using a species exotic to the country.

A Member Country requested clarification on risk related to vertical transmission of ISAV in relation to Article 10.5.17. The Aquatic Animals Commission noted that an article on disinfection of eggs had been included in chapters on ISA, VHS and IHN. For these three diseases, egg surface-associated transmission has been demonstrated, but no true vertical transmission is known. Details of these assessments were provided in the March 2009 Report of the Aquatic Animals Commission (Annex V of Annex XVI :

[http://www.oie.int/fileadmin/Home/eng/International\\_Standard\\_Setting/docs/pdf/Mar2009\\_English\\_.pdf](http://www.oie.int/fileadmin/Home/eng/International_Standard_Setting/docs/pdf/Mar2009_English_.pdf)). To the knowledge of the Commission there is no new evidence that would warrant reassessment.

The revised Chapter 10.5. is attached as Annex 10 for adoption at the 81<sup>st</sup> General Session in May 2013.

## **EU position**

**The EU thanks the OIE and in general supports the adoption of this modified chapter. Comments are inserted in the text of Annex 10.**

## **2. Other Tasks**

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

At the September 2012 meeting, the Aquatic Animals Commission agreed that a new chapter on Criteria for determining susceptibility of aquatic animals to specific pathogenic agents should be included in the *Aquatic Code* to ensure consistency in the way susceptible species are listing in *Code* and *Manual* chapters.

The Aquatic Animals Commission developed a new draft Chapter X.X. 'Criteria for determining susceptibility of aquatic animals to specific pathogenic agents' for the *Aquatic Code* based on the criteria developed by the OIE *ad hoc* Group on Assessing the Criteria for Listing Aquatic Animal Species as Susceptible to Infection with a Specific Pathogen (provided in the March 2012 Report of the Aquatic Animals Commission in Annex 23:

<http://web.oie.int/aac/eng/FDC%20reports/Oct%202010%20%28English%29.pdf>)

The new Chapter X.X. is attached as Annex 15 for Member Countries' comments.

**EU comments**

**The EU commends the OIE Aquatic Animals Commission and in general supports this new chapter. Specific comments are inserted in the text of Annex 15.**

**3. Manual of Diagnostic Tests for Aquatic Animals**

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

The Aquatic Animals Commission wished to thank authors of the revised *Aquatic Manual* chapters and the disease card for Infection with salmonid alphavirus for their work.

**3.1. Review of the draft *Aquatic Manual* chapters****3.1.1. Epizootic ulcerative syndrome (Chapter 2.3.2.)**

The Aquatic Animals Commission revised the *Aquatic Manual* chapter for Epizootic ulcerative syndrome (Chapter 2.3.2.) to ensure consistency with the proposed amended name for this disease (see Items 1.8. and 1.4.3.).

The revised Chapter 2.3.2. is attached as [Annex 11](#) for adoption at the 81<sup>st</sup> General Session in May 2013.

**EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter.**

**3.1.2. Infectious salmon anaemia (Chapter 2.3.5.)**

The Aquatic Animals Commission reviewed the revised *Aquatic Manual* chapter for Infectious salmon anaemia (Chapter 2.3.5.) provided by the author and made amendments as appropriate to ensure consistency with the amended *Aquatic Code* chapters (see Items 1.4.2. and 1.9.).

The revised Chapter 2.3.5. is attached as [Annex 12](#) for adoption at the 81<sup>st</sup> General Session in May 2013.

**EU position**

**The EU thanks the OIE and in general supports the adoption of this modified chapter. Specific comments are inserted in the text of Annex 12.**

**3.1.3. Viral encephalopathy and retinopathy (Chapter 2.3.11.)**

The Aquatic Animals Commission reviewed the revised *Aquatic Manual* chapter for Viral encephalopathy and retinopathy (Chapter 2.3.11.) provided by the authors. The Aquatic Animals Commission noted that this is a de-listed disease, but is included in the *Manual* to provide information for Member Countries.

The revised Chapter 2.3.11. is attached as [Annex 13](#) for adoption at the 81<sup>st</sup> General Session in May 2013.

**EU position**

**The EU supports the adoption of this modified chapter.**

**3.1.4. Infection with ostreid herpesvirus-1 (Chapter 2.4.9.)**

The Aquatic Animals Commission reviewed the revised *Aquatic Manual* chapter for Infection with ostreid herpesvirus-1 (Chapter 2.4.9.) provided by the author and made amendments as

appropriate to ensure consistency with the proposed listing of infection with ostreid herpesvirus-1 microvariant in the *Aquatic Code* (see Item 1.4.1.).

The revised Chapter 2.4.9. is attached as Annex 14 for adoption at the 81<sup>st</sup> General Session in May 2013.

### **EU position**

**The EU thanks the OIE and in general supports the adoption of this modified chapter.**

**The EU notes that the revised title of the chapter indicates that this chapter deals with OsHV1- microvariant, the strain of OsHV1 which has caused significant mortality in *C. gigas* in both the northern and southern hemisphere since 2007. The text which has been presented however, has references to both the microvariant and the reference strain throughout, making it difficult to determine in places, which characteristics refer to the microvariant, which is the subject of the chapter.**

**Furthermore, the EU notes that the abbreviation "µVar" is still used in some places in the text of this chapter. For reasons of consistency, the EU suggests the word "microvariant" be used throughout the text instead of the abbreviation.**

**Specific comments are inserted in the text of Annex 14.**

#### **3.2. OIE Disease card for infection with salmonid alphavirus**

The Aquatic Animals Commission reviewed the disease card for infection with salmonid alphavirus and requested that it be uploaded onto the OIE website (see Item 1.4.4.).

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

The Aquatic Animals Commission reviewed the three disease-specific guidance documents on surveillance for a fish, a mollusc and a crustacean disease. The Commission agreed that the documents contained valuable information for Member Countries and proposed that they be made public on the OIE website. The Commission suggested that an external editor be engaged to ensure that the three documents are in line with the *Aquatic Manual* and *Aquatic Code*

### **4. OIE Reference Centres**

#### **4.1. Update on procedures: Resolutions**

The Aquatic Animals Commission was informed that the OIE Council would present a Resolution at the 81<sup>st</sup> General Session in May 2013 that would formalise the role of the OIE Delegate in the procedure to replace experts at OIE Reference Laboratories. Under the proposed procedure, nominations would receive final approval by the Council at one of its three annual meetings. This would streamline the process so that any new experts could be approved and the on-line list of OIE Reference Centres updated after each Council meeting rather than waiting for approval at the General Session in May of each year. As the Commission's meetings often take place after Council's meetings, the Commission members agreed to review any nominations received electronically and provide their endorsement of a nominee to the Council in time for the latter's meeting. The Council would also present Resolutions for both the designation and the withdrawal of Reference Centres at the General Session in May 2013.

#### **4.2. New applications for OIE Reference Centre status**

No applications had been received.

#### **4.3. Review nominations for replacement experts**

The Aquatic Animals Commission reviewed the following nomination for a replacement expert at an OIE Reference Laboratory and recommended his acceptance:

*Infectious salmon anaemia*

Dr Knut Falk will replace Dr Birgit Dannevig at the National Veterinary Institute, Sentrum, Oslo, Norway.

This nomination will be proposed for adoption at the 81<sup>st</sup> General Session in May 2013.

The OIE was saddened to learn that Dr Birgit Dannevig died in September 2012. She had been the OIE Designated Expert at the Reference Laboratory since its designation in 1999. Dr Dannevig had always been very helpful, cooperative and conscientious; her contribution was highly valued.

#### **4.4. Review of annual reports on Reference Centre activities in 2012**

Ms Min-Kyung Park (Intern, OIE Scientific and Technical Department) joined the meeting to present an analysis of the activities of the Reference Laboratories and Collaborating Centres for aquatic animal diseases. The Commission was reminded that the new annual report template had been used by the Reference Laboratories for the first time. An electronic version of the template should be available for the 2013 reports.

The Commission noted that the percentage of laboratories that did not maintain a quality management system that had been certified according to international standards was 55% and requested that these laboratories be contacted to find out what type of quality system they have in place. Low levels of international networking and low participation in inter-laboratory proficiency testing were also recognised.

The Commission believed that the report indicated important findings that should be addressed at the third International Conference of the OIE Reference Centres to be held in 2014.

#### **4.5. International Conference of the OIE Reference Centres, Seoul (Republic of Korea, 2014)**

The Aquatic Animals Commission noted that the third International Conference of the OIE Reference Centres will be held in Seoul (Rep. of Korea) from 7 to 9 October 2014. The Commission strongly supports the Conference and requested that one of its members be invited to participate in the Conference's Scientific Committee. The Commission recommended that there be a session to focus on issues relevant to aquatic animals.

### **5. OIE Procedure for Registration of Diagnostic Kits: review of applications**

Dr François Diaz joined the meeting for this agenda item and updated the Aquatic Animals Commission on the current status of the dossier on "IQ Plus<sup>TM</sup> WSSV DNA Kit" submitted according to the OIE Procedure for Registration of Diagnostic Kits.

He informed the Commission that the evaluation of this dossier had been completed and a final report had been received from the panel of experts with a few remaining questions to the applicant. Based on this final report and in the expectation that the applicant will reply satisfactorily and on time to these final questions, the Commission was favourable to the proposal to include this diagnostic kit in the OIE register with the following purposes:

IQ Plus<sup>TM</sup> WSSV DNA Kit is fit for the diagnosis of white spot disease in muscle-containing tissues of *Litopenaeus vannamei* and for the following purposes:

1. To certify freedom from infection (<20 virions/sample) in individual animals or products for trade/movement purposes;
2. To confirm diagnosis of suspect or clinical cases (confirmation of a diagnosis by histopathology or clinical signs);
3. To estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control).

### **6. OIE List of antimicrobial agents of veterinary importance**

Dr Diaz informed the Aquatic Animals Commission that the *ad hoc* Group on Antimicrobial Resistance had updated the OIE List of antimicrobial agents of veterinary importance during their January 2013 meeting. He noted that the List also includes antimicrobial agents used in aquatic animals. Dr Diaz informed the Commission that the amended List had been endorsed by the Scientific Commission and would be proposed for adoption at the 81<sup>st</sup> General Session by Resolution. The Commission had no comments and endorsed the List.

## 7. Cooperation with FAO

### 6.1. Joint FAO/OIE Aquatic Animal Health Coordination Group Meeting Report, January 2013

Dr Berthe informed the Aquatic Animals Commission that the Joint FAO/OIE Aquatic Animal Health Coordination Group (JAAHCG) had been convened following an agreement between Dr Vallat and the FAO Director General, Dr Jose Graziano da Silva, to renew institutional commitments to jointly address mutual issues on aquatic animal health and aquaculture. The JAAHCG held its first meeting in January 2013 to develop the terms of reference and a work plan for this group. In addition, the JAAHCG reviewed the FAO/OIE Chart and Vade Mecum (that describes FAO and OIE competencies and complementarities in the field of animal health) and agreed that the complementarities and synergies in the FAO/OIE Chart apply equally to aquatic animal health. The JAAHCG proposed that the Chart be amended to include aquatic animal health.

The report of the JAAHCG is presented at [Annex 16](#) for Member Country information.

## 8. 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 upcoming activities.

The detailed Commission's Work Plan is presented in [Annex 17](#).

### EU comments

**The EU thanks the Aquatic Animals Commission for providing this updated work plan and for having taken its comment as to the need for concurrent work on relevant Aquatic Code and Manual chapters into account, and supports the work plan outlined in Annex 17.**

## 9. Other business

### 8.1. User's Guide

Dr Mylrea informed the Aquatic Animals Commission of the work being undertaken by Dr Etienne Bonbon to revise the User's guide in the *Terrestrial Code* with a view to addressing Member Country requests for clarification on the role, scope and correct use of the *Terrestrial Code*. The Commission agreed that the User's Guide for the *Aquatic Code* should be revised to clarify the role, scope and correct use of the *Aquatic Code*.

### 8.2. 'Standards' versus 'guidelines and recommendations'

Dr Mylrea reported on discussions held during the February 2013 meeting of the Code Commission where it was noted that there had been confusion among Member Countries regarding the terms 'standards', 'guidelines' and 'recommendations'. While recognising that the Agreement on the Application of Sanitary and Phytosanitary Measures of the World Trade Organization does not distinguish between these terms, the Code Commission considers that there should be clear differentiation of how they are used for OIE texts: 'standards' should mean any texts that have been subjected to the official procedure of the OIE for adoption by the World Assembly of Delegates, and thus are found in *Codes* and *Manuals*, while 'guidelines' and 'recommendations' are used for other texts. The Aquatic Animals Commission noted this approach.

### 8.3. Acute hepatopancreatic necrosis syndrome

The Aquatic Animals Commission discussed acute hepatopancreatic necrosis syndrome (AHPNS), a disease syndrome in shrimp aquaculture in China (People's Republic of), Malaysia, Thailand and Vietnam. This disease syndrome has had significant impact on shrimp production in some affected countries. The aetiological agent has not been identified.

The Commission noted that the disease has been included in the list of diseases for reporting through the NACA/FAO/OIE quarterly aquatic animal disease reporting system (for the Asia and Pacific region) commencing 1<sup>st</sup> January 2013.

The Network of Aquaculture Centres in the Asia-Pacific (NACA) disease card and other information on AHPNS is available on the NACA website:

<http://www.enaca.org/publications/health/disease-cards/ahpns-disease-advisory-update-march-2013.pdf>

## 10. Next meeting

The Aquatic Animals Commission proposed to hold their next meeting from 30<sup>th</sup> September to 4<sup>th</sup> October 2013.

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.../Annexes





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

**Paris, 11–15 March 2013**

**List of participants**

**MEMBERS OF THE COMMISSION**

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**Dr Franck Berthe**

*President*  
Senior Scientific Officer  
European Food Safety Authority - EFSA  
Head of Animal Health and Animal Welfare  
Unit  
Via Carlo Magno 1, Parma  
ITALY  
Tel.: + 39 052 1 036 870  
Fax: + 39 052 1 036 0870  
Franck.Berthe@efsa.europa.eu

**Dr Jie Huang**

*Vice President*  
Maricultural Organism Diseases Control &  
Molecular Pathology Laboratory,  
Yellow Sea Fisheries Research Institute,  
Chinese Academy of Fishery Sciences  
106 Nanjing Road  
Qingdao, SD 266071  
PR CHINA  
Tel.: +86 532 582 3062  
Fax: +86-532-5811514  
huangjie@ysfri.ac.cn

**Dr Victor Manuel Vidal Martínez**

*Vice President*  
Centro de Investigación y de  
Estudios Avanzados del Instituto  
Politécnico Nacional  
Carretera Antigua a Progreso Km. 6  
Apartado Postal 73 Cordemex  
Mérida,  
Yucatán C.P. 97310  
MÉXICO  
Tel: +52 99 99 42 94 72  
Fax: +52 99 81 29 17  
vvidal@mda.cinvestav.mx

**Dr Ingo Ernst**

Director, Aquatic Animal Health  
Animal Health Policy Branch  
Australian Government Department of  
Agriculture, Fisheries and Forestry  
GPO Box 858  
Canberra ACT 2601  
Tel: 02 627 256 15  
Fax: 02 627 231 50  
ingo.ernst@daff.gov.au

**Dr Brit Hjeltnes**

Deputy Director, Fish and Shellfish Health  
National Veterinary Institute  
PO Box 750 Sentrum, N-0106  
Bergen  
NORWAY  
Tel.: +47 918 893 76  
brit.hjeltnes@vetinst.no

**Dr Alicia Gallardo Lagno**

Jefa Unidad de Salud Animal  
Servicio Nacional de Pesca y  
Acuicultura  
Calle Victoria 2832  
CHILE  
Tel.: +56 32 281 9282  
agallardol@sernapesca.cl

**OTHER PARTICIPANTS**

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**Dr Melba B. Reantaso**

Aquaculture Officer  
Aquaculture Service (FIRA)  
Fisheries and Aquaculture Resources Use and  
Conservation Division (FIM)  
Fisheries and Aquaculture Department  
Food and Agriculture Organization of the  
United Nations (FAO)  
Viale Terme di Caracalla, 00153, Rome, ITALY  
Phone: + 39 06 570 54843; Fax: + 39 06 570  
53020  
Melba.Reantaso@fao.org

Annex 1 (contd)**OIE HEADQUARTERS**

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**Dr Bernard Vallat**  
Director General  
OIE  
12, rue de Prony  
75017 Paris  
FRANCE  
Tel.: 33 - (0)1 44 15 18 88  
Fax: 33 - (0)1 42 67 09 87  
oie@oie.int

**Dr Derek Belton**  
Head  
International Trade Department  
OIE  
d.belton@oie.int

**Ms Sara Linnane**  
Scientific editor  
Scientific and Technical Department  
OIE  
s.linnane@oie.int

**Dr Gillian Mylrea**  
Deputy Head  
International Trade Department  
OIE  
g.mylrea@oie.int

UNOFFICIAL VERSION

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

**Paris, 11–15 March 2013**

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**Adopted agenda**

Meeting with the OIE Director General

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 aquatic animal diseases (Chapter 1.2.)
  - 1.4. Diseases listed by the OIE (Chapter 1.3.)
    - 1.4.1. Infection with ostreid herpesvirus-1  $\mu$ var as an emerging disease
    - 1.4.2. Infectious salmon anaemia
    - 1.4.3. Epizootic ulcerative syndrome
    - 1.4.4. Infection with salmonid alphavirus (previously referred to as pancreas disease)
  - 1.5. Import risk analysis (Chapters 2.1. and 2.2.)
  - 1.6. Control of hazards in aquatic animal feeds (Chapter 6.1.)
  - 1.7. Killing for disease control purposes (Chapter 7.4.)
  - 1.8. Epizootic ulcerative syndrome (Chapter 10.2.)
  - 1.9. Infectious salmon anaemia (Chapter 10.5.)
2. Other Tasks
  - 2.1. Criteria for determining susceptibility of aquatic animals to specific pathogenic agents (new chapter)
3. *Manual of Diagnostic Tests for Aquatic Animals*
  - 3.1. Review of the draft *Manual* chapters:
    - 3.1.1. Epizootic ulcerative syndrome (Chapter 2.3.2.)
    - 3.1.2. Infectious salmon anaemia (Chapter 2.3.5.)
    - 3.1.3. Viral encephalopathy and retinopathy (Chapter 2.3.11.)

Annex 2 (contd)

- 3.1.4. Infection with ostreid herpesvirus-1 (Chapter 2.4.9.)
- 3.2. OIE Disease Card for Infection with salmonid alphavirus
- 3.3. Disease specific guidance documents on surveillance for a fish, a mollusc and a crustacean disease
4. OIE Reference Centres
  - 4.1. Update on procedures: Resolutions
  - 4.2. New applications for OIE Reference Centre status
  - 4.3. Review nominations for replacement experts
  - 4.4. Review of annual reports on Reference Centre activities in 2012
  - 4.5. International Conference of the OIE Reference Centres, Seoul, Korea (Rep. of), 2014
5. OIE Procedure for registration of diagnostic kits: review of applications
6. OIE List of antimicrobial agents of veterinary importance
7. Cooperation with FAO
  - 7.1. Joint FAO/OIE Aquatic Animal Health Coordination Group Meeting Report, January 2013
8. Aquatic Animals Commission Work Plan 2013
9. Other business
  - 9.1. User's Guide
  - 9.2. 'Standards' versus 'guidelines and recommendations'
  - 9.3. Acute hepatopancreatic necrosis syndrome
10. Next meeting

## GLOSSARY

### EU position

**The EU thanks the OIE for having taken some of its comments into account and in general supports the adoption of the modified glossary. A comment is inserted in the text below.**

#### **Aquatic animal health professional**

means a person who, for the purposes of the *Aquatic Animal Health Code*, is authorised by the *Competent Authority* to carry out certain designated tasks in a *territory*, ~~is under the responsibility and direction of the *Competent Authority*~~ and has the appropriate qualifications and training to perform the designated tasks.

#### ***Aquatic Animal Health Services***

means the governmental and non-governmental organisations that implement animal health and welfare measures and other standards and recommendations in the *Aquatic Code* in the territory. The Aquatic Animal Health Services are under the overall control and direction of the *Competent Authority*. Private sector organisations, *veterinarians*, or aquatic animal health professionals ~~or veterinary paraprofessionals~~ are normally accredited or approved by the *Competent Authority* to deliver the delegated functions.

### EU comment

**The EU in general supports the above modified definition. However, the term "aquatic animal health professionals" should be italicised, if the new definition proposed for that term be adopted at the General Session in May, to refer to that new glossary definition.**

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— Text deleted



## CHAPTER 1.1.

NOTIFICATION OF DISEASES AND  
EPIDEMIOLOGICAL INFORMATION**EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter. The EU notes that several of the amendments proposed are in line with the proposed changes to the corresponding Terrestrial Code chapter, which the EU supports. Furthermore, the EU acknowledges that the changes proposed to this chapter aim at harmonising the text as far as possible with that of the relevant chapter in the Terrestrial Code, which the EU strongly supports. Specific EU comments made on the Terrestrial Code Chapter 1.1. equally apply to the Aquatic Code and are therefore repeated in the text below for ease of reference.**

## Article 1.1.1.

For the purposes of the *Aquatic Code* and in terms of Articles 1.1.5, 1.1.9 and 1.1.10 of the *OIE Organic Statutes*, every Member of the OIE shall recognise the right of the *Headquarters* to communicate directly with the Veterinary Competent Authority of its *territory or territories*.

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

## Article 1.1.2.

- 1) ~~Countries~~ Members shall make available to other ~~countries~~ Members, through the OIE, whatever information is necessary to minimise the spread of important aquatic animal diseases of aquatic animals and their aetiological pathogenic agents and to assist in achieving better world-wide control of these *diseases*.
- 2) To achieve this, ~~countries~~ Members shall comply with the ~~reporting~~ 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 diseases is constantly ~~evolving~~ developing and that the presence of an infectious agent does not necessarily imply the presence of a clinical disease, ~~countries~~ Members shall ensure through their reports that they comply with the spirit and intention of paragraph point 1 above. This means that the presence detection of an infectious agent of a listed disease in an aquatic animal, should be reported, even in the absence of ~~clinical disease signs of disease, should be reported~~.
- 5) In addition to notifying findings in accordance with Article 1.1.3., ~~countries~~ Members 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 ~~that 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 ~~described~~ specified.

## Article 1.1.3.

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

- 1) in accordance with relevant provisions in the *disease* specific chapters, **immediate 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) ~~for diseases listed by the OIE, the first occurrence or re-occurrence of a disease in a country or zone or compartment of the country, if the country or zone or compartment of the country was previously considered to be free of that particular disease; or~~
  - b) ~~for diseases listed by the OIE, if the disease has occurred in a new host species; or~~
  - c) ~~for diseases listed by the OIE, if the disease has occurred with a new pathogen strain or in a new disease manifestation; or~~
  - d) ~~for diseases listed by the OIE, if the disease has a newly recognised zoonotic potential; or~~
  - e) ~~for diseases not listed by the OIE, if there is a case of an emerging disease or pathogenic agent should there be findings that are of epidemiological significance to other countries.~~
- 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 report that declared an 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.
  - d) a sudden and unexpected increase in the distribution, incidence, morbidity or mortality of a listed disease prevalent within a country, a zone or a compartment.
  - e) evidence of change in the epidemiology of a listed disease (including host range, pathogenicity, strain) in particular if there is a zoonotic impact;
  - f) an emerging disease or the pathogenic agent with significant morbidity or mortality, or zoonotic potential.

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

- 2) Weekly reports subsequent to a *notification* under ~~paragraph point 1~~ above, to provide further information on the evolution of ~~an the event~~ incident that ~~which~~ justified ~~immediate the~~ **immediate 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 ~~country~~ Member to the OIE; in ~~each any~~ case, a final report on the ~~event~~ incident should be submitted.
- 3) Six-monthly reports on the absence or presence and evolution of ~~diseases listed diseases by the OIE~~, and information of epidemiological significance to other ~~countries~~ Members ~~with respect to diseases that are not listed.~~
- 4) ~~An~~ Annual questionnaire reports concerning any other information of significance to other ~~countries~~ Members.

Although Members 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.

### EU comment

**The EU strongly supports this additional sentence, which is in line with its previous general comment on the need to clearly differentiate between the mandatory notification of listed or emerging diseases, and information on other animal health events provided to the OIE on a voluntary basis. Such a clear distinction that would be based on the case definitions of the disease specific chapters of the Aquatic Code where they already exist also needs to be made in WAHID, including in its disease maps. This would ultimately**



**increase Member Countries' willingness to share such voluntary information with the OIE.**

Article 1.1.4.

- 1) The Veterinary 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* of a for a particular disease shall be considered as such until a period exceeding the known infective period for the disease in question specified in the Aquatic Code has elapsed after the last reported case, outbreak and when full prophylactic and appropriate sanitary animal health measures have been applied to prevent possible reappearance or spread of the *disease*. These measures will be found in detail in the various chapters of Section 8 to 11 of the Aquatic Code.
- 3) A Member may be considered to regain freedom again declare itself free (i.e. self-declaration of freedom from disease) from a specific *disease* when it complies with all the relevant conditions given in the corresponding relevant chapters of Section 8 to 11 of the Aquatic Code have been fulfilled.
- 4) ~~The *Veterinary Authority* of a Country in which one or more *free zones* or *compartments* have been established may wish to inform the *Headquarters*, giving necessary particulars, of the *zones* or *compartments* and describing their location (e.g. by a map or other precise locators such as GPS [Global Positioning System] co ordinates). The *Headquarters* may publish this information. The Veterinary Competent Authority of a Country Member in which sets up one or more several free zones or compartments have been established may wish to shall inform the *Headquarters*, giving necessary particulars details, of the *zones* or *compartments* and describing their location (e.g. by a map or other precise locators such as GPS [Global Positioning System] co ordinates). The *Headquarters* may publish this information, 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.~~

~~Article 1.1.5.~~ Article 1.1.5.

- ~~1. The *Headquarters* shall~~ The *Headquarters* shall communicate send by fax, e-mail or e-mail or World Animal Health Information Database ~~Disease Information to to the *Veterinary Authorities*~~ Competent Authorities concerned, ~~all notifications received as provided in Article 1.1.2. to 1.1.4.~~ all notifications received as provided in Articles 1.1.2. to 1.1.4. and other relevant information.

**EU comment**

**The EU supports the amendments proposed in Article 1.1.5., including the proposed new text on "other relevant information", understanding the OIE will make a clear distinction between mandatory notification and voluntary information when communicating to Veterinary Authorities (cf. EU comment above).**

- ~~2) The *Headquarters* shall notify Members through *Disease Information* of any event of exceptional epidemiological significance reported by a Member.~~

— Text deleted



## CHAPTER 1.2.

CRITERIA FOR LISTING  
AQUATIC ANIMAL DISEASES**EU position**

The EU can in principle support the adoption of this modified chapter. While the new text added at the end of the proposed new Article 1.2.1. takes into account some of the EU concerns expressed in previous EU comments, the EU's general position as to the listing of emerging diseases remains unchanged. For instance, the EU is of the opinion that article 1.2.3. is not necessary. Indeed, member countries are already obliged to notify an emerging disease as per Article 1.1.3. point 1 f), thus making the listing of emerging diseases complying only with the criteria in Article 1.2.3. superfluous. Only diseases meeting the criteria of Article 1.2.2 should be listed on the OIE list. However, the EU is aware that the OIE is currently undertaking an important review of the entire concept of "emerging disease" within the context of the Terrestrial Code, a process which is very much welcomed and supported by the EU. The EU therefore suggests revisiting the concept of "emerging diseases", including the need to list them, in the Aquatic Code once that process has been completed in the Terrestrial Code.

Article 1.2.1.Introduction

This chapter describes the criteria for listing diseases in Chapter 1.3 of the Aquatic Code. The objective of listing is to support Members' 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 highlight emerging diseases and collect relevant epidemiological information to improve understanding of an emerging disease. 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. Members 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 1.2.1. 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

No.		Criteria for listing	Explanatory notes
			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.	Wild aquatic animal populations can be populations that are commercially harvested (wild fisheries) and hence are an economic asset. However, the asset could be ecological or environmental in nature, for example, if the population consists of an endangered species of aquatic animal or an aquatic animal potentially endangered by the disease.
3.	Or	The agent is of public health concern.	
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.

## Annex 5 (contd)

No.	Criteria for listing	Explanatory notes
<b>And B. Spread</b>		
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. of the <i>Aquatic Code</i> .	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 ~~1.2.2.~~ 1.2.3.**Criteria for listing an emerging aquatic animal disease**

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

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

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 — Text deleted



## CHAPTER 1.3.

## DISEASES LISTED BY THE OIE

**EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter. As regards infection with salmonid alphavirus, the EU encourages the OIE to draft chapters for the Aquatic Code and Manual with the relevant recommendations for trade, including on safe commodities, to be presented for adoption by the World Assembly as soon as possible.**

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

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

## Article 1.3.1.

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

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

## Article 1.3.2.

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

- Infection with abalone herpes virus
- Infection with *Bonamia ostreae*
- Infection with *Bonamia exitiosa*
- Infection with *Marteilia refringens*
- Infection with *Perkinsus marinus*
- Infection with *Perkinsus olseni*
- Infection with *Xenohaliotis californiensis*
- Infection with ostreid herpesvirus-1 microvariant *μvar* (OsHV-1 *μvar*)<sup>1</sup>.

## Article 1.3.3.

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

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

## Article 1.3.4.

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

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

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- Text deleted

<sup>1</sup> Listed according to Article 1.2.3.



## CHAPTER 2.1.

**GENERAL CONSIDERATIONS**  
**IMPORT RISK ANALYSIS****EU position**

**The EU in general supports the adoption of this modified chapter. A comment is inserted in the text below.**

Article 2.1.1.

**Introduction**

The importation of *aquatic animals* and aquatic animal products, ~~whether of aquatic or terrestrial origin,~~ involves a degree of *disease risk* to the *importing country*. This ~~risk, which may be to humans or animals,~~ may be represented by one or several *diseases or infections* ~~not present in the importing country.~~

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

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

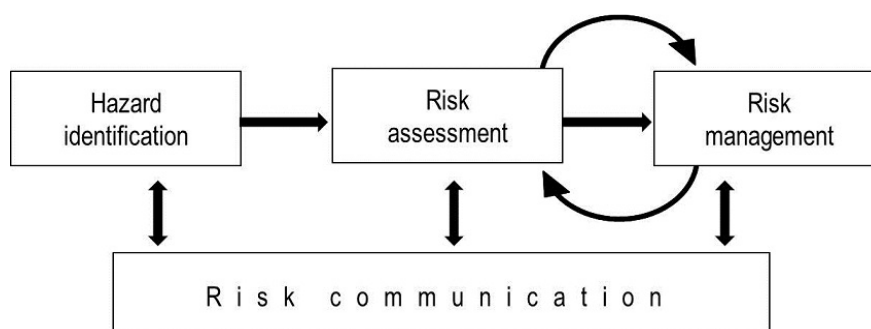
This chapter outlines the role of the OIE with respect to the Agreement on the Application of Sanitary and Phytosanitary Measures (the so-called SPS Agreement) of the World Trade Organization (WTO) and describes the OIE procedure for settlement of disputes.

**EU comment**

**The EU suggests removing the paragraph above, since the SPS agreement is now discussed in a proposed new chapter (5.X.).**

~~This Chapter 2-2~~ provides recommendations and principles for conducting transparent, objective and defensible *risk analyses* for *international trade*. However, it cannot provide details on the means by which a *risk analysis* is carried out as the purpose of the *Aquatic Code* is simply to outline the necessary basic steps. The components of *risk analysis* described in ~~this Chapter 2-2~~ are *hazard identification*, *risk assessment*, *risk management* and *risk communication* (Figure 1).

Fig. 1. The four components of risk analysis



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

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

~~Article 2.1.2.~~

~~The Agreement on the Application of Sanitary and Phytosanitary Measures and role and responsibility of the OIE~~

~~The SPS Agreement encourages WTO Members to base their *sanitary measures* on international standards, guidelines and recommendations, where they exist. Members may choose to adopt a higher level of protection than that provided by international texts if there is a scientific justification or if the level of protection provided by the relevant international texts is considered to be inappropriate. In such circumstances, Members are subject to obligations relating to *risk assessment* and to a consistent approach to *risk management*.~~

~~The SPS Agreement encourages Governments to make a wider use of *risk analysis*: WTO Members shall undertake an assessment as appropriate to the circumstances of the actual *risk* involved.~~

~~The SPS Agreement, in Article 7, obliges WTO Members to notify changes in, and provide relevant information on, sanitary measures which may, directly or indirectly, affect international trade.~~

~~The SPS Agreement recognises the OIE as the relevant international organisation responsible for the development and promotion of international animal health standards, guidelines, and recommendations affecting trade in live animals and animal products, whether aquatic or terrestrial in origin.~~

~~Article 2.1.3.~~

~~The OIE in-house procedure for settlement of disputes~~

~~The OIE shall maintain its existing voluntary in-house mechanisms for assisting Members to resolve differences. In-house procedures that will apply are that:~~

- ~~1. Both parties agree to give the OIE a mandate to assist them in resolving their differences.~~
- ~~2. If considered appropriate, the Director General of the OIE recommends an expert, or experts, and a chairman, as requested, agreed by both parties.~~
- ~~3. Both parties agree on the terms of reference and working programme, and to meet all expenses incurred by the OIE.~~
- ~~4. The expert or experts are entitled to seek clarification of any of the information and data provided by either country in the assessment or consultation processes, or to request additional information or data from either country.~~
- ~~5. The expert or experts should submit a confidential report to the Director General, who will transmit it to both parties.~~

~~CHAPTER 2.2.~~  
**IMPORT RISK ANALYSIS**

~~Article 2.2.1.~~

**Introduction**

An import *risk analysis* begins with a description of the *commodity* proposed for import and the likely annual quantity of trade. It should be recognised that whilst an accurate estimate of the anticipated quantity of trade is desirable to incorporate into the *risk* estimate, it may not be readily available, particularly where such trade is new.

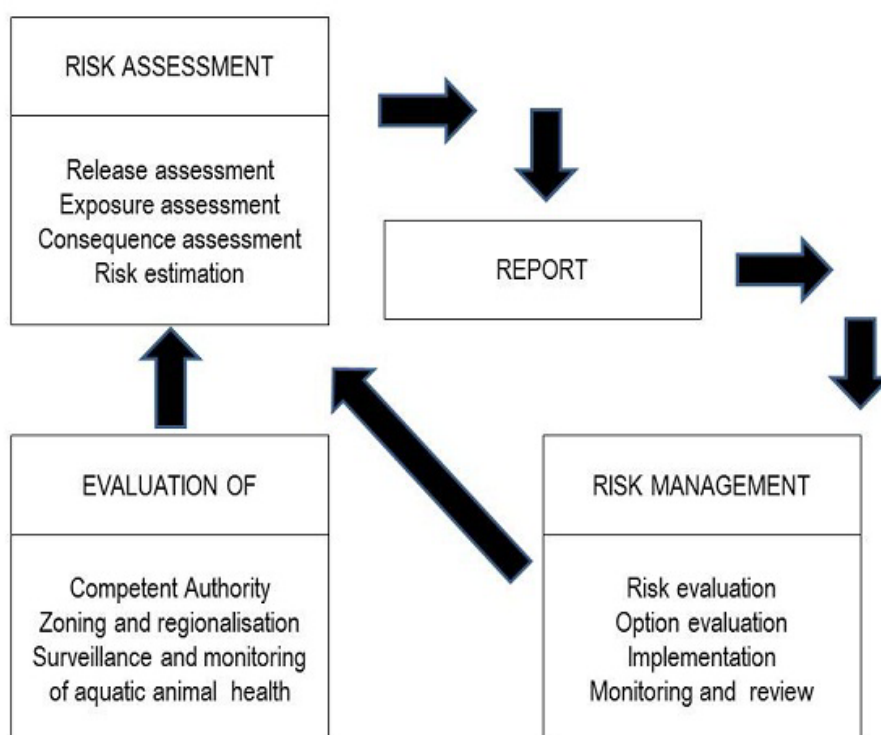
*Hazard identification* is an essential step that should be conducted before the *risk assessment*.

The *risk assessment* process consists of four interrelated steps. These steps clarify the stages of the *risk assessment*, describing them in terms of the events necessary for the identified potential *risk(s)* to occur, and facilitate understanding and evaluation of the conclusions (or 'outputs'). The product is the *risk assessment report*, which is used in *risk communication* and *risk management*.

The relationships between *risk assessment* and *risk management* processes are outlined in Figure 1.

Fig. 1. **The relationship between risk assessment and risk management processes**

**DELETE TABLE**



Article 2.12.2.

**Hazard identification**

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

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

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

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

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

Article 2. 12.3.

#### Principles of risk assessment

- 1) *Risk assessment* should be flexible in order to deal with the complexity of real-life situations. No single method is applicable in all cases. *Risk assessment* should be able to accommodate the variety of aquatic animal commodities, the multiple *hazards* that may be identified with an importation and the specificity of each *disease*, detection and *surveillance* systems, exposure scenarios and types and amounts of data and information.
- 2) Both qualitative risk assessment and quantitative *risk assessment* methods are valid.
- 3) The *risk assessment* should be based on the best available information that is in accord with current scientific thinking. The assessment should be well documented and supported with references to the scientific literature and other sources, including expert opinion.
- 4) Consistency in *risk assessment* methods should be encouraged and transparency is essential in order to ensure fairness and rationality, consistency in decision-making and ease of understanding by all the interested parties.
- 5) *Risk assessments* should document the uncertainties, the assumptions made, and the effect of these on the final *risk* estimate.
- 6) *Risk* increases with increasing volume of *commodity* imported.
- 7) The *risk assessment* should be amenable to updating when additional information becomes available.

Article 2. 12.4.

#### Risk assessment steps

##### 1. Entry assessment

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

- a) Biological factors
  - i) Species, strain or genotype, and age of *aquatic animal*
  - ii) Strain of agent

- iii) Tissue sites of *infection* and/or contamination
  - iv) Vaccination, testing, treatment and *quarantine*.
- b) Country factors
- i) *Incidence or prevalence*
  - ii) Evaluation of *Aquatic Animal Health Services, surveillance* and control programmes, and zoning and compartmentalisation systems of the *exporting country*.
- c) Commodity factors
- i) Whether the *commodity* is alive or dead
  - ii) Quantity of *commodity* to be imported
  - iii) Ease of contamination
  - iv) Effect of the various processing methods on the *pathogenic agent* in the *commodity*
  - v) Effect of storage and transport on the *pathogenic agent* in the *commodity*.

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

## 2. Exposure assessment

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

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

- a) Biological factors
- i) ~~Presence of potential vectors or intermediate hosts~~
  - ii) ~~Genotype of host~~
  - iii) Properties of the agent (e.g. virulence, pathogenicity and survival parameters)
  - iv) Genotype of host.
- b) Country factors
- i) Presence of potential vectors or intermediate hosts
  - ii) *Aquatic animal* demographics (e.g. presence of known susceptible and carrier species, distribution)
  - iii) Human and terrestrial animal demographics (e.g. possibility of scavengers, presence of piscivorous birds)
  - iv) Customs and cultural practices
  - v) Geographical and environmental characteristics (e.g. hydrographic data, temperature ranges, water courses).

Annex 7 (contd)

## c) Commodity factors

- i) Whether the *commodity* is alive or dead
- ii) Quantity of *commodity* to be imported
- iii) Intended use of the imported *aquatic animals* or *products* (e.g. domestic consumption, restocking, incorporation in or use as *aquaculture feed* or bait)
- iv) Waste disposal practices.

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

3. Consequence assessment

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

## a) Direct consequences

- i) *Aquatic animal infection, disease*, production losses and facility closures
- ii) Public health consequences.

## b) Indirect consequences

- i) *Surveillance* and control costs
- ii) Compensation costs
- iii) Potential trade losses
- iv) Adverse, and possibly irreversible, consequences to the environment
- ~~iv) Adverse consumer reaction.~~

4. Risk estimation

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

For a quantitative assessment, the final outputs may include:

- i) The various populations of *aquatic animals* and/or estimated numbers of *aquaculture establishments* or people likely to experience health impacts of various degrees of severity over time
- ii) Probability distributions, confidence intervals, and other means for expressing the uncertainties in these estimates
- iii) Portrayal of the variance of all model inputs

Annex 7 (contd)

- iv) A sensitivity analysis to rank the inputs as to their contribution to the variance of the *risk* estimation output
- v) Analysis of the dependence and correlation between model inputs.

Article 2. 12.5.

**Principles of risk management**

- 1) *Risk management* is the process of deciding upon and implementing measures to achieve the Member's appropriate level of protection, whilst at the same time ensuring that negative effects on trade are minimised. The objective is to manage *risk* appropriately to ensure that a balance is achieved between a country's desire to minimise the likelihood or frequency of *disease* incursions and their consequences and its desire to import *commodities* and fulfil its obligations under international trade agreements.
- 2) The international standards of the OIE are the preferred choice of *sanitary measures* for *risk management*. The application of these *sanitary measures* should be in accordance with the intentions of the standards ~~or other recommendations of the SPS Agreement~~.

Article 2. 12.6.

**Risk management components**

- 1) *Risk evaluation* - the process of comparing the *risk* estimated in the *risk assessment* with the Member's appropriate level of protection.
- 2) Option evaluation - the process of identifying, evaluating the efficacy and feasibility of, and selecting measures to reduce the *risk* associated with an importation in order to bring in line with the Member's appropriate level of protection. The efficacy is the degree to which an option reduces the likelihood or magnitude of adverse health and economic consequences. Evaluating the efficacy of the options selected is an iterative process that involves their incorporation into the *risk assessment* and then comparing the resulting level of *risk* with that considered acceptable. The evaluation for feasibility normally focuses on technical, operational and economic factors affecting the implementation of the *risk management* options.
- 3) Implementation - the process of following through with the *risk management* decision and ensuring that the *risk management* measures are in place.
- 4) Monitoring and review - the ongoing process by which the *risk management* measures are continuously audited to ensure that they are achieving the results intended.

Article 2. 12.7.

**Principles of risk communication**

- 1) *Risk communication* is the process by which information and opinions regarding *hazards* and *risks* are gathered from potentially affected and interested parties during a *risk analysis*, and by which the results of the *risk assessment* and proposed *risk management* measures are communicated to the decision-makers and interested parties in the *importing* and *exporting countries*. It is a multidimensional and iterative process and should ideally begin at the start of the *risk analysis* process and continue throughout.
- 2) A *risk communication* strategy should be put in place at the start of each *risk analysis*.
- 3) The *communication of risk* should be an open, interactive, iterative and transparent exchange of information that may continue after the decision on importation.
- 4) The principal participants in *risk communication* include the authorities in the *exporting country* and other stakeholders such as domestic aquaculturists, recreational and commercial fishermen, conservation and wildlife groups, consumer groups, and domestic and foreign industry groups.
- 5) The assumptions and uncertainty in the model, model inputs and the *risk* estimates of the *risk assessment* should be communicated.

Annex 7 (contd)

- 6) Peer review of *risk analyses* is an essential component of *risk communication* ~~in order to for~~ obtaining a scientific critique ~~and to aimed at~~ ensuring that the data, information, methods and assumptions are the best available.

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UNOFFICIAL VERSION



## CHAPTER 5.X.

**OIE PROCEDURES RELEVANT TO THE AGREEMENT  
ON THE APPLICATION OF SANITARY AND  
PHYTOSANITARY MEASURES OF THE WORLD  
TRADE ORGANIZATION**

**EU position**

**The EU supports the adoption of this new chapter.**

## Article 5.X.1.

**The Agreement on the Application of Sanitary and Phytosanitary Measures and role and responsibility of the OIE**

The Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) encourages the Members of the World Trade Organization to base their *sanitary measures* on international standards, guidelines and recommendations, where they exist. Members may choose to adopt a higher level of protection than that provided by international texts if there is a scientific justification or if the level of protection provided by the relevant international texts is considered to be inappropriate. In such circumstances, Members are subject to obligations relating to *risk assessment* and to a consistent approach of *risk management*.

The SPS Agreement encourages Governments to make a wider use of *risk analysis*: WTO Members shall undertake an assessment as appropriate to the circumstances of the actual *risk* involved.

The SPS Agreement, in Article 7, obliges WTO Members to notify changes in, and provide relevant information on, sanitary measures which may, directly or indirectly, affect international trade.

The SPS Agreement recognises the OIE as the relevant international organisation responsible for the development and promotion of international animal health standards, guidelines, and recommendations affecting trade in live animals and animal products, including *aquatic animals* and their products.

## Article 5.X.2.

**Introduction on the judgement of the equivalence of sanitary measures**

The importation of *aquatic animals* and *aquatic animal products* involves a degree of *risk* to the *aquatic animal health status* of an *importing country*. The estimation of that *risk* and the choice of the appropriate *risk management* option(s) are made more difficult by differences among the *aquatic animal* health and production systems in OIE Members. It is now recognised that significantly different *aquatic animal* health and production systems can provide equivalent *aquatic animal* and human health protection for the purpose of *international trade*, with benefits to both the *importing country* and the *exporting country*.

These recommendations are to assist OIE Members to determine whether *sanitary measures* arising from different *aquatic animal* health and production systems may provide the same level of *aquatic animal* and human health protection. They discuss principles which might be utilised in a judgement of equivalence, and outline a step-wise process for trading partners to follow in facilitating a judgement of equivalence. These provisions are applicable whether equivalence applies at the level of specific measures or on a systems-wide basis, and whether equivalence applies to specific areas of trade or *commodities*, or generally.

## Article 5.X.3.

**General considerations on the judgement of the equivalence of sanitary measures**

Before trade in *aquatic animals* or their products may occur, an *importing country* must be satisfied that its *aquatic animal health status* will be appropriately protected. In most cases, the *risk management* measures drawn up will rely in part on judgements made about the *aquatic animal* health and production system(s) in the *exporting country* and the effectiveness of sanitary procedures undertaken there. Systems operating in the *exporting country* may differ from those in the *importing country* and from those in other countries with which the *importing*

country has traded. Differences may be with respect to infrastructure, policies and/or operating procedures, laboratory systems, approaches to the pests and diseases present, border security and internal movement controls.

International recognition of the legitimacy of different approaches to achieving the *importing country's appropriate level of protection* (ALOP) has led to the principle of equivalence being included in trade agreements, including the SPS Agreement of the WTO.

Benefits of applying equivalence may include:

- 1) minimising costs associated with *international trade* by tailoring *aquatic animal* health measures to local circumstances;
- 2) maximising *aquatic animal* health outcomes for a given level of resource input;
- 3) facilitating trade by achieving the required health protection through less trade restrictive *sanitary measures*; and
- 4) decreased reliance on relatively costly *commodity* testing and isolation procedures in bilateral or multilateral agreements.

The *Aquatic Code* recognises equivalence by recommending alternative *sanitary measures* for many *diseases* and pathogenic agents. Equivalence may be gained, for example, by enhanced *surveillance* and monitoring, by the use of alternative test, treatment or isolation procedures, or by combinations of the above. To facilitate the judgement of equivalence, Members should base their *sanitary measures* on the standards, guidelines and recommendations of the OIE.

It is essential to apply a scientific *risk analysis* to the extent practicable in establishing the basis for a judgement of equivalence.

#### Article 5.X.4.

#### Prerequisite considerations in a judgement of equivalence

##### 1. Application of risk assessment

Application of the discipline of *risk assessment* provides a structured basis for judging equivalence among different *sanitary measures* as it allows a close examination to be made of the effect of a measure(s) on a particular step(s) in the importation pathway, and the relative effects of proposed alternative measure(s) on the same or related steps.

A judgement of equivalence needs to assess the *sanitary measure* in terms of its effectiveness regarding the particular *risk* or group of *risks* against which the measure is designed to protect. Such an assessment may include the following elements: the purpose of the measure, the level of protection achieved by the measure and the contribution the measure makes to achieving the ALOP of the *importing country*.

##### 2. Categorisation of sanitary measures

Proposals for equivalence may be in terms of a measure comprising a single component of a measure (e.g. an isolation procedure, a test or treatment requirement, a certification procedure) or multiple components (e.g. a production system for *commodity*), or a combination of measures. Multiple components or combinations of measures may be applied consecutively or concurrently.

*Sanitary measures* are those described in each chapter of the *Aquatic Code* which are used for *risk* reduction and are appropriate for particular *diseases*. *Sanitary measures* may be applied either alone or in combination and include test requirements, processing requirements, inspection or certification procedures, quarantine confinements, and sampling procedures.

For the purposes of judging equivalence, *sanitary measures* can be broadly categorised as:

- a) infrastructure: including the legislative base (e.g. *aquatic animal* health law) and administrative systems (e.g. organisation of national and regional *aquatic animal* health authorities, emergency response organisations);

- b) programme design/implementation: including documentation of systems, performance and decision criteria, *laboratory* capability, and provisions for certification, audit and enforcement;
- c) specific technical requirement: including requirements applicable to the use of secure facilities, treatment (e.g. retorting of cans), specific test (e.g. PCR) and procedures (e.g. pre-export inspection).

A *sanitary measure(s)* proposed for a judgement of equivalence may fall into one or more of these categories, which are not mutually exclusive.

In some cases, a comparison of specific technical requirements may suffice. In many instances, however, a judgement as to whether the same level of protection is likely to be achieved may only be able to be determined through an evaluation of all relevant components of an *exporting country's aquatic animal* health and production system. For example, a judgement of equivalence for a specific *sanitary measure* at the programme design/implementation level may require a prior examination of infrastructure while a judgement of equivalence for a specific measure at the specific technical requirement level may require that the specific measure be judged in its context through examination of infrastructure and programmes.

#### Article 5.X.5.

#### Principles for judgement of equivalence

In conjunction with the above considerations, judgement of the equivalence of *sanitary measures* should be based on application of the following principles:

- 1) an *importing country* has the right to set the level of protection it deems appropriate (its ALOP) in relation to human and animal life and health in its territory; this ALOP may be expressed in qualitative or quantitative terms;
- 2) the *importing country* should be able to describe the reason for each *sanitary measure* i.e. the level of protection intended to be achieved by application of the identified measure against a *hazard*;
- 3) an *importing country* should recognise that *sanitary measures* different from the ones it has proposed may be capable of providing the same level of protection;
- 4) the *importing country* should, upon request, enter into consultations with the *exporting country* with the aim of facilitating a judgement of equivalence;
- 5) any *sanitary measure* or combination of *sanitary measures* can be proposed for judgement of equivalence;
- 6) an interactive process should be followed that applies a defined sequence of steps, and utilises an agreed process for exchange of information, so as to limit data collection to that which is necessary, minimise administrative burden, and facilitate resolution of claims;
- 7) the *exporting country* should be able to demonstrate objectively how the alternative *sanitary measure(s)* proposed as equivalent will provide the same level of protection;
- 8) the *exporting country* should present a submission for equivalence in a form that facilitates judgement by the *importing country*;
- 9) the *importing country* should evaluate submissions for equivalence in a timely, consistent, transparent and objective manner, and according to appropriate *risk assessment* principles;
- 10) the *importing country* should take into account any knowledge of and prior experience with the *Veterinary Authority* or other *Competent Authority* of the *exporting country*;
- 11) the *exporting country* should provide access to enable the procedures or systems which are the subject of the equivalence judgement to be examined and evaluated upon request of the *importing country*;
- 12) the *importing country* should be the sole determinant of equivalence, but should provide to the *exporting country* a full explanation for its judgement;
- 13) to facilitate a judgement of equivalence, OIE Members should base their *sanitary measures* on relevant OIE standards;

- 14) to allow the judgement of equivalence to be reassessed if necessary, the *importing country* and the *exporting country* should keep each other informed of significant changes to infrastructure, health status or programmes which may bear on the judgement of equivalence; and
- 15) an *importing country* should give positive consideration to a request by an exporting developing country for appropriate technical assistance that would facilitate the successful completion of a judgement of equivalence.

#### Article 5.X.6.

#### Sequence of steps to be taken in judgement of equivalence

There is no single sequence of steps which must be followed in all judgements of equivalence. The steps that trading partners choose will generally depend on the circumstances and their trading experience. The interactive sequence of steps described below may be useful for all *sanitary measures* irrespective of their categorisation as infrastructure, programme design/implementation or specific technical requirement components of an *aquatic animal* health and production system.

This sequence assumes that the *importing country* is meeting its obligations under the WTO SPS Agreement and has in place a transparent measure based either on an international standard or a *risk analysis*.

Recommended steps are:

- 1) the *exporting country* identifies the measure(s) for which it wishes to propose an alternative measure(s), and requests from the *importing country* a reason for its *sanitary measure* in terms of the level of protection intended to be achieved against a *hazard(s)*;
- 2) the *importing country* explains the reason for the measure(s), in terms which would facilitate comparison with an alternative *sanitary measure(s)* and consistent with the principles set out in these provisions;
- 3) the *exporting country* demonstrates the case for equivalence of an alternative *sanitary measure(s)* in a form which facilitates analysis by an *importing country*;
- 4) the *exporting country* responds to any technical concerns raised by the *importing country* by providing relevant further information;
- 5) judgement of equivalence by the *importing country* takes into account as appropriate:
  - a) the impact of biological variability and uncertainty;
  - b) the expected effect of the alternative *sanitary measure(s)* on all relevant *hazards*;
  - c) OIE standards;
  - d) application of solely qualitative frameworks where it is not possible or reasonable to conduct *quantitative risk assessment*;
- 6) the *importing country* notifies the *exporting country* of its judgement and the underlying reasons within a reasonable period of time:
  - a) recognition of the equivalence of the *exporting country's* alternative *sanitary measure(s)*;
  - b) request for further information; or
  - c) rejection of the case for equivalence of the alternative *sanitary measure(s)*;
- 7) an attempt should be made to resolve any differences of opinion over judgement of a case, either interim or final, by using an agreed mechanism to reach consensus (e.g. the OIE informal procedure for dispute mediation), or by referral to an agreed expert;

Annex 7 (contd)

- 8) depending on the category of measures involved, the *importing country* and the *exporting country* may enter into a formal equivalence agreement giving effect to the judgement or a less formal acknowledgement of the equivalence of a specific measure(s) may suffice.

An *importing country* recognising the equivalence of an *exporting country's* alternative *sanitary measure(s)* needs to ensure that it acts consistently with regard to applications from third countries for recognition of equivalence applying to the same or very similar measure(s). Consistent action does not mean however that a specific measure(s) proposed by several *exporting countries* should always be judged as equivalent as a measure(s) should not be considered in isolation but as part of a system of infrastructure, policies and procedures.

## Article 5.X.7.

**Sequence of steps to be taken in establishing a zone/compartiment and having it recognised for international trade purposes**

There is no single sequence of steps which should be followed in establishing a *zone* or a *compartiment*. The steps that the *Veterinary Services* or *Aquatic Animal Health Services* of the *importing country* and the *exporting country* choose and implement will generally depend on the circumstances existing within the countries and at their borders, and their trading history. The recommended steps are:

1. For zoning

- a) The *exporting country* identifies a geographical area within its territory, which it considers to contain an *aquatic animal subpopulation* with a distinct health status with respect to a specific *disease/specific diseases*, based on *surveillance*.
- b) The *exporting country* describes in the *biosecurity plan* for the *zone* the measures which are being, or will be, applied to distinguish such an area epidemiologically from other parts of its territory, in accordance with the recommendations in the *Aquatic Code*.
- c) The *exporting country* provides:
  - i) the above information to the *importing country*, with an explanation of why the area can be treated as an epidemiologically separate *zone* for *international trade* purposes;
  - ii) access to enable the procedures or systems that establish the *zone* to be examined and evaluated upon request by the *importing country*.
- d) The *importing country* determines whether it accepts such an area as a *zone* for the importation of *aquatic animals* and *aquatic animal products*, taking into account:
  - i) an evaluation of the *exporting country's* *Veterinary Services* or *Aquatic Animal Health Services*;
  - ii) the result of a *risk assessment* based on the information provided by the *exporting country* and its own research;
  - iii) its own *aquatic animal* health situation with respect to the *disease(s)* concerned; and
  - iv) other relevant OIE standards.
- e) The *importing country* notifies the *exporting country* of its determination and the underlying reasons, within a reasonable period of time, being:
  - i) recognition of the *zone*; or
  - ii) request for further information; or
  - iii) rejection of the area as a *zone* for *international trade* purposes.
- f) An attempt should be made to resolve any differences over recognition of the *zone*, either in the interim or finally, by using an agreed mechanism to reach consensus such as the OIE informal procedure for dispute mediation (Article 5.3.8.bis).
- g) The *Veterinary Authorities* or other Competent Authorities of the *importing* and *exporting countries* should enter into a formal agreement recognizing the *zone*.

Annex 7 (contd)2. For compartmentalisation

- a) Based on discussions with the relevant industry, the *exporting country* identifies within its territory a *compartment* comprising an *aquatic animal subpopulation* contained in one or more *establishments* or other premises operating under common management practices related to biosecurity. The *compartment* contains an identifiable *aquatic animal subpopulation* with a distinct health status with respect to specific *disease(s)*. The *exporting country* describes how this status is maintained through a partnership between the relevant industry and the *Veterinary Authority* or other Competent Authority of the *exporting country*.
- b) The *exporting country* examines the *compartment's biosecurity plan* and confirms through an audit that:
  - i) the *compartment* is epidemiologically closed throughout its routine operating procedures as a result of effective implementation of its *biosecurity plan*; and
  - ii) the *surveillance* and monitoring programme in place is appropriate to verify the status of such a *subpopulation* with respect to such *disease(s)*.
- c) The *exporting country* describes the *compartment*, in accordance with the recommendations in the *Aquatic Code*.
- d) The *exporting country* provides:
  - i) the above information to the *importing country*, with an explanation of why such a *subpopulation* can be treated as an epidemiologically separate *compartment* for *international trade* purposes; and
  - ii) access to enable the procedures or systems that establish the *compartment* to be examined and evaluated upon request by the *importing country*.
- e) The *importing country* determines whether it accepts such a *subpopulation* as a *compartment* for the importation of *aquatic animals* and *aquatic animal products*, taking into account:
  - i) an evaluation of the *exporting country's Veterinary Services or Aquatic Animal Health Services*;
  - ii) the result of a *risk assessment* based on the information provided by the *exporting country* and its own research;
  - iii) its own *aquatic animal* health situation with respect to the *disease(s)* concerned; and other relevant OIE standards.
- f) The *importing country* notifies the *exporting country* of its determination and the underlying reasons, within a reasonable period of time, being:
  - i) recognition of the *compartment*; or
  - ii) request for further information; or
  - iii) rejection of such a *subpopulation* as a *compartment* for *international trade* purposes.
- g) An attempt should be made to resolve any differences over recognition of the *compartment*, either in the interim or finally, by using an agreed mechanism to reach consensus such as the OIE informal procedure for dispute mediation (Article 5.3.8.).
- h) The *Veterinary Authorities* or other Competent Authority of the *importing* and *exporting countries* should enter into a formal agreement recognizing the *compartment*.
- i) The *Veterinary Authority* or other Competent Authority of the *exporting country* should promptly inform *importing countries* of any occurrence of a *disease* in respect of which the *compartment* was defined.

## Article 5.X.8.

**The OIE informal procedure for dispute mediation**

OIE shall maintain its existing voluntary in-house mechanisms for assisting OIE Members to resolve differences. In-house procedures which will apply are that:

Annex 7 (contd)

- 1) Both parties agree to give the OIE a mandate to assist them in resolving their differences.
  - 2) If considered appropriate, the Director General of the OIE recommends an expert, or experts, and a chairman, as requested, agreed by both parties.
  - 3) Both parties agree on the terms of reference and working programme, and to meet all expenses incurred by the OIE.
  - 4) The expert or experts are entitled to seek clarification of any of the information and data provided by either country in the assessment or consultation processes, or to request additional information or data from either country.
  - 5) The expert or experts shall submit a confidential report to the Director General of the OIE, who will transmit it to both parties.
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## CHAPTER 7.4.

**KILLING OF FARMED FISH  
FOR DISEASE CONTROL PURPOSES**

[...]

**EU position**

**The EU cannot support the adoption of this revised chapter at the General Session in May as current scientific knowledge on pain in fish justifies the wording of the existing chapter. Scientific findings are summarised below together with the references.**

Article 7.4.2.

**General principles**

- 1) Fish welfare considerations should be addressed within *contingency plans* for disease control (refer to Chapter 4.4.).
- 2) The killing method should be selected taking into consideration fish welfare and biosecurity requirements as well as safety of the personnel.
- 3) When fish are killed for disease control purposes, methods used should result in immediate death or immediate loss of consciousness lasting until death; when loss of consciousness is not immediate, induction of unconsciousness should be non-aversive or the least aversive possible and should not cause avoidable stress pain, distress or suffering in fish.

**EU comment**

**The EU asks the OIE to maintain the original wording of "... should not cause avoidable pain, distress or suffering in fish."**

**Justification**

**To date there are a number of scientific studies (see below) which infer that fish have the ability to feel pain and to have an awareness of this pain. Such is the documentation on this issue that it is relevant to require that fish are treated as if they can feel pain and can suffer.**

**The scientific findings supporting pain perception and fish consciousness are outlined below:**

**There is considerable evidence of a nociceptive system in fish (Sneddon 2002; Sneddon 2003; Sneddon *et al.* 2003ab; Ashley *et al.*, 2007), and painful stimulation applied to the periphery will be transmitted to the fish brain, the telencephalon included (Dunlop & Laming 2005; Nordgreen *et al.* 2007). The question of fish consciousness, and of whether they are aware of pain and therefore can be said to suffer is more controversial. It is important to bear in mind that consciousness in most non-human animals can only be inferred, but equally important to acknowledge that this inference can be done in fish just as it is done in mammals (Edelman *et al.*, 2005). First of all, it is important to note that fully developed self-consciousness is not necessary to be aware of pain, and will not**

be discussed here. However, phenomenal consciousness (as defined by Ned Block and elaborated in an animal welfare context by Dawkins) is necessary to be conscious of hunger, thirst, fear, pain and pleasure, and is the level of consciousness that is most relevant for animal welfare and for the question of pain in fish.

There are three main groups of scientific findings that can be used to infer consciousness in fish; limited attention, brain anatomy and studies of learning and emotion.

### 1. LIMITED ATTENTION

It is well known that one can only be conscious of very few things at any time, and 'limited attention' is a hallmark of consciousness (Baars) which fish also demonstrate. Clinical studies have shown that pain may interfere with other tasks in humans, and a study indicates that this is the case also for fish: Pain will capture the attention of fish, so that they will not flee from a novel object (Sneddon *et al.* 2003b). When treated with a pain-killer (morphine), the fish will resume their normal avoidance of the novel object. Fish thus fulfil the requirement of phenomenal consciousness.

### 2. BRAIN ANATOMY

Importantly, we do not understand how the human brain gives rise to consciousness, and thus cannot determine the question of consciousness in non-human animals merely by studying their brains. The fish brain was previously thought simpler than it is partly due to its ontogeny: the fish brain develops by eversion, not evagination, so that structures found medially in the mammalian brain will be found laterally in the teleost brain. There is also a growing understanding that a cerebral cortex is merely a way to organise a brain, not a prerequisite for consciousness or complex cognition. Titles of newer publications such as: *Insight without cortex: lessons from the avian brain* (Kirsch *et al.* 2008) and *Non-laminar cerebral cortex in teleost fishes?* (Ito and Yamamoto 2009) are good examples of this updated view on comparative neuroanatomy. Based on telencephalic connectivity in teleosts, Ito and Yamamoto hypothesized that the dorsal telencephalon in fish contains neurons with similar properties to parts of the mammalian neocortex. A lot of research on the fish brain remains to be done, but it is important to be aware that fish do possess pallial telencephalic areas. These are the same type of areas that constitute the cortex in mammals, consequently demonstrating that fish possess the neuronal structures that may be necessary for the experience of pain.

### 3. LEARNING AND EMOTIONS

Fish can learn by methods that are known to require consciousness in humans: In trace classical conditioning, there is a "silent" trace interval between the termination of the conditioned stimulus (CS) and the delivery of the unconditioned stimulus (US), whereas in delay conditioning the CS and US overlap. In humans, delay conditioning can proceed after damage to the hippocampus (Clark and Squire 1998), whereas in trace conditioning it cannot, since the task is dependent on the hippocampus, and on conscious awareness of the stimulus contingencies (Clark and Squire 1998, 1999). Hence, in trace classical conditioning, the test individuals become able to expect the US after CS

exposure (Clark *et al.* 2002) and learn the fact that the US follows the CS. Trace conditioning is thus a simple example of declarative learning. The difference in the anatomical substrate for delay and trace conditioning has also been confirmed in other mammals such as the rabbit (Christian and Thompson 2003). It has therefore been suggested that trace conditioning can be used to investigate the declarative (i.e. conscious, fact-finding) system in non-human animals (Clark and Squire 1998). Cod (*Gadus morhua*) (Nilsson *et al.* 2008) and goldfish (*Carassius auratus*) (Portavella *et al.* 2004) are able to learn by trace conditioning, and lesions to the proposed hippocampus homologue in goldfish impair trace conditioning (Portavella *et al.* 2004), without affecting delay conditioning (Gomez Garcia *et al.* 2004). Rainbow trout can also learn by trace classical conditioning (Nordgreen *et al.* 2010). Furthermore, when the nature of the association the rainbow trout had made was tested further, it was confirmed that trace conditioning was of a declarative "facts-learning" rather than a "reflex-learning" type. The fish were able to associate food with a light cue, and would approach the light also in absence of food. When food was associated with electric shock, the fish changed their response to the light, no longer approaching it even though the light itself was never paired with shock. Put simply, the fish in a trace conditioning regime learnt that "light predicts food, but food predicts shock, therefore, I will no longer approach the light". Recently, trace conditioning has been demonstrated in Atlantic salmon, and the learning process was linked to the emotional response of frustration (Vindas *et al.* 2012).

#### Reference list:

- Ashley PJ., Sneddon LU. & McCrohan CR. (2007) **Nociception in fish: stimulus-response properties of receptors on the head of trout *Oncorhynchus mykiss***. *Brain Research* **1166**, 47-54.
- Sneddon LU. (2002) **Anatomical and electrophysiological analysis of the trigeminal nerve in a teleost fish, *Oncorhynchus mykiss***. *Neuroscience Letters* **319**, 167-171.
- Sneddon LU. (2003a) **The evidence for pain in fish: the use of morphine as an analgesic**. *Applied Animal Behaviour Science* **83**, 153-162.
- Sneddon LU. (2003b) **Trigeminal somatosensory innervation of the head of a teleost fish with particular reference to nociception**. *Brain Research* **972**, 44-52.
- Sneddon LU., Braithwaite VA. & Gentle MJ. (2003a) **Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system**. *Proceedings of the Royal Society of London B* **270**, 1115-1121.
- Sneddon LU., Braithwaite VA. & Gentle MJ. (2003b) **Novel object test: Examining nociception and fear in the rainbow trout**. *Journal of pain* **4**(8), 431-440.
- Dunlop R. & Laming P. (2005) **Mechanoreceptive and nociceptive responses in the central nervous system of goldfish (*Carassius auratus*) and trout (*Oncorhynchus mykiss*)**. *The Journal of Pain* **6**, 561-568.
- Nordgreen J.; Horsberg T.E.; Ranheim B.; Chen A.C.N. (2007) **Somatosensory evoked potentials in the telencephalon of Atlantic salmon (*Salmo salar*) following galvanic stimulation of the tail**. *Journal of Comparative Physiology A* **193**, 1235-1242.
- Edelman, D.B.; Baars, B.J.; Seth, A.K. (2005) **Identifying hallmarks of consciousness in non-mammalian species**. *Consciousness and Cognition* **14**, 169-187.
- Dawkins MS. (2001) **Who needs consciousness?** *Animal Welfare* **10**, S19-S29.
- Block N (1991) **Evidence against epiphenomenalism**. *Behavioral and Brain Sciences* **14**: 670-672.

- Baars, BJ. (1997) **What is consciousness?** Commentary. Journal of the American Psychoanalytic Association, 45, 707-714.
- Kirsch, J.A.; Güntürkün, O.; Rose, J. (2008) **Insight without cortex: lessons from the avian brain.** Consciousness and cognition 17, 475-483.
- Ito, H.; Yamamoto, N. (2009) **Non-laminar cerebral cortex in teleost fishes?** Biology Letters 5, 117-121.
- Clark RE, Squire LR (1998) **Classical conditioning and brain systems: the role of awareness.** Science 280:77-81
- Clark RE, Squire LR (1999) **Human eyeblink classical conditioning: Effects of manipulating awareness of the stimulus contingencies.** Psychol Sci 10:14-18
- Clark RE, Manns JR, Squire LR (2002) **Classical conditioning, awareness and brain systems.** Trends Cogn Sci 6:524-531
- Christian KM, Thompson RF (2003) **Neural substrates of eyeblink conditioning: acquisition and retention.** Learn Mem 10:427-455
- Nilsson J, Kristiansen TS, Fosseidengen JE, Ferno A, van den Bos R (2008) **Learning in cod (*Gadus morhua*): long trace interval retention.** Anim Cogn 11:215-222
- Portavella M, Torres B, Salas C (2004) **Avoidance response in gold-fish: emotional and temporal involvement of medial and lateral telencephalic pallium.** J Neurosci 24:2335-2342
- Gomez Garcia A, Álvarez E, Durán E, Ocaña FM, Broglio C, Jiménez- Moya F, Salas C, Rodriguez F (2004) **Delay vs trace conditioning following pallium ablation in goldfish.** FENS Abstr 2:A042.10
- Nordgreen J, Janczak AM, Hovland A-L., Ranheim B, Horsberg TE (2010) **Trace classical conditioning in rainbow trout (*Oncorhynchus mykiss*): what do they learn?** Animal Cognition 13, 303-309.
- Vindas, MA; Folkedal, O; Kristiansen, TS; Stien, LH; Braastad, BO; Mayer, I; Overlie O (2012) **Omission of expected reward agitates Atlantic salmon (*Salmo salar*).** Animal Cognition 15, 903-911.

- 4) The methods described in Chapter 7.3. can also be used for disease control purposes.
- 5) Some of the methods recommended for disease control purposes (e.g. anaesthetic overdose, maceration) may render the fish unsuitable for human consumption, and this should be specified in the *contingency plan*.
- 6) Depending on the situation, emergency killing of fish may be carried out on site or after fish are transported to an approved killing facility.

#### Article 7.4.3.

#### Operational guidelines for affected premises and approved killing facilities

The following should apply when killing fish:

- 1) Operational procedures should be adapted to the specific circumstances on the premises and should address fish welfare and biosecurity specific to the *disease* of concern.
- 2) Killing of fish should be carried out without delay by appropriately qualified personnel with all due consideration made to increased biosecurity protocols.
- 3) Handling of fish should be kept to a minimum to avoid stress and to prevent spread of *disease*. This should be done in accordance with the articles described below.
- 4) Methods used to kill the fish should render them unconscious until death or kill them in the shortest time possible, and should not cause avoidable **stress pain or distress**.

#### EU comment

**The EU asks the OIE to maintain the original wording of "... should not cause avoidable pain or distress in fish."**

**Justification**

**See above**

- 5) There should be continuous monitoring of the procedures to ensure they are consistently effective with regard to biosecurity and fish welfare.
- 6) Standard operating procedures (SOP's) should be available and followed at the premises.

Article 7.4.4.

**Competencies and responsibilities of the operational team**

The operational team is responsible for planning, implementation of, and reporting on the killing of the fish.

1. Team leader

a) Competencies

- i) Ability to assess fish welfare, especially relating to the effectiveness of the stunning and killing techniques selected and utilised in the fish killing operations, to detect and correct any deficiencies;
- ii) ability to assess biosecurity risks and mitigation measures being applied to prevent spread of *disease*;
- iii) skills to manage all activities on premises and deliver outcomes on time;
- iv) awareness of the psychological impact on fish farmers, team members and general public;
- v) effective communication skills.

b) Responsibilities

- i) Determine most appropriate killing method(s) to ensure that the fish are killed without avoidable **stress pain and distress** while balancing biosecurity considerations;

**EU comment**

**The EU asks the OIE to maintain the original wording of "... should not cause avoidable pain or distress in fish."**

**Justification**

**See above**

- ii) plan overall operations on the affected premises;
- iii) determine and address requirements for fish welfare, operator safety and biosecurity;
- iv) organise, brief and manage a team of people to facilitate killing of the relevant fish in accordance with national *contingency plans* for disease control;

[...]

— Text deleted



## CHAPTER 10.2.

**INFECTION WITH APHANOMYCES INVADANS**  
**(EPIZOOTIC ULCERATIVE SYNDROME)**

**EU position**

**The EU supports the adoption of this modified chapter.**

## Article 10.2.1.

For the purposes of the *Aquatic Code*, ~~epizootic ulcerative syndrome (EUS) means infection with the Oomycete fungus *Aphanomyces invadans* means all infections caused by the Oomycete fungus *A. invadans* (syn. *A. piscicida*). The disease was previously referred to as epizootic ulcerative syndrome.~~

Standards for information on methods for diagnostic tests are described ~~are provided~~ in the *Aquatic Manual*.

## Article 10.2.2.

**Scope**

The recommendations in this chapter apply to: yellowfin seabream (*Acanthopagrus australis*), climbing perch (*Anabas testudineus*), eels (Anguillidae), bagrid catfishes (Bagridae), silver perch (*Bidyanus bidyanus*), Atlantic menhaden (*Brevoortia tyrannus*), jacks (*Caranx* spp.), catla (*Catla catla*), striped snakehead (*Channa striatus*), mrigal (*Cirrhinus mrigala*), torpedo-shaped catfishes (*Clarius* spp.), halfbeaks flying fishes (*Exocoetidae*), tank goby (*Glossogobius giurus*), marble goby (*Oxyeleotris marmoratus*), gobies (Gobiidae), rohu (*Labeo rohita*), rhinofishes (*Labeo* spp.), barramundi and giant sea perch (*Lates calcarifer*), striped mullet (*Mugil cephalus*), mullets [Mugilidae] (*Mugil* spp. and *Liza* spp.), ayu (*Plecoglossus altivelis*), pool barb (*Puntius sophore*), barcoo grunter (*Scortum barcoo*), sand whiting (*Sillagociliata*), wells catfishes (Siluridae), snakeskin gourami (*Trichogaster pectoralis*), common archer fish (*Toxotes chatareus*), silver barb (*Puntius gonionotus*), spotted scat (*Scatophagus argus*), giant gourami (*Osphronemus goramy*), dusky flathead (*Platycephalus fuscus*), spiny turbot (*Psettodes* sp.), Tairiku-baratanago (*Rhodeus ocellatus*), Keti-Bangladeshi (*Rohtee* sp.), rudd (*Scaridinius erythrophthalmus*), tharapon (*Tharapon* sp.) and three-spot gouramy (*Trichogaster trichopterus*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

## Article 10.2.3.

**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 *A. invadans* epizootic ulcerative syndrome**

- 1) *Competent Authorities* should not require any infection with *A. invadans* EUS-related conditions, regardless of the *A. invadans* EUS 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 10.2.2. intended for any purpose and complying with Article 5.3.1.:
  - 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 heat treatment at 90°C for at least 10 minutes (or any time/temperature equivalent which has been demonstrated to inactivate *A. invadans*);
  - c) mechanically dried eviscerated fish (i.e. a heat treatment at 100°C for at least 30 minutes or any time/temperature equivalent which has been demonstrated to inactivate *A. invadans*);
  - d) fish oil;
  - e) fish *meal*;
  - f) frozen eviscerated fish; and

- g) frozen fillets or steaks.
- 2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 10.2.2., other than those referred to in point 1 of Article 10.2.3., *Competent Authorities* should require the conditions prescribed in Articles 10.2.7. to 10.2.12. relevant to the infection with *A. invadans* EUS 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 *A. invadans* EUS of a species not covered in Article 10.2.2. but which could reasonably be expected to pose a *risk* of transmission for *A. invadans* EUS, *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 10.2.4.

Infection with *A. invadans* ~~Epizootic ulcerative syndrome-free country~~

A country may make a *self-declaration of freedom* from infection with *A. invadans* EUS 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 *A. invadans* EUS if all the areas covered by the shared water are declared EUS-free countries or *zones free from infection with *A. invadans* (see Article 10.2.5.).*

- 1) A country where ~~the species referred to in Article 10.2.2. are present but~~ there has been no observed occurrence of the infection with *A. invadans* ~~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 infection with *A. invadans* EUS when *basic biosecurity conditions* have been continuously met in the country for at least the past ten years.

OR

- 2) A country where the last observed occurrence of the infection with *A. invadans* ~~disease~~ was within the past ten years or where the *infection* status prior to *targeted surveillance* was unknown (e.g. ~~because of the absence of conditions conducive to clinical expression as described in the corresponding chapter of the *Aquatic Manual*~~) may make a *self-declaration of freedom* from infection with *A. invadans* EUS when:
- basic biosecurity conditions* have been continuously met for at least the past two years; and
  - targeted surveillance*, as described in Chapter 1.4. of the *Aquatic Code*, has been in place for at least the last two years without detection of infection with *A. invadans*.

OR

- 3) A country that has made a *self-declaration of freedom* from infection with *A. invadans* EUS but in which the *disease* is subsequently detected may make a *self-declaration of freedom* from infection with *A. invadans* EUS again when the following conditions have been met:
- on detection of the *disease*, the affected area was declared an *infected zone* and a *protection zone* was established; and
  - 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
  - targeted surveillance*, as described in Chapter 1.4. of the *Aquatic Code*, has been in place for at least the last two years without detection of *A. invadans*; and
  - previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least ~~the past~~ two years following 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 2 of Article 10.2.5.

Article 10.2.5.



**Infection with *A. invadans* ~~Epizootic ulcerative syndrome~~ free zone or free compartment**

A zone or compartment within the territory of one or more countries not declared free from infection with *A. invadans* EUS 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.

If a zone or compartment extends over more than one country, it can only be declared an infection with *A. invadans* EUS free zone or compartment if all the *Competent Authorities* confirm that the conditions have been met.

- 1) A zone or compartment where the species referred to in Article 10.2.2. 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 infection with *A. invadans* EUS when *basic biosecurity conditions* have been continuously met in the zone or compartment for at least the past ten years.

OR

- 2) A zone or compartment where the last observed occurrence of the disease was within the past ten years or where the infection status prior to *targeted surveillance* was unknown (e.g. ~~because of the absence of conditions conducive to clinical expression as described in the corresponding chapter of the *Aquatic Manual*~~) may be declared free from infection with *A. invadans* EUS 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. of the *Aquatic Code*, has been in place for at least the last two years without detection of *A. invadans*.

OR

- 3) A zone previously declared free from infection with *A. invadans* EUS but in which the disease is detected may be declared free from infection with *A. invadans* EUS 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. of the *Aquatic Code*, has been in place for at least the last two years without detection of *A. invadans*; and
  - d) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least ~~the past two years~~ following eradication of the disease.

Article 10.2.6.

**Maintenance of free status**

A country, zone or compartment that is declared free from infection with *A. invadans* EUS following the provisions of point 1 of Articles 10.2.4. or 10.2.5. (as relevant) may maintain its status as infection with *A. invadans* EUS free provided that *basic biosecurity conditions* are continuously maintained.

A country, zone or compartment that is declared free from infection with *A. invadans* EUS following the provisions of point 2 of Articles 10.2.4. or 10.2.5. (as relevant) may discontinue *targeted surveillance* and maintain its status as infection with *A. invadans* EUS free provided that conditions that are conducive to clinical expression of the disease EUS, as described in the corresponding chapter of the *Aquatic Manual*, exist and *basic biosecurity conditions* are continuously maintained.

However, for declared free zones or compartments in infected countries and in all cases where conditions are not conducive to clinical expression of infection with *A. invadans* EUS, *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 10.2.7.

**Importation of live aquatic animals from a country, zone or compartment declared free from infection with *A. invadans* ~~epizootic ulcerative syndrome~~**

When importing live *aquatic animals* of the species referred to in Article 10.2.2. from a country, zone or compartment declared free from infection with *A. invadans* EUS, 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 10.2.4. or 10.2.5. (as applicable, the place of production of the *aquatic animal* is a country, zone or compartment declared free from infection with *A. invadans* EUS).

The *certificate* should be in accordance with the Model Certificate in Chapter 5.10.

This Article does not apply to *commodities* referred to in point 1 of Article 10.2.3.

## Article 10.2.8.

**Importation of live aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with *A. invadans* ~~epizootic ulcerative syndrome~~**

- 1) When importing, for *aquaculture*, live *aquatic animals* of the species referred to in Article 10.2.2. from a country, zone or compartment not declared free from infection with *A. invadans* EUS, 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 *A. invadans* EUSV.
- 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: [www.ices.dk/pubs/Miscellaneous/ICESCodeofPractice.pdf](http://www.ices.dk/pubs/Miscellaneous/ICESCodeofPractice.pdf)) 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 *A. invadans* EUSV, 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 *A. invadans* EUSV and perform general examinations for pests and general health/disease status;
  - g) if *A. invadans* EUSV 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 *A. invadans* EUS-free or specific pathogen free (SPF) for *A. invadans* EUSV;

## Annex 9 (contd)

- h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.
- 3) With respect to point 3(e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

This Article does not apply to *aquatic animals* referred to in point 1 of Article 10.2.3.

## Article 10.2.9.

**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 *A. invadans* epizootic ulcerative syndrome**

When importing, for processing for human consumption, *aquatic animals* or *aquatic animal products* of species referred to in Article 10.2.2. from a country, *zone* or *compartment* not declared free from infection with *A. invadans* EUS, 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 10.2.3., or products described in point 1 of Article 10.2.12., 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 *A. invadans* or is disposed in a manner that prevents contact of waste with *susceptible species*.

For these *commodities* Members 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 10.2.10.

**Importation of live aquatic animals intended for use in animal feed, or for agricultural, industrial or pharmaceutical use from a country, zone or compartment not declared free from infection with *A. invadans* epizootic ulcerative syndrome**

When importing, for use in animal *feed*, or for agricultural, industrial or pharmaceutical use, live *aquatic animals* of the species referred to in Article 10.2.2. from a country, *zone* or *compartment* not declared free from infection with *A. invadans* EUS, 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 *A. invadans*.

This Article does not apply to *commodities* referred to in point 1 of Article 10.2.3.

## Article 10.2.11.

**Importation of aquatic animal products from a country, zone or compartment declared free from infection with *A. invadans* epizootic ulcerative syndrome**

When importing *aquatic animal products* of the species referred to in Article 10.2.2. from a country, *zone* or *compartment* declared free from infection with *A. invadans* EUS, 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 10.2.4. or 10.2.5. (as applicable), the place of production of the *commodity* is a country, *zone* or *compartment* declared free from infection with *A. invadans* EUS.

The *certificate* should be in accordance with the Model Certificate in Chapter 5.10.

Annex 9 (contd)

This Article does not apply to *commodities* referred to in point 1 of Article 10.2.3.

*Article 10.2.12.*

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 *A. invadans* ~~epizootic ulcerative syndrome~~

- 1) *Competent Authorities* should not require any infection with *A. invadans* EUS related conditions, regardless of the infection with *A. invadans* EUS 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 5.3.2.:
  - a) fillets or steaks (chilled).

Certain assumptions have been made in assessing the safety of *aquatic animals* and *aquatic animal products* listed above. Members should refer to these assumptions at Article 5.3.2. and consider whether the assumptions apply to their conditions.

For these *commodities* Members 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 10.2.2. from a country, zone or compartment not declared free from infection with *A. invadans* EUS, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk mitigation* measures.

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## CHAPTER 10.5.

**INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS****EU position**

**The EU thanks the OIE and in general supports the adoption of this modified chapter. Comments are inserted in the text below.**

## 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. This includes infection with HPR (highly polymorphic region) deleted ISAV or HPR0 ISAV. Both genotypes should be notified in accordance with the Aquatic Code.

There is a link between non-pathogenic HPR0 ISAV and the emergence of 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 There are 3 possible levels of *disease* status with respect to ISAV:

(i) HPR deleted ISAV and HPR0 ISAV free;

(ii) HPR0 ISAV endemic (but HPR deleted ISAV free);

(iii) HPR deleted ISAV and HPR0 ISAV endemic.

(i) HPR0 ISAV and HPR-deleted ISAV free;

(ii) HPR0 ISAV endemic (but HPR-deleted ISAV free);

(iii) 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 (*Oncorhynchus mykiss*). These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

**EU comment**

**The EU would like to ask the OIE to consider adding coho salmon to the list of susceptible species.**

## 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 related conditions, 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 5.3.1.:
  - a) heat sterilised, hermetically sealed fish products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent);
  - b) pasteurised fish products that have been subjected to a heat treatment at 90°C for at least 10 minutes (or to any time/temperature equivalent which has been demonstrated to inactivate ISAV);
  - c) mechanically dried, eviscerated fish (i.e. a heat treatment at 100°C for 30 minutes or any time/temperature equivalent which has been demonstrated to inactivate ISAV);
  - d) fish oil;
  - e) fish *meal*; and
  - f) fish skin leather.
- 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 ISA-free countries or zones free from infection with ISAV (see Article 10.5.56.).

- 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 any 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. of the *Aquatic Code*, 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 any 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:

- 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
- bc) *targeted surveillance*, as described in Chapter 1.4. of the *Aquatic Code*, has been in place for at least the last two years without detection from infection with ISAV; and
- ed) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least ~~the past~~ two years following 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* of infection with HPR-deleted ISAV 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 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 10.5.6Z.).

- 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

- 2) 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 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 infection with HPR-deleted ISAV 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 infection with HPR-deleted ISAV the disease was within the past ten years or where the infection-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. of the *Aquatic Code*, has been in place for at least the last two years without detection of HPR-deleted ISAV.

OR

- 4) A country that has made a *self-declaration of freedom* from infection with HPR-deleted ISAV but in which infection with HPR-deleted ISAV the disease 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 disease, the affected area was declared an *infected zone* and a *protection zone* was established; and

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- 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 the disease, and the appropriate *disinfection* procedures (see *Aquatic Manual*) have been completed; and
- c) *targeted surveillance*, as described in Chapter 1.4. of the *Aquatic Code*, has been in place for at least the last two years without detection of 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 ~~the past~~ two years following 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 10.5.67.

## 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 (including HPR0) 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. of the *Aquatic Code*, has been in place for at least the last two years without detection of ISAV.

OR

- 3) A *zone* or *compartment* previously declared free from any 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:
  - a) on detection of 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. of the *Aquatic Code*, has been in place for at least the last two years without detection of ISAV; and
  - ed) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place for at least ~~the past~~ two years following eradication of the disease.



## Article 10.5.7.

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

In this article, all statements referring 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, 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

- 2) 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 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 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

- 3) A *zone* or *compartment* where the last observed occurrence of infection with HPR-deleted ISAV the disease was within the past ten years or where the infection 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. of the *Aquatic Code*, has been in place for at least the last two years without detection of HPR-deleted ISAV.

OR

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

- a) on detection of infection with HPR-deleted ISAV 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 HPR-deleted ISAV the disease, and the appropriate *disinfection* procedures (see *Aquatic Manual*) have been completed; and
- c) *targeted surveillance*, as described in Chapter 1.4. of the *Aquatic Code*, has been in place for at least the last two years without detection of 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 ~~the past~~ two years following eradication of the disease.

Annex 10 (contd)

## 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 10.5.76. (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 10.5.76. (as relevant) ~~may must continue targeted surveillance~~ to 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 10.5.45. or 10.5.47. (as relevant) may maintain its free status 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 10.5.45. or 10.5.67. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions that are conducive to clinical expression of ISA, 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 of ISA, *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), 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 5.10.

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), 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 5.10.

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 a country, zone or compartment free from 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.
- 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/pubs/Miscellaneous/ICESCodeofPractice.pdf>) 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;

Annex 10 (contd)

- h) release SPF F-1 stock from *quarantine* for *aquaculture* or stocking purposes in the country, *zone* or *compartment*.
- 4) With respect to point 3(e), *quarantine* conditions should be conducive to multiplication of the pathogen and eventually to clinical expression. If *quarantine* conditions are not suitable for pathogen multiplication and development, the recommended diagnostic approach might not be sensitive enough to detect low *infection* level.

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

## 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 a country, zone or compartment free from ISAV are for any detectable ISAV, including HPR0 ISAV.

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

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

For these *commodities* Members 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 a country, zone or compartment free from 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* 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 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 a country, zone or compartment free from 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. (as applicable), 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 5.10.

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 a country, zone or compartment free from ISAV are for any detectable ISAV, including HPR0 ISAV.

- 1) *Competent Authorities* should not require any infection with ISAV related conditions, regardless of the ISA 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 5.3.2.:
  - a) fish fillets or steaks (frozen or chilled).

**EU position**

**The EU suggests replacing the "a)" in the point above by an "-", as there is no "b)" below.**

Certain assumptions have been made in assessing the safety of aquatic animals and aquatic animal products listed above. Members should refer to these assumptions at Article 5.3.2. and consider whether the assumptions apply to their conditions.

For these *commodities* Members 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 ISA, 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 a country, zone or compartment free from 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:
- 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.
- OIE Members 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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## CHAPTER 2.3.2.

**INFECTION WITH APHANOMYCES INVADANS**  
**(EPIZOOTIC ULCERATIVE SYNDROME)**

**EU position**

**The EU thanks the OIE and supports the adoption of this modified chapter.**

## 1. Scope

For the purposes of this chapter infection with *Aphanomyces invadans* means all infections caused by the oomycete fungus *A. invadans* (syn. *A. piscicida*).

~~Epizootic ulcerative syndrome (EUS) is considered to be an infection with the oomycete known as *Aphanomyces invadans* (synonym: *A. piscicida*) and characterised histologically by penetrating hyphae surrounded by granulomatous inflammation. It is an epizootic condition of wild and farmed freshwater and estuarine fish.~~

## 2. Disease information

## 2.1. Agent factors

## 2.1.1. Aetiological agent, agent strains

~~EUS infection with *A. invadans* is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish. It has a complex infectious aetiology and is clinically characterised by the presence of invasive *A. invadans* infection and necrotising ulcerative lesions, typically leading to a granulomatous response. Infection with *A. invadans* is most commonly known as epizootic ulcerative syndrome (EUS). It is also known as red spot disease (RSD), mycotic granulomatosis (MG) and ulcerative mycosis (UM). In 2005, scientists proposed that EUS—the disease should be named as epizootic granulomatous aphanomycosis or EGA (Baldock *et al.*, 2005); however, the term EUS continues to be ~~has been~~ used by most scientists. The disease is caused by the oomycete fungus that causes EUS is known as *Aphanomyces invadans*. ~~EUS infection with *A. invadans* has spread widely since the first outbreak in 1971 in Japan and to date only one genotype has been recorded. Parasites and rhabdoviruses have also been associated with particular outbreaks, and secondary Gram-negative bacteria invariably infect EUS-lesions caused by *A. invadans*.~~~~

The genus *Aphanomyces* is a member of a group of organisms commonly known as the water moulds. Although long regarded as a fungus because of its characteristic filamentous growth, this group, the Oomycetida, is not a member of the Eumycota, but is classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

## 2.1.2. Survival outside the host

How *A. invadans* survives outside the host is still unclear. If the motile zoospore cannot find suitable substrates, it will encyst. There is no suitable method to recover or isolate the encysted zoospore from affected in EUS infected fish ponds. How long the encysted spore can survive in water or on a non-fish substrate is still unclear. In an *in-vitro* experiment, the encysted zoospore survived for at least 19 days (Lilley *et al.*, 2001).

## 2.1.3. Stability of the agent (effective inactivation methods)

*Aphanomyces invadans* grows best at 20–30°C; it does not grow *in-vitro* at 37°C. Water salinity over 2 parts per thousand (ppt) can stop spread of the agent. Preparing fish ponds by sun-drying and liming are effective disinfection methods for EUS *A. invadans*. Similar to other oomycetes or water molds,

general disinfection chemicals effectively destroy any *A. invadans* that might contaminate farms, fish ponds or fishing gear.

#### 2.1.4. Life cycle

*Aphanomyces invadans* (Saprolegniales, Oomycetes) has an aseptate fungal-like mycelia structure. This oomycete has two typical zoospore forms. The primary zoospore consists of round cells that develop inside the sporangium. The primary zoospore is released to the tip of the sporangium where it forms a spore cluster. It quickly transforms into the secondary zoospore, which is reniform with laterally biflagellate cells and can swim freely in the water. The secondary zoospore remains motile for a period that depends on the environmental conditions and presence of the fish host or substratum. Typically, the zoospore encysts and germinates to produce new hyphae, although further tertiary generations of zoospores may be released from cysts (polyplanetism) (Lilley *et al.*, 1998).

## 2.2. Host factors

### 2.2.1. Susceptible host species

EUS-*A. invadans* causes disease and mortality in farmed and wild fish, worldwide. Around 94 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS-*A. invadans* as shown in Table 2.1. Suspect cases of natural infection with *A. invadans* in species other than those listed should be referred immediately to the appropriate OIE Reference Laboratory, whether or not clinical signs are associated with the findings. Some fish, such as common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and milk fish (*Chanos chanos*), have been considered to be naturally resistant to EUS-infection with *A. invadans* (Lilley *et al.*, 1998).

**Table 2.1.** Fish species susceptible to EUS-infection with *Aphanomyces invadans*

Scientific name	Common name	Scientific name	Common name
<i>Acanthopagrus australis</i>	yellowfin sea bream	<i>Macquaria ambigua</i>	golden perch
<i>Acanthopagrus berda</i>	black bream	<i>Macquaria novemaculeata</i>	Australian bass
<i>Alosa sapidissima</i>	American shad	<i>Marcusenius macrolepidotus</i>	bulldog
<i>Ambassis agassiz</i>	chanda perch	<i>Melanotaenia splendida</i>	rainbow fish
<i>Ameiurus melas</i>	black bullhead	<i>Micralestes acutidens</i>	silver robber
<i>Ameiurus nebulosus</i>	Brown bullhead	<i>Micropterus salmoides</i>	largemouth black bass
<i>Amniataba percoides</i>	striped grunter	<i>Mugil cephalus</i>	grey mullet or striped mullet
<i>Anabas testudineus</i>	climbing perch	<i>Mugil curema</i>	white mullet
<i>Archosargus probatocephalus</i>	sheepshead	<i>Mugilidae (Mugil spp.; Liza spp.)</i>	mullet
<i>Arius sp.</i>	fork-tailed catfish	<i>Myxus petard</i>	mullet
<i>Aseraggodes macleayanus</i>	narrow banded sole	<i>Nematalosa erebi</i>	bony bream
<i>Bairdiella chrysoura</i>	drums or croakers	<i>Oncorhynchus mykiss</i>	rainbow trout
<i>Barbus peludinosus</i>	straightfin barb	<i>Oreochromis andersoni</i>	three-spotted tilapia
<i>Barbus poecheii</i>	dashtail barb	<i>Oreochromis machrochir</i>	greenhead tilapia
<i>Barbus thamalakaneensis</i>	Thamalakane barb	<i>Osphronemus goramy</i>	giant gourami
<i>Barbus unitaeniatus</i>	longbeard barb	<i>Oxyeleotris lineolatus</i>	sleepy cod
<i>Bidyanus bidyanus</i>	silver perch	<i>Oxyeleotris marmoratus</i>	marble goby
<i>Brevoortia tyrannus</i>	Atlantic menhaden	<i>Petrocephalus catostoma</i>	churchill
<i>Brycinus lateralis</i>	striped robber	<i>Platycephalus fuscus</i>	dusky flathead
<i>Carassius auratus auratus</i>	goldfish	<i>Plecoglossus altivelis</i>	ayu
<i>Catla catla</i>	catla	<i>Pogonias cromis</i>	black drum
<i>Channa marulius</i>	great snakehead fish	<i>Psettodes sp.</i>	spiny turbot
<i>Channa striatus</i>	striped snakehead	<i>Puntius gonionotus</i>	silver barb
<i>Cirrhinus mrigala</i>	mrigal	<i>Puntius sophore</i>	pool barb
<i>Clarias gariepinus</i>	sharp-tooth African catfish	<i>Rohtee sp.</i>	keti-Bangladeshi
<i>Clarias ngamensis</i>	blunt-toothed African catfish	<i>Sargochromis carlottae</i>	rainbow bream
<i>Clarius batrachus</i>	walking catfish	<i>Sargochromis codringtonii</i>	green bream



**Table 2.1. cont.** Fish species susceptible to EUS-infection with *Aphanomyces invadans*

Scientific name	Common name	Scientific name	Common name
<i>Colisa lalia</i>	dwarf gourami	<i>Sargochromis giardi</i>	pink bream
<i>Esomus</i> sp.	flying barb	<i>Scatophagus argus</i>	spotted scat
<i>Fluta alba</i>	swamp eel	<i>Schilbe intermedius</i>	silver catfish
<i>Glossamia aprion</i>	mouth almighty	<i>Schilbe mystus</i>	African butter catfish
<i>Glossogobius giurus</i>	bar-eyed goby	<i>Scleropages jardini</i>	saratoga
<i>Glossogobius</i> sp.	goby	<i>Scortum barcoo</i>	Barcoo Grunter
<i>Helostoma temmincki</i>	kissing gourami	<i>Selenotoca multifasciata</i>	striped scat
<i>Hepsetus odoe</i>	African pike	<i>Serranochromis angusticeps</i>	thinface largemouth
<i>Hydrocynus vittatus</i>	tigerfish	<i>Serranochromis robustus</i>	Nembwe
<i>Ictalurus punctatus</i>	channel catfish	<i>Sillago ciliata</i>	sand whiting
<i>Kurtus gulliveri</i>	nursery fish	<i>Siluridae</i> (genus)	wells catfish
<i>Labeo cylindricus</i>	red-eye labeo	<i>Strongylura krefftii</i>	long tom
<i>Labeo lunatus</i>	upper Zambezi labeo	<i>Therapon</i> sp.	therapon
<i>Labeo rohita</i>	rohu	<i>Tilapia rendalli</i>	redbreast tilapia
<i>Lates calcarifer</i>	barramundi or sea bass	<i>Tilapia sparrmanii</i>	banded tilapia
<i>Leiopotherapon unicolor</i>	spangled perch	<i>Toxotes chatareus</i>	common archer fish
<i>Lepomis macrochirus</i>	bluegill	<i>Toxotes lorentzi</i>	primitive acher fish
<i>Lutjanus argentimaculatus</i>	mangrove jack	<i>Trichogaster pectoralis</i>	snakeskin gourami
<i>Macchullochella peelii</i>	Murray cod	<i>Trichogaster trichopterus</i>	three-spot gourami
<i>Maccullochela ikei</i>	freshwater cod	<i>Tridentiger obscures</i>	dusky tripletooth goby
		<i>obscures</i>	

#### 2.2.2. Susceptible stages of the host

The susceptible life stages of the fish are usually juvenile and young adults. There is no report of EUS *A. invadans* being found in fish fry or fish larvae.

An experimental injection of *A. invadans* into the yearling life stage of Indian major carp, catla, rohu and mrigal, revealed resistance to EUS-*A. invadans* (Pradhan *et al.*, 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish are susceptible (Hatai *et al.*, 1977; 1994), but common carp (Wada *et al.*, 1996), Nile tilapia (Khan *et al.*, 1998) and European eel, *Anguilla anguilla*, (Oidtmann *et al.*, 2008) are resistant.

#### 2.2.3. Species or subpopulation predilection (probability of detection)

EUS-*Aphanomyces invadans* can be readily detected with histological techniques from diseased fish specimens that are collected from affected EUS-infected areas using histological techniques. However, *A. invadans* can be isolated only from fish with mild or moderate clinical signs of EUS-infection with *A. invadans*, exhibiting red spots or small ulcers. Fish with severe clinical signs or large ulcers are not suitable for isolation.

#### 2.2.4. Target organs and infected tissue

The motile zoospore plays an important role in the spread of the disease. Once the motile spore attaches to the skin of the fish, the spore will germinate under suitable conditions and its hyphae will invade the fish skin, muscular tissue and reach the internal organs. Fish skeletal muscle is the target organ and exhibits major EUS-clinical signs of infection with *A. invadans* with mycotic granulomas.

Annex 11 (contd)**2.2.5. Persistent infection with lifelong carriers**

There is no information to indicate that fish can be lifelong carriers of *A. invadans*. Generally, most infected fish die during an outbreak. Although some fish with mild or moderate EUS-infected fish infections could recover, they are unlikely to be lifelong carriers of *A. invadans*.

**2.2.6. Vectors**

No data available.

**2.2.7. Known or suspected wild aquatic animal carriers**

No data available.

**2.3. Disease pattern****2.3.1. Transmission mechanisms**

Infection with *A. invadans* is transmitted horizontally. The ~~*Aphanomyces*~~ *A. invadans* zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the secondary zoospores or free-swimming stage zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the secondary zoospores cannot find the susceptible species or encounter unfavourable conditions, they can encyst in the pond environment. The cysts may wait for conditions that favour their transformation into tertiary generations of zoospores that are also in the free-swimming stage. The encysting property of *A. invadans* ~~the *Aphanomyces* pathogen~~ may play an importance role in the cycle of outbreaks in endemic areas.

**2.3.2. Prevalence**

The prevalence of EUS-infection with *A. invadans* in the wild and in aquaculture farms may be high in endemic areas when high levels of mortality are observed, but can vary substantially. There is very limited data on prevalence of disease or infection at other times. Uncontrolled water exchange in fish farms in endemic areas will result in EUS-infection with *A. invadans* outbreaks in most of the farms that culture susceptible fish species.

**2.3.3. Geographical distribution**

EUS-Infection with *A. invadans* was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Oita Prefecture, Kyushu Island, Japan in 1971 (Egusa & Masuda, 1971). It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972 (Fraser *et al.*, 1992; McKenzie & Hall, 1976). EUS-Infection with *A. invadans* has extended its range through Papua New Guinea into South-East and South Asia, and into West Asia, where it has reached Pakistan (Lilley *et al.*, 1998; Tonguthai, 1985). Outbreaks of ulcerative disease in menhaden (*Brevoortia tyrannus*) in the United States of America (USA) had the same aetiological agent as the EUS-disease observed in Asia (Blazer *et al.*, 1999; Lilley *et al.*, 1997a; Vandersea *et al.*, 2006). The first confirmed outbreaks of infection with *A. invadans* on the African continent occurred in 2007 in Botswana, Namibia and Zambia, and were connected to the Zambezi-Chobe river system (FAO, 2009). ~~Recently~~ In 2010 and 2011, EUS-infection with *A. invadans* appeared in wild freshwater fish in Western Cape Province, South Africa and in wild brown bullhead fish in Lake Ontario in the Province of Ontario, Canada. EUS Infection with *A. invadans* has been reported from more than 20 countries in four continents: North America, Southern Africa, Asia and Australia.

**2.3.4. Mortality and morbidity**

When EUS-infection with *A. invadans* spreads into a fish culture pond, such as a snakehead fish pond, high morbidity (>50%) and high mortality (>50%) might be observed in those years that have a long cold season, with water temperatures between 18 and 22°C. However, mortality and morbidity may vary greatly depending on the fish species. Some infected fish may recover when the cold period is over.

### 2.3.5. Environmental factors

EUS-infection with *A. invadans* occurs mostly at water temperatures of 18–22°C and after periods of heavy rainfall (Bondad-Reantaso *et al.*, 1992). These conditions favour sporulation of *A. invadans* (Lumanlan-Mayo *et al.*, 1997), and temperatures of 17–19°C have been shown to delay the inflammatory response of fish to oomycete infection (Catap & Munday, 1998; Chinabut *et al.*, 1995). In some countries, outbreaks occur in wild fish first and then spread to fish ponds. Normally, a bath infection of *A. invadans* in healthy susceptible fish species does not result in clinical signs of disease. The *A. invadans* Aphanomyces-oomycete needs predisposing factors that lead to skin damage, such as parasites, bacteria or virus infection or acid water, to initiate the clinical signs of the EUS disease (Lilley *et al.*, 1998).

EUS-infection with *A. invadans* has been reported from more than 20 countries on four continents. Movements of live ornamental fish from EUS-infection with *A. invadans* - infected countries might spread the disease as was the case with the outbreak in Sri Lanka (Balasuriya, 1994). Flooding also caused the spread of EUS-infection with *A. invadans* in Bangladesh and Pakistan (Lilley *et al.*, 1998). Once an outbreak occurs in rivers/canals, the disease can spread downstream as well as upstream where the susceptible fish species exist. Ensuring that water from infected rivers does not come into contact with fish culture ponds could prevent the spread of EUS-the disease.

## 2.4. Control and prevention

### 2.4.1. Vaccination

There is no protective vaccine available. However, snakehead fish that had been immunised with a crude extract of the *A. invadans* elicited humoral immune response as detected by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and Western blot analysis (Thompson *et al.*, 1997).

### 2.4.2. Chemotherapy

There is no effective treatment for EUS *A. invadans*-infected fish in the wild and in aquaculture ponds. To minimise fish losses in infected fish ponds water exchange should be stopped and lime or hydrated lime and/or salt should be applied (Lilley *et al.*, 1998). Attempts at using green water, ash, lime, and neem seeds or branches (*Azadirachta indica*) for prophylactic treatments of the *A. invadans* EUS-infected fish in fish ponds gave variable results (Inland Aquatic Animal Health Research Institute [AAHRI], Thailand, internal report, 2001).

### 2.4.3. Immunostimulation

Preliminary experiments showed that intraperitoneal injection of the immunostimulant, Salar-bec (containing 300 g kg<sup>-1</sup> vitamin C, 150 g kg<sup>-1</sup> vitamin B and trace quantities of vitamins B1, B2, B6 and B12), into snakehead fish can increase serum inhibition of germination and growth of the zoospore *in vitro*. Snakehead fish fed on normal pellet feed and Salar-bec-supplemented feed still exhibited clinical signs of EUS-infection with *A. invadans* after challenge with *A. invadans*. However, snakehead fish that received the immunostimulant, Salar-bec, showed a relative per cent survival of 59.2% higher than the control group that received normal feed (Miles *et al.*, 2001).

### 2.4.4. Resistance breeding

No data available.

### 2.4.5. Restocking with resistant species

Some important culture species, including Nile tilapia, milk fish and Chinese carp, have been shown to be resistant to EUS-infection with *A. invadans* and could be cultured in endemic areas. Introducing resistant indigenous fish species is recommended.

Annex 11 (contd)**2.4.6. Blocking agents**

No data available.

**2.4.7. Disinfection of eggs and larvae**

Routine disinfection of fish eggs and larvae against water molds is ~~equally~~ effective against *A. invadans*. It should be noted that there is no report of the presence of *A. invadans* in fish eggs or larvae.

**2.4.8. General husbandry practices**

Control of ~~EUS-*A. invadans*~~ in natural waters is probably impossible. In outbreaks occurring in small, closed water-bodies or fish ponds, liming water with agricultural limes and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease. Ensuring no leakage of water from ~~EUS-*A. invadans*~~-infected areas into fish ponds is a normal practice that easily prevents the spread of ~~EUS-the disease~~ into farms. Sodium chloride or salt and agricultural lime are safe and effective chemicals for treating or preventing the spread of ~~EUS-*A. invadans*~~.

**3. Sampling****3.1. Selection of individual specimens**

Scoop net, cast net or seine net represent the best choices for catching ~~EUS-infected-diseased~~ fish in natural waters or in fish ponds. For outbreak investigations, diseased fish with ulcerative lesions or red spots on the body should be sampled.

**3.2. Preservation of samples for submission**

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Fish collected from remote areas should be anaesthetised and can be fixed in normal 10% formalin or 10% phosphate-buffered formalin for at least 1–2 days. The fixed specimens are then transferred to double-layer plastic bags with formalin-moistened tissue paper. The bags containing moist specimens are sealed and sent to the laboratory ~~in semi-dry conditions~~.

**3.3. Pooling of samples**

Ten diseased fish specimens are sampled from the site affected by infection with *A. invadans* ~~EUS-infected-site~~. Diagnosis is achieved using the histological technique and oomycete isolation on individual fish or a group of a few fish.

**3.4. Best organs or tissues**

Fish with minor clinical signs are recommended and the muscle tissue next to or underneath the ulcer is best for oomycete isolation. The best tissue for histopathological examination is muscle tissue at the edge of the ulcers.

**3.5. Sample/tissues that are not suitable**

Severely diseased or dead fish are not suitable for oomycete isolation.

**4. Diagnostic methods**

Diagnosis of EUS-infection with *A. invadans* in clinically affected fish may be achieved by histopathology, oomycete isolation or polymerase chain reaction amplification.

#### 4.1. Field diagnostic methods

EUS-infection with *A. invadans* outbreaks have been associated with mass mortality of various species of freshwater fish in the wild (including rice-fields, estuaries, lakes and rivers) and in farms during periods of low temperatures and after periods of heavy rainfall.

##### 4.1.1. Clinical signs

Fish usually develop red spots or small to large ulcerative lesions on the body.

##### 4.1.2. Behavioural changes

The early signs of the disease include loss of appetite and fish become darker. Infected fish may float near the surface of the water, and become hyperactive with a very jerky pattern of movement.

#### 4.2. Clinical methods

##### 4.2.1. Gross pathology

Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Most species other than striped snakeheads and mullet will die at this stage. In highly susceptible species, such as snakehead, the lesions are more extensive and can lead to complete erosion of the posterior part of the body, or to necrosis of both soft and hard tissues of the cranium, so that the brain is exposed in the living animal.

##### 4.2.2. Clinical chemistry

No information available.

##### 4.2.3. Microscopic pathology

Early EUS-lesions are caused by erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesion progresses from a mild chronic active dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae. Lesion scrapes from fish body or ulcers generally show secondary fungal, bacterial and/or parasitic infections.

##### 4.2.4. Wet mounts

Not suitable for EUS-infection with *A. invadans* diagnosis.

##### 4.2.5. Smears

Not suitable for EUS-infection with *A. invadans* diagnosis.

##### 4.2.6. Electron microscopy/cytopathology

Not suitable for EUS-infection with *A. invadans* diagnosis.

#### 4.3. Agent detection and identification methods

##### 4.3.1. Direct detection methods

###### 4.3.1.1. Microscopic methods

The squash preparation can be carried out as follows:

- i) Remove ulcer surface using a sharp scalpel blade.

Annex 11 (contd)

- ii) Cut the muscular tissue at the edge of the ulcer.
- iii) Place the pieces of tissue on a cutting board then make thin slices using a sharp scalpel blade.
- iv) Place the thinly sliced tissue between two glass slides and squeeze gently with fingers.
- v) Remove one of the glass slides and cover the tissue with a cover-slip. View under a light microscope to find the nonseptate hyphae structure of *A. invadans* (12–25 µm in diameter).

4.3.1.1.1. *Wet mounts*

Not suitable for ~~EUS~~ infection with *A. invadans* diagnosis

4.3.1.1.2. *Smears*

Not suitable for ~~EUS~~ infection with *A. invadans* diagnosis

4.3.1.1.3. *Fixed sections*

## Sampling procedure

- i) Sample only live or moribund specimens of fish with clinical lesions.
- ii) Take samples of skin/muscle (<1 cm<sup>3</sup>), including the leading edge of the lesion and the surrounding tissue.
- iii) Fix the tissues immediately in 10% formalin. The amount of formalin should be 10 times the volume of the tissue to be fixed.

*Histological procedure*

Processing the fixed tissue involves dehydration through ascending alcohol grades, clearing in a wax-miscible agent and impregnation with wax. The blocks of fish tissue are cut at about 5 µm and mounted on a glass slide. Before staining, the section must be completely de-waxed and stained in haematoxylin and eosin (H&E) (Chinabut & Roberts, 1999). H&E and general fungus stains (e.g. Grocott's stain) will demonstrate typical granulomas and invasive hyphae.

**4.3.1.2. Agent isolation and identification**4.3.1.2.1. *Isolation of *Aphanomyces invadans* from internal tissues*

The following are two methods of isolation of *A. invadans* or *A. piscicida* adapted from Lilley *et al.* (1998) and Willoughby & Roberts (1994).

*Method 1:* Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through the stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 mm<sup>3</sup>, and place on a Petri dish containing glucose/peptone (GP) agar (see Table 4.1) with penicillin G (100 units ml<sup>-1</sup>) and streptomycin (100 µg ml<sup>-1</sup>). Seal plates, incubate at room temperature or at 25°C and examine daily. Repeatedly transfer emerging hyphal tips on to fresh plates of GP agar with antibiotics until cultures are free of contamination.

## Annex 11 (contd)

*Method 2:* Lesions located on the flank or tail of fish <20 cm in length can be sampled by cutting the fish in two using a sterile scalpel, and slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2–4 mm<sup>3</sup>) from beneath the lesion and place it in a Petri dish of GP medium (see Table 4.1) with 100 units ml<sup>-1</sup> penicillin G and 100 µg ml<sup>-1</sup> streptomycin. Instruments should not contact the contaminated external surface of the fish. Incubate inoculated media at approximately 25°C and examine under a microscope (preferably an inverted microscope) within 12 hours. Repeatedly transfer emerging hyphal tips to plates of GP medium with 12 g litre<sup>-1</sup> technical agar, 100 units ml<sup>-1</sup> penicillin G and 100 µg ml<sup>-1</sup> streptomycin until axenic cultures are obtained. The oomycete isolate can also be maintained at 25°C on GY agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

#### 4.3.1.2.2. Identification of *Aphanomyces invadans*

*Aphanomyces invadans* does not produce any sexual structures and should thus not be diagnosed by morphological criteria alone. However, the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces* spp., as described in Lilley *et al.*, 1998. *Aphanomyces invadans* is characteristically slow growing in culture and fails to grow at 37°C on GPY agar (Table 4.1). Detailed temperature–growth profiles are given in Lilley & Roberts (1997). Two procedures that can be used to confirm *A. invadans* are bioassay and polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

#### 4.3.1.2.3. Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing GPY broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water (Table 4.1), and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope.

#### 4.3.1.2.4. Bioassay

Fish can be experimentally infected by intramuscularly injecting a 0.1 ml suspension of 100+ motile zoospores into ~~EUS-susceptible fish~~ susceptible to infection with *A. invadans* (preferably *Channa striata* or other susceptible species) at 20°C. Histological growth of aseptate hyphae, 12–25 µm in diameter, should be demonstrated in the muscle of fish sampled after 7 days, and typical mycotic granulomas should be demonstrated in the muscle of fish sampled after 10–14 days.

#### 4.3.1.2.5. Antibody-based antigen detection methods

Polyclonal antibodies against *A. invadans* or *Aphanomyces* saprophyte showed cross reactivity to each other using protein gel electrophoresis and Western blot analysis and immunohistochemistry. (Lilley *et al.*, 1997b). However a specific monoclonal antibody against *A. invadans* developed later was found to have high specificity and high sensitivity to ~~the *Aphanomyces* pathogens of the EUS~~ *A. invadans* using immunofluorescence. This monoclonal antibody could detect *A. invadans* ~~*Aphanomyces*~~-hyphae at the early stage of ~~the EUS~~ infection (Miles *et al.*, 2003).

#### 4.3.1.2.6. Molecular techniques

##### 4.3.1.2.6.1. Polymerase chain reaction amplification of the DNA of *A. invadans*

##### DNA preparation from *A. invadans* isolate

DNA is extracted from an actively growing colony of *A. invadans* culture in GY broth at about 4 days or when young mycelia reach 0.5–1.0 cm in diameter. The mycelia are transferred to sterile 100-mm Petri dishes, washed twice with PBS and then placed on tissue paper for liquid removal. Hyphal tips (~50–250 mg) are excised with a sterile scalpel blade and transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Commercial DNA extraction kits have been used successfully (Phadee *et al.*, 2004b; Vandersea *et al.*, 2006).

Annex 11 (contd)*DNA preparation from A. invadans ~~EUS~~-infected tissue*

Small pieces of *A. invadans* ~~EUS~~-infected tissue (25–50 mg) are suitable for DNA extractions (Phadee *et al.*, 2004a).

*Diagnostic PCR technique*

Three published techniques are specific to *A. invadans*. It is recommended to use ultra-pure water for all chemical dilutions in the PCR reaction.

*Method 1:* The species-specific forward primer site is located near the 3' end of the SSU (small subunit) gene and a species-specific reverse primer site is located in the ITS1 region for Ainvad-2F (5'-TCA-TTG-TGA-GTG-AAA-CGG-TG-3') and Ainvad-ITSR1 (5'-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3'). The PCR mixture contained 25 pM of each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase and 20 ng of genomic DNA (either from an *Aphanomyces* isolate or from infected tissue) for a total volume of 50 µl. DNA is amplified in a thermocycle machine under the following cycle conditions: 2 minutes at 95°C; 35 cycles, each consisting of 30 seconds at 95°C, 45 seconds at 56°C, 2.5 minutes at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 234 bp (Vandersea *et al.*, 2006).

*Method 2:* The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is ITS11 (5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3') and the reverse is ITS23 (5'-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3'). The PCR mixture contains 0.5 µM of each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl<sub>2</sub>, 0.6 U of *Taq* DNA polymerase and 20 ng of genomic DNA (from an *Aphanomyces* isolate) for a total volume of 25 µl. The DNA is amplified under the following cycle conditions: 5 minutes at 94°C; 25 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 550 bp. PCR amplification using the DNA template from the infected tissue is similar to the above protocol except that 5 ng of the DNA template is used for 35 cycles (Phadee *et al.*, 2004b).

*Method 3:* The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is BO73 (5'-CTT-GTG-CTG-AGC-TCA-CAC-TC-3') and the reverse is BO639 (5'-ACA-CCA-GAT-TAC-ACT-ATC-TC-3'). The PCR mixture contains 0.6 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl<sub>2</sub>, 0.625 units of *Taq* DNA polymerase, and approximately 5 ng of genomic DNA (or 2.5 µl of DNA template extracted from 25 mg of infected tissue and suspended in 100 µl buffer) in a 50 µl reaction volume (Oidtmann *et al.*, 2008). The DNA is amplified under the following cycle conditions: 96°C for 5 minutes; 35 cycles of 1 minute at 96°C, 1 minute at 58°C and 1 minute at 72°C; followed by a final extension at 72°C for 5 minutes (Oidtmann, pers. comm.). The PCR product is analysed by agarose gel electrophoresis and the target product is 564 bp.

All *A. invadans* isolates found so far belong to a single genotype, and this facilitates identification. Alternatively, sequencing of the PCR products can be performed and the results can be compared with the sequence deposited in the public gene data banks. *A. invadans* has similar characteristics to *A. astaci*, the aetiological agent of crayfish plague. Both pathogenic oomycetes can be differentiated using molecular tools (Diéguez-Urbeondo *et al.*, 2009; Lilley *et al.*, 2003; Phadee *et al.*, 2004b; Vandersea *et al.*, 2006).

4.3.1.2.6.2. *Fluorescent peptide nucleic acid in-situ hybridisation (FISH)*

A fluorescent peptide nucleic acid *in-situ* hybridisation (FISH) technique has demonstrated a high specificity for *A. invadans*. The technique can directly detect the mycelia-like structure of the oomycete in thinly sliced tissues of affected organs of the susceptible fish. The fluorescein (FLU) probe designed to hybridise the small subunit of the rRNA *A. invadans* (bp 621 to 635; GenBank acc. AF396684) is 5'-FLU-GTA-CTG-ACA-TTT-CGT-3' or Ainv-FLU3.



The EUS-*A. invadans*-affected tissue is fixed and hybridised as soon as possible after the fish are collected to minimise RNA degradation. Tissue (~20 mg) is dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtitre plate. One ml ethanol-saline fixative (44 ml of 95% ethanol, 10 ml of deionised H<sub>2</sub>O, and 6 ml of 25 × SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-enesorbite monolaurate (Tween 20) is added to enhance tissue permeabilization. The microtitre plate is gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 hours. The fixed tissues are rinsed (twice for 15 minutes each time) with 0.5 ml of hybridisation buffer (5 × SET, 0.1% [v/v] Igepal-CA630 and 25 µg ml<sup>-1</sup> poly[A]) containing 3% Tween 20. The hybridisation buffer is removed, and the tissues are resuspended in 0.5 ml of hybridisation buffer containing 3% Tween 20 and 100 nM Ainv-FLU3 probe. "No-probe" control specimens are incubated with 0.5 ml of hybridisation buffer/3% Tween 20. All tissues are incubated at 60°C for 1 hour in the dark. Following incubation, the tissues are rinsed twice with 1 ml of pre-warmed (60°C) 5 × SET buffer containing 3% Tween 20 to remove residual probe. The tissue specimens are mounted onto poly-L-lysine-coated microscope slides. One drop of the light anti-fade solution is placed on the specimens, which are then overlaid with a cover-slip. Analyses are performed by light and epifluorescence microscopy. The camera and microscope settings for epifluorescent analyses are held constant so that comparative analyses of relative fluorescence intensity can be made between probed and non-probed specimens. The fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The above detailed protocols are published by Vandersea *et al.* (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed tissue.

**Table 4.1.** Media for isolation, growth and sporulation of *Aphanomyces invadans* cultures

GP (glucose/peptone) medium	GPY (glucose/peptone/yeast) broth	GPY agar	GY agar	Autoclaved pond water
3 g litre <sup>-1</sup> glucose 1 g litre <sup>-1</sup> peptone 0.128 g litre <sup>-1</sup> MgSO <sub>4</sub> ·7H <sub>2</sub> O 0.014 g litre <sup>-1</sup> KH <sub>2</sub> PO <sub>4</sub> 0.029 g litre <sup>-1</sup> CaCl <sub>2</sub> ·2H <sub>2</sub> O 2.4 mg litre <sup>-1</sup> FeCl <sub>3</sub> ·6H <sub>2</sub> O 1.8 mg litre <sup>-1</sup> MnCl <sub>2</sub> ·4H <sub>2</sub> O 3.9 mg litre <sup>-1</sup> CuSO <sub>4</sub> ·5H <sub>2</sub> O 0.4 mg litre <sup>-1</sup> ZnSO <sub>4</sub> ·7H <sub>2</sub> O	GP broth + 0.5 g litre <sup>-1</sup> yeast extract	GPY broth + 12 g litre <sup>-1</sup> technical agar	1% glucose, 0.25% yeast extract, 1.5% agar	Sample pond/lake water known to support oomycete growth. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave. pH to 6–7.

#### 4.3.1.2.7. Agent purification

Maintaining *A. invadans* in the axenic culture is necessary. As it is characteristically slow growing, it easily becomes contaminated with other micro-organisms, such as bacteria and other fast-growing oomycetes and fungi. Attempts to purify or isolate *A. invadans* from contaminated cultures usually fail.

#### 4.3.2. Serological methods

Serological methods for detection and identification of *A. invadans* in EUS-diseased specimens are not practical. If necessary, the monoclonal antibody offers a better specificity and sensitivity than polyclonal antibody for serological detection or identification of *A. invadans* in diseased specimens or in pathogen isolates.

## Annex 11 (contd)

## 5. Rating of tests against purpose of use

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

*Table 5.1. Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Fish fry	Juveniles	Adults		
Gross signs	c	c	c	b	c
Direct LM; observation of the oomycete hyphae in tissues, fresh squash	d	d	d	b	b
FISH; observation of the oomycete hyphae in tissues	d	d	d	a	a
Histopathology	c	c	c	a	a
Isolation of <i>A. invadans</i> and confirmatory identification by bioassay or PCR	d	d	d	a	a
PCR of tissue extracts	d	d	d	a	a
Sequence analysis	d	d	d	d	a
Transmission EM of tissues	d	d	d	d	d

LM = light microscopy; FISH = fluorescent peptide nucleic acid *in-situ* hybridisation;  
PCR = polymerase chain reaction; EM = electron microscopy.

## 6. Test(s) recommended for targeted surveillance to declare freedom from epizootic ulcerative syndrome

The test for targeted surveillance to declare freedom from EUS-infection with *A. invadans* is examination of gross signs. Targeted surveillance is conducted twice a year to cover the range of seasonal variation, at least once during the season that favours EUS-infection with *A. invadans* occurrence or when water temperatures are about 18–25°C. Biosecurity measures should be implemented to maintain disease-free status in controlled aquaculture facilities or compartments.

Using the gross sign test for targeted surveillance, a large number of the fish should be examined without killing them. Fish on farms, in compartments or in natural water bodies should be sampled carefully using suitable gear or nets. The suitable numbers of fish specimens examined should be based on details described in the OIE *Guide for Aquatic Animal Health Surveillance* (2009).

Once fish show similar gross signs to EUS-infection with *A. invadans*, they should be categorised as suspect EUS fish, and the location/farm/compartments/zone should be considered suspect. Suspect specimens should be further tested using the methods listed under presumptive diagnosis followed by confirmative diagnosis as described in the Table 5.1.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

~~A suspect case of EUS disease is defined as the presence of typical clinical signs, a single or multiple red spot(s) or ulcer(s) on the body, in a population of susceptible fish at water temperatures between 18 and 25°C OR the presence of mycotic granulomas in muscle tissue OR the presence of branching non-septate oomycete hyphae in a muscle squash preparation OR the isolation of slow-growing *Aphanomyces* without further identification of the agent.~~

A positive result obtained by any of the diagnostic techniques described in Section 4. should be considered suspect.

### 7.2. Definition of confirmed case

~~A confirmed case of EUS is defined as a suspect case that has been identified as positive by the PCR or FISH detection techniques OR that *Aphanomyces invadans* has been isolated and confirmed by either bioassay, PCR, or sequence analysis.~~

In susceptible species within the known geographical range of infection with *A. invadans*, a confirmed case of infection with *A. invadans* is a positive result by observation of mycotic granulomas in histopathology.

In other host species or outside the known range of *A. invadans*, confirmation by histopathology and PCR is recommended.

## 8. References

BALDOCK F.C., BLAZER V., CALLINAN R., HATAI K., KARUNASAGAR I., MOHAN C.V. & BONDAD-REANTASO M.G. (2005). Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): Re-examination of causal factors, case definition and nomenclature. *In: Diseases in Asian Aquaculture V*, Walker P., Laster R. & Bondad-Reantaso M.G., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 555–585.

BALASURIYA L.K.S.W. (1994). Epizootic ulcerative syndrome in fish in Sri Lanka, country status report. *In: Proceeding of the ODA Regional Seminar on Epizootic Ulcerative*, Robert R.J., Campbell B. & MacRae I.H., eds. Aquatic Animal Health Research Institute, Bangkok, Thailand, pp 39–47.

BLAZER V.S., VOGELBEIN W.K., DENSMORE C.L., MAY E.B., LILLEY J.H. & ZWERNER D.E. (1999). *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. *J. Aquat. Anim. Health*, **11**, 340–349.

BONDAD-REANTASO M.G., LUMANLAN S.C., NATIVIDAD J.M. & PHILLIPS M.J. (1992). Environmental monitoring of the epizootic ulcerative syndrome (EUS) in fish from Munoz, Nueva Ecija in the Philippines. *In: Diseases in Asian Aquaculture 1*, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 475–490.

CATAP E.S. & MUNDAY B.L. (1998). Effects of variations of water temperature and dietary lipids on the expression of experimental epizootic ulcerative syndrome (EUS) in sand whiting, *Sillago ciliata*. *Fish Pathol.*, **33**, 327–335.

CHINABUT S. & ROBERTS R.J. (1999). Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS). Aquatic Animal Health Research Institute, Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33 pp. ISBN 974-7604-55-8.

CHINABUT S., ROBERTS R.J., WILLOUGHBY G.R. & PEARSON M.D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.*, **18**, 41–47.

Annex 11 (contd)

DIÉGUEZ-URIBEONDO J., GARCIA M.A., CERENIUS L., KOZUBÍKOVÁ E., BALLESTEROS I., WINDELS C., WEILAND J., KATOR H., SÖDERHÄLL K. & MARTÍN M.P. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genetics and Biology*, **46**, 365–376.

EGUSA S. & MASUDA N. (1971). A new fungal disease of *Plecoglossus altivelis*. *Fish Pathol.*, **6**, 41–46.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (2009). Report of the international emergency disease investigation task force on a serious fish disease in Southern Africa, 18-26 May 2007, FAO, Rome, Italy, 70 pp.

FRASER G.C., CALLINAN R.B. & CALDER L.M. (1992). *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.*, **15**, 173–181.

HATAI K. & EGUSA S. (1979). Studies on pathogenic fungus of mycotic granulomatosis III. Development of the medium for MG-fungus. *Fish Pathol.*, **13**, 147–152.

HATAI K., EGUSA S., TAKAHASHI S. & OOE K. (1977). Study on the pathogenic fungus of mycotic granulomatosis – I. Isolation and pathogenicity of the fungus from cultured-ayu infected with the disease. *Fish Pathol.*, **12**, 129–133.

HATAI K., NAKAMURA K., AN RHA S., YUASA K. & WADA S. (1994). *Aphanomyces* infection in dwarf gourami (*Colisa lalia*). *Fish Pathol.*, **29**, 95–99.

KHAN M.H., MARSHALL L., THOMPSON K.D., CAMPBELL R.E. & LILLEY J.H. (1998). Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. *Bull. Eur. Assoc. Fish Pathol.*, **18**, 192–197.

LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J. (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Bangkok, Thailand.

LILLEY J.H., HART D., PANYAWACHIRA V., KANCHANAKHAN S., CHINABUT S., SÖDERHÄLL K. & CERENIUS L. (2003). Molecular characterization of the fish-pathogenic fungus *Aphanomyces invadans*. *J. Fish Dis.*, **26**, 263–275.

LILLEY J.H., HART D., RICHARDS R.H., ROBERTS R.J., CERENIUS L. & SÖDERHALL K. (1997a). Pan-Asian spread of single fungal clone results in large scale fish kills. *Vet. Rec.*, **140**, 653–654.

LILLEY J.H., PETCHINDA T. & PANYAWACHIRA V. (2001). *Aphanomyces invadans* zoospore physiology: 4. *In vitro* viability of cysts. *The AAHRI Newsletter*, **10**, 1–4.

LILLEY J.H. & ROBERTS R.J. (1997). Pathogenicity and culture studies comparing the *Aphanomyces* INVOLVED IN EPIZOOTIC ULCERATIVE SYNDROME (EUS) WITH OTHER SIMILAR FUNGI. *J. Fish Dis.*, **20**, 135–144.

LILLEY J.H., THOMPSON K.D. & ADAMS A. (1997b). Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis. *Dis. Aquat. Org.*, **30**, 187–197.

LUMANLAN-MAYO S.C., CALLINAN R.B., PACLIBARE J.O., CATAP E.S. & FRASER, G.C. (1997). Epizootic ulcerative syndrome (EUS) in rice-fish culture systems: an overview of field experiments 1993-1995. *In: Diseases in Asian Aquaculture III*, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 129–138.

MCKENZIE R.A. & HALL W.T.K. (1976). Dermal ulceration of mullet (*Mugil cephalus*). *Aust. Vet. J.*, **52**, 230–231.

MILES D.J.V., POLCHANA J., LILLEY J.H., KANCHANAKHAN S., THOMPSON K.D. & ADAMS A. (2001). Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture*, **195**, 1–15.

MILES D.J.C., THOMPSON K.D., LILLEY J.H. & ADAMS A. (2003). Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody. *Dis. Aquat. Org.*, **55**, 77–84.

Annex 11 (contd)

OIDTMANN B., STEINBAUER GEIGER S. & HOFFMANN R.W. (2008). Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. *Dis. Aquat. Org.*, **82**, 185–207.

PHADEE P., KURATA O. & HATAI K. (2004a). A PCR method for the detection of *Aphanomyces piscicida*. *Fish Pathol.*, **39**, 25–31.

PHADEE P., KURATA O., HATAI K., HIRONO I. & AOKI T. (2004b). Detection and identification of fish-pathogenic *Aphanomyces piscicida* using polymerase chain reaction (PCR) with species-specific primers. *J. Aquat. Anim. Health*, **16**, 220–230.

PRADHAN P.K., MOHAN C.V., SHANKAR K.M., KUMAR B.M. & DEVARAJA G. (2007). Yearlings of Indian major carps resist infection against the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*. *Current Science*, **92**, 1430–1434.

THOMPSON K.D., LILLEY J.H., CHINABUT S. & ADAMS A. (1997). The antibody response of snakehead, *Channa striata* Bloch, to *Aphanomyces invadans*. *Fish & Shellfish Immunology*, **7**, 349–353.

TONGUTHAI K. (1985). A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, Thailand, 39 pp.

VANDERSEA M.W., LITAKER R.W., YONNISH B., SOSA E., LANDSBERG J.H., PULLINGER C., MOON-BUTZIN P., GREEN J., MORRIS J.A., KATOR H., NOGA E.J. & TESTER P.A. (2006). Molecular assays for detecting *Aphanomyces invadans* in ulcerative mycotic fish lesions. *Appl. Environ. Microbiol.*, **72**, 1551–1557.

WADA S., AN RHA S., KONDOH T., SUDA H., HATAI K. & ISHII H. (1996). Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida*. *Fish Pathol.*, **31**, 71–80.

WILLOUGHBY L.G. & ROBERTS R.J. (1994). Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. *J. Fish Dis.*, **17**, 541–543.

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\* \*

**NB:** There is an OIE Reference Laboratory for infection with *Aphanomyces invadans* Epizootic ulcerative syndrome

(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:

<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on infection with *Aphanomyces invadans* Epizootic ulcerative syndrome



## CHAPTER 2.3.5.

## INFECTION WITH INFECTIOUS SALMON ANAEMIA VIRUS

### EU position

**The EU thanks the OIE and in general supports the adoption of this modified chapter. Specific comments are inserted in the text below.**

#### 1. Scope

Infectious salmon anaemia (ISA) is an orthomyxovirus infection of sea-farmed Atlantic salmon (*Salmo salar*) (Thorud & Djupvik, 1988) inducing 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 systemic 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, but cumulative mortality may become very high for a period lasting several months if nothing is done to limit disease dissemination (Rimstad *et al.*, 2011). For the purpose of this chapter, ISA is considered to be infection with salmon anaemia virus (ISAV) (Kawaoka *et al.*, 2005).

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; Kibenge *et al.*, 2004; Mjaaland *et al.*, 1997; Rimstad *et al.*, 2011).

The morphological, physiochemical and genetic properties of ISAV are consistent with those of the *Orthomyxoviridae* (Falk *et al.*, 1997; Mjaaland *et al.*, 1997), and ISAV has recently been classified as the type species of the new genus *Isavirus* (Kawaoka *et al.*, 2005) within this virus family. The nucleotide sequences of all eight genome segments, have been described. The viral genome encodes encoding at least ten proteins, have been described (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002; 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 nonstructural protein with type I interferon antagonistic properties, while ORF2 has been suggested to encode for a nuclear export protein (NEP). The possibility of a third ORF has been discussed. 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

(Anonymous, 2007; Garcia-Rosado *et al.*, 2008; Kibenge *et al.*, 2004; 2007; Rimstad & Mjaaland, 2002).

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002). According to sequence differences in the 5'-region terminal end of the HE gene, ISAV isolates have been divided into two major groups, one European and one North American group. The European group may be further divided into three major groups (Anonymous, 2007). A small highly polymorphic region (HPR) of near the transmembrane domain haemagglutinin gene has been identified (Mjaaland *et al.*, 2002a). This region is characterised by the presence of gaps rather than single-nucleotide substitutions (Cunningham *et al.*, 2002; Mjaaland *et al.*, 2002). However, there is no direct correlation between phylogenetic groups and deletion patterns in the HPR. A full-length gene (HPR0) has been suggested to represent a precursor an ancient variant from which all ISAV HPR-deleted (pathogenic) variants of ISAV originate with deletions in the HPR region have been derived. The presence of non-pathogenic HPR0 ISAV has been reported in both apparently healthy wild and farmed Atlantic salmon (Anonymous, 2007; Kibenge *et al.*, 2004), 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, between virus isolates have been suggested to be important for virulence, as all diseased fish contain deletions in this region. However, other gene segments may be of importance for in virulence because isolates with identical HPRs vary significantly in development of ISA. A putative and severity of disease (Mjaaland *et al.*, 2005). Recently, a potential virulence marker has been identified in segment 5 encoding the fusion (F) protein. Here, a single amino acid substitution, or a sequence insertion, near and involving the protease recognition pattern at the the protein's putative cleavage site of the fusion protein has been found to be a prerequisite for virulence (Kibenge *et al.*, 2007; Markussen *et al.*, 2008). Furthermore, evidence for reassortment and nonhomologous recombination of ISAV has been provided. 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 RNA 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, UV irradiation and temperature. 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 (Anonymous, 2007). 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. Cell culture isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes exposure at 56°C (Falk *et al.*, 1997). 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 (Anonymous, 2007; 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 (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002). This has recently been confirmed by immunohistochemical examination of several organs (National Veterinary Institute Norway, unpublished results) and by immunohistochemistry and in-situ hybridisation (Kibenge *et al.*, 2004). 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 (Aarnefot *et al.*, 2012; Rimstad *et al.*, 2011) several organs. In-situ hybridisation studies have indicated the most extensive and prolonged replication to occur in the heart tissue (Kibenge *et al.*, 2004).

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 virus binds to glycoproteins 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 (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002; 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, but the virus has been isolated from rainbow trout in Ireland (Geoghegan, 2002) and there is a report of isolation of ISAV from and in Coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge *et al.*, 2004-2001). 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 (Kibenge *et al.*, 2004). In these studies, only weak positive results were obtained, and gills were included in the tissue samples examined. Contamination of virus present in surrounding water cannot, therefore, be excluded, and corroborative studies are needed before these species can be identified as possible hosts for ISAV. (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).

Following experimental infection, replication of ISAV has been demonstrated in several fish species, including brown trout, sea trout, rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), herring (*Clupea harengus*) (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002) and Atlantic cod (*Gadus morhua*) (Grove *et al.*, 2007). Mortality and histopathological changes have been introduced in rainbow trout by experimental infection, although the lesion characteristics were different from those in Atlantic salmon (Macwilliams *et al.*, 2007). Attempts have been made to induce infection or disease in *P. virens*, but with negative results (Kibenge *et al.*, 2004).

### 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 (Anonymous, 2007; Rimstad *et al.*, 2011). Furthermore, 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. (Anonymous, 2007). Furthermore, a functional association between disease resistance and major histocompatibility (MHC) class I and II polymorphism has been demonstrated (Grimholt *et al.*, 2003). The use of MHC compatible Atlantic salmon indicated that the ability to mount a strong proliferative response correlated to survival and virus clearance, while induction of a humoral response was less protective (Mjaaland *et al.*, 2005).

Annex 12 (contd)**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 many 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 ~~cause any recognisable disease problems~~. 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) (Anonymous, 2007; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002). See Section 2.4.1 for the possible creation of virus carriers through vaccination.

**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 (Anonymous, 2007; Plarre *et al.*, 2005; 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**

~~The disease is spread horizontally by water borne transmission as shown by experimental infection studies. There is no strong evidence for vertical transmission through infected gonadal products. It has been suggested that ISAV is spread over long distances by transportation of smolt, either infected prior to shipping or by well boats contaminated with ISAV. Contamination of well boats may be due either to previous transport of infected fish or through intake water from areas with farms harbouring diseased fish.~~

Epidemiological Studies of recurrent epidemics of ISA in different salmon-producing areas conclude have shown that the risk of ISA transmission is closely linked to husbandry practices in aquaculture and horizontal transmission. Geographical or hydrological (via prevailing currents) proximity (<5 km) to farms that the virus spreads locally between adjoining sites. Proximity to sites with ISA outbreaks or slaughterhouses/processing plants releasing contaminated water, numerous smolt deliveries and the use of well boats, and sharing staff and equipment are all considered significant risk factors (Anonymous, 2007; Gustafson *et al.*, 2007; Lyngstad *et al.*, 2008; Scheel *et al.*, 2007) 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 of ISAV biosecurity measures are not adequate (Rimstad *et al.*, 2011).

#### EU comment

**In the paragraph above, please replace "of ISAV biosecurity" by "if ISAV biosecurity" (typographical error).**

Other horizontal pathways have also been suggested, such as transmission through sea lice, infected wild fish and various harvesting methods (Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002). According to Nylund *et al.* (2007) vertical or transgenerational transmission may occur. Carryover or stocking of multiple year classes on a given site, or within a region connected hydrologically, may also influence occurrence of ISA (Gustafson *et al.*, 2007).

#### 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 appears to be a seasonal and transient infection (Christiansen *et al.*, 2011). HPR0 variants of ISAV has 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) and Nova Scotia in 2000), the United Kingdom (Scotland in 1998), the Faroe Islands (2000 report to OIE), the USA (Maine in 2001) and in Chile (2007) (Anonymous, 2007; Godoy *et al.*, 2008; Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002 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. The virus has been reported from rainbow trout in Ireland in 2002 (Geoghegan, 2002) and from Coho salmon in Chile (Kibenge *et al.*, 2004).

#### 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 experienced during a period of, for example, 3 several months. Initially, an outbreak of ISA may be limited to one or two net pens over a long time period and the spread to other. In such cases, if net pens with clinical ISA are slaughtered immediately, further development of clinical ISA at the site may be prevented take months but is slowed by early detection and depopulation of infected pens. 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.

Annex 12 (contd)**2.3.5. Environmental factors**

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

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

Vaccination against ISA has been carried out in North America since 1999 and the Faroe Islands during the past 5 years 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. The vaccines, which are inactivated, whole virus vaccines, do not give virus clearance in immunised fish, and they may thus become virus carriers (Kibenge *et al.*, 2004).

**2.4.2. Chemotherapy**

Not applicable. 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.

**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
<del>Immunocytochemistry</del>	
<u>Immunofluorescence</u> (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 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.

#### **EU comment**

**In the above paragraph, the EU suggests the OIE to consider replacing the word verification by the word confirmation.**

**Furthermore, the EU would like to suggest that pooling of samples cannot be used for surveillance of HPR0 as the level of viral RNA in samples is typically so low that relatively high CT values are generated in the QPCR when HPR0 had been detected. Instead, individual fish should be used in surveillance for HPR0.**

### 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. ~~For surveillance purposes using PCR, gills should also be included;~~

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

~~Immunocytochemistry~~ 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

#### ~~3.5. Samples/tissues that are not suitable~~

~~For virus isolation, gills are not recommended as it is quite difficult to avoid microbial contamination of cell cultures inoculated with gill tissue homogenate even after appropriate filtration.~~

## 4. Diagnostic methods

The diagnosis of ISA was initially based on clinical and pathological findings only (Kibenge *et al.*, 2004; Rimstad & Mjaaland, 2002). Following the isolation of the causative agent, a number of direct methods for detection of virus and confirmation of the diagnosis have been established. These include isolation of the virus in cell culture followed by immunological identification, immunological demonstration of ISAV antigen in tissues and PCR techniques. Differential diagnoses are: other anaemic and haemorrhagic conditions, and winter ulcer and septicæmias caused by infections with *Moritella viscosa*.

#### 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.

Nutritional status is usually quite normal, but diseased fish has no feed in the digestive tract.

##### 4.1.2. Behavioural changes

Generally, naturally infected Atlantic salmon with HPR-deleted ISAV ISA 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 ~~Clinical methods~~

##### 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.

~~A haematocrit value below 10 is not a unique finding for ISA. Fish with disease conditions such as ulcerations and erythrocytic inclusion body syndrome, may regularly demonstrate haematocrit values this low.~~

#### 4.2.3. Microscopic pathology ~~Histopathology~~

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 throughout the body~~ 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.

Annex 12 (contd)

## 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) ~~Preparations of cryosections~~

~~Tissue samples from kidney, liver and heart are collected from moribund fish, frozen in isopentane, chilled in liquid nitrogen, and stored at -80°C. Sections are cut on a cryostat, placed on poly-L-lysine coated slides, fixed in chilled 100% acetone for 10 minutes and stored at -80°C until use.~~

## ii) Staining procedure

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

4.3.1.1.2. *Fixed sections*4.3.1.1.3.1 *Immunohistochemistry (IHC)*

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

## i) Preparation of tissue sections

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

## ii) Staining procedure for IHC

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

a) Antigen retrieval is done by boiling sections in 0.1 M citrate buffer pH 6.0 for 2 × 6 minutes followed by blocking with 5% non-fat dry milk and 2% goat serum in 50 mM TBS (TBS; Tris/HCl 50 mM, NaCl 150 mM, pH 7.6) for 20 minutes.

b) Sections are then incubated overnight with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein) diluted in TBS with 1% non-fat dry milk, followed by three washes in TBS with 0.1% Tween 20.

c) For detection of bound antibodies, sections are incubated with Alkaline phosphatase-conjugated antibodies to rabbit IgG for 60 minutes. Following a final wash, Fast Red (1 mg ml<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

~~SHK-1 (Dannevig *et al.*, 1995), 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), TO and CHSE 214 (Kibenge *et al.*, 2004),~~ may be used. However, strain variability and the ability to replicate in different cell lines should be taken into consideration. The ~~SHK-1 and 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-mercapto-ethanol (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 ~~24-multi-well cell culture plates (see above).~~ Inoculate monolayers (25 cm<sup>2</sup> tissue culture flasks) with 0.1–1.5 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, fully L-15 medium supplemented growth medium with 2–5% FCS. Alternatively, a 1/1000 dilution and direct inoculation without medium replacement can be used.

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

##### ii) Monitoring incubation

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

Annex 12 (contd)

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 ~~on a rocking platform~~ 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 ~~plastic 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 ~~containing 0.5% dry skimmed milk~~ for 1 hour, and rinse twice with PBS/0.05% Tween 20. If unspecific binding is observed, incubate with PBS containing 0.5% dry skimmed milk.
- v) Incubate with FITC-conjugated goat anti-mouse immunoglobulin for 1 hour (or if antibody raised in rabbits is used as the primary antibody, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity, FITC-conjugated goat anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with the described rinsing in between the additional step. Rinse once with PBS/0.05% Tween 20, as described above. The nuclei can be stained with propidium 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), if the plates cannot be examined immediately, add 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. Examine under UV light.

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.

## Annex 12 (contd)

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

RT-PCR may be used for detection of ISAV in tissue samples or in samples from cell cultures. Care should be taken for the interpretation of results from cell culture, as detected virus does not necessarily indicate that virus replication has taken place, but may represent virus remaining in the cell culture after inoculation with a tissue sample.

Total RNA (or total nucleic acid) is extracted from tissues, tissue homogenates or from infected cell layers. The concentration and purity of the extracted nucleic acid can be estimated by measuring the optical density at 260 nm and at 280. An alternative approach is to include internal controls directed against host RNAs. For detection of viral nucleic acids in fish tissue, primers against 18s rRNA, elongation factor 1 alpha (EF1A) or RNA polymerase 1 have been used successfully as internal controls.

Since the first RT-PCR for ISAV was reported in 1997, several attempts have been made to optimise the method (see Mjaaland *et al.*, 2002b for a review). A two-step RT-PCR can be performed whereby the RT and PCR steps are run in separate tubes. The introduction of one-step procedures, where the two reactions are run in a single tube, has been successful regarding sensitivity of the test. However, in this case, no cDNA is left for use in additional amplifications, which may be a disadvantage if several primer sets need to be included in the examination.

Several primer sets for ISAV RT-PCR have been reported and some are presented in the table below. The primer sets derived from genomic segment 8 (ILA1/ILA2 and FA3/RA3) have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in carrier fish. The ILA2 reverse primer does not match isolates from North America and alternative primer sets should be used. In these cases, a modified FA3 primer together with RA3 can be used. The segment 6 primers may be useful for verification of PCR results based on segment 8 primers as an alternative to sequencing the PCR product.

<u>RT-PCR: Primer sequences</u>	<u>Named</u>	<u>Genomic segment</u>	<u>Product size</u>	<u>Reference</u>
<u>5'-GGC-TAT-CTA-CCA-TGA-ACG-AAT-C-3'</u> <u>5'-GCC-AAG-TGT-AAG-TAG-CAC-TCC-3'</u>	<u>ILA1</u> <u>ILA2</u>	<u>8</u>	<u>155</u>	<u>Mjaaland <i>et al.</i>, 2002b</u>
<u>5'-GAA-GAG-TCA-GGA-TGC-CAA-GAC-G-3'</u> <u>5'-GAA-GTC-GAT-GAA-CTG-CAG-CGA-3'</u>	<u>FA3</u> <u>RA3</u>	<u>8</u>	<u>211</u>	<u>Devold <i>et al.</i>, 2000</u>
<u>5'-GGA-ATC-TAC-AAG-GTC-TGC-ATT-G-3'</u> <u>5'-CTT-CAA-AGG-TGT-CTG-ACA-CGT-A-3'</u>	<u>Seg6U</u> <u>Seg6L</u>	<u>6</u>	<u>130</u>	<u>Designed by</u> <u>OIE Ref.</u> <u>Lab.</u>

The use of real time RT-PCR may increase the specificity and, probably, also the sensitivity of the test, especially when including a sequence-specific probe (Piarre *et al.*, 2005; Snow *et al.*, 2006; 2009). This method is more rapid compared with conventional one-tube RT-PCR, the risk of contamination may be reduced and it is possible to estimate the relative amount of viral RNA in the sample. Primer and probe sequences that have been used for screening for ISAV by real time RT-PCR are presented in the table below. Both primers and probes listed in this table target conserved regions of and ensure detection of all documented ISAV strains (Snow *et al.*, 2006).

## Annex 12 (contd)

Real time RT-PCR: Primer and probe sequences	Named	Genome segment	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-MGB-MGBNFQ-3'	forward primer reverse primer Taqman@probe	7	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-TAMRA-3'	forward primer reverse primer Taqman@probe	8	Snow <i>et al.</i> , 2006

As an alternative, ILAS7 probe listed in the table below has been shown to be effective at detecting European ISAV isolates.

Real time RT-PCR: Primer and probe sequences	Named	Genome segment	Reference
5'-TGG-GAT-CAT-GTG-TTT-CCT-GCT-A-3' 5'-GAA-AAT-CCA-TGT-TCT-CAG-ATG-CAA-3' 5'-6FAM-CAG-ATG-ACC-CCT-CGT-C-MGBNFQ-3'	ILAS7-F1 ILAS7-R1 ILAS7-probe	7	Plarre <i>et al.</i> , 2005

## 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 infectious salmon anaemia-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 ISA diagnosis	Confirmatory ISA diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	c	b
Histopathology	d	d	d	b	b	b
IFAT on kidney imprints	e-d	e-d	e-d	e-d	b	a
Immunohistochemistry	e-d	e-d	e-d	e-d	b	a
Transmission-EM	d	d	d	d	e	d
Isolation in cell culture with virus identification	a	a	a	a	a	a
RT-PCR or real-time RT-PCR followed by (sequencing for genotyping)	a	a	a	a	b	a

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

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

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

~~None of the methods described above have been evaluated for the purpose of declaration of freedom of virus or disease, as data on prevalence and distribution of ISAV in subclinically infected fish population are lacking.~~ 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~~ may be an efficient way of obtaining data on the occurrence-prevalence of ISA in farmed populations. Alternatively, In addition to regular health inspections, testing for HPR-deleted ISAV, preferentially by PCR-based methodology, at certain intervals may be carried out in addition to regular health inspections. 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 carefully interpreted 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)
- ~~iv) Detection of antibodies to ISAV.~~

##### 7.2. Definition of confirmed case (HPR-deleted ISAV)

###### 7.2.1. Definition of confirmed ISA

The following criteria ~~in i)~~ should be met for confirmation of ISA: ~~The criteria given in ii) and iii) should be met for the confirmation of ISAV infection.~~

- ~~i)~~—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:
  - a) 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
  - ~~b) i)~~ 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.

## 8. References

AAMELFOT M., DALE O.B., WELI S., KOPPANG E.O. & FALK K. (2012). Expression of 4-O-acetylated sialic acids on Atlantic salmon endothelial cells correlates with cell tropism of Infectious salmon anemia virus. *J. Virol.*, **86**, 10571–10578.

ALDRIN M., LYGSTAD T.M., KRISTOFFERSEN A.B., STORVIK B., BORGAN O. & JANSEN P.A. (2011). Modelling the spread of infectious salmon anaemia among salmon farms based on seaway distances between farms and genetic relationships between infectious salmon anaemia virus isolates. *J.R. Soc. Interface*, **8**, 1346–1356.

ANONYMOUS (2007). Which risk factors relating to spread of Infectious Salmon Anaemia (ISA) require development of management strategies? Norwegian Scientific Committee for Food Safety. <http://www.vkm.no/eway/library/openForm.aspx?param1=17005&param5=read>

CHRISTIANSEN D.H., ØSTERGAARD P.S., SNOW M., DALE O.B & FALK K. (2011). A low-pathogenic variant of infectious salmon anemia virus (ISAV1 - HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (*Salmo salar* L.) in the Faroe Islands. *J. Gen. Virol.*, **92**, 909–918.

COTTET L., RIVAS-ARAVENA A., CORTEZ-SAN MARTIN M., SANDINO A.M. & SPENCER E. (2011) Infectious salmon anemia virus – genetics and pathogenesis. *Virus Res.*, **155**, 10-19.

CUNNINGHAM C.O., GREGORY A., BLACK J., SIMPSON I. & RAYNARD R.S. (2002). A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. *Bull. Eur. Assoc. Fish Pathol.*, **22**, 366–374.

DANNEVIG, B.H., FALK, K. & NAMORK E. (1995). Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J. Gen. Virol.*, **76**, 1353–1359.

DEVOLD M., KARLSEN M. & NYLUND A. (2006). Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. *J. Gen. Virol.*, **87**, 2031–2040.

DEVOLD M., KROSSOY B., ASPEHAUG V. & NYLUND A. (2000). Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. *Dis. Aquat. Org.*, **40**, 9–18.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2012) EFSA Panel on Animal Health and Welfare (AHAW); Scientific Opinion on infectious salmon anaemia. *EFSA Journal*, **10** (11), 2971.

FALK K., NAMORK E., RIMSTAD E., MJAALAND S. & DANNEVIG B.H. (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *J. Virol.*, **71**, 9016–9023.

GARCIA-ROSADO E., MARKUSSEN T., KILENG Ø., BAEKKEVOLD E.S., ROBERTSEN B., MJAALAND S. & RIMSTAD E. (2008). Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity. *Virus Res.*, **133**, 228–238.

GEOGHEGAN F. (2002). First isolation and identification of ISAV in Ireland. 6th Annual Meeting of EU National Reference Laboratories for Fish Diseases, Brussels, Belgium, 23–24 September 2002.

GODOY M.G., AEDO A., KIBENGE M.J.T., GROMAN D.B., YASON C.V., GROTHUSEN H., LISPERCUER A., CALBUCURA M., AVENDAÑO F., IMLÁN M., JARPA M. & KIBENGE F.S.B. (2008). First detection, isolation and molecular characterization of ISAV associated with clinical disease in farmed Atlantic salmon in Chile. *BMC Vet. Res.*, **4**, 28.

GRIMHOLT U., LARSEN S., NORDMO R., MIDTLYNG P., KJOEGLUM S., STORSET A., SAEBO S. & STET R.J. (2003). MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogen.*, **55**, 210–219.

GROVE S., HJORTAAS M.J., REITAN L.J. & DANNEVIC B.H. (2007). Infectious salmon anaemia virus (ISAV) in experimentally challenged Atlantic cod (*Gadus morhua*). *Arch. Virol.*, **152**, 1829–1837.

GUSTAFSON L.L., ELLIS S.K., BEATTIE M.J., CHANG B.D., DICKEY D.A., ROBINSON T.L., MARENGHI F.P., MOFFETT P.J. & PAGE F.H. (2007). Hydrographics and the timing of infectious salmon anemia outbreaks among Atlantic salmon (*Salmo salar* L.) farms in the Quoddy region of Maine, USA and New Brunswick, Canada. *Prev. Vet. Med.*, **78**, 35–56.

KAWAOKA Y., COX N.J., HALLER O., HONGO S., KAVERIN N., KLENK H.D., LAMB R.A., MCCAULEY J., PALESE P., RIMSTAD E. & WEBSTER R.G. (2005). Infectious Salmon Anaemia Virus. In: *Virus Taxonomy – Eight Report of the International Committee on Taxonomy Viruses*, Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A., eds. Elsevier Academic Press, New York, USA, pp 681–693.

KIBENGE F.S., XU H., KIBENGE M.J., QIAN B. & JOSEPH T. (2007). Characterization of gene expression on genomic segment 7 of infectious salmon anaemia virus. *Viol. J.*, **4**, 34.

KIBENGE F.S.B., GARATE O.N., JOHNSON G., ARRIAGADA K., KIBENGE M.J.T. & WADOWAKA D. (2001). Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. *Dis. Aquat. Org.*, **45**, 9–18.

KIBENGE F.S.B., GODOY M.G., WANG Y., KIBENGE M.J.T., GHERARDELLI V., MANSILLA S., LISPERGER A., JARPA M., LARROQUETE G., AVENDAÑO F., LARA M. & GALLARDO A. (2009). Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. *Viol. J.*, **6**, 88.

KIBENGE F.S.B., KIBENGE M.J.T., WANG Y., QIAN B., HARIHARAN S. & MCGEACHY S. (2007). Mapping of putative virulence motifs on infectious salmon anaemia virus surface glycoprotein genes. *J. Gen. Virol.*, **88**, 3100–3111.

KIBENGE F.S.B., MUNIR K., KIBENGE M.J.T., MONEKE T.J. & MONEKE E. (2004). Infectious salmon anemia virus: causative agent, pathogenesis and immunity. *Anim. Health Res. Rev.*, **5**, 65–78.

KULSHRESHTHA V., KIBENGE M., SALONIUS K., SIMARD N., RIVEROLL A. & KIBENGE F. (2010). Identification of the 3' and 5' terminal sequences of the 8 RNA genome segments of European and North American genotypes of infectious salmon anaemia virus (an orthomyxovirus) and evidence for quasispecies based on the non-coding sequences of transcripts. *Viol. J.*, **7**, 338.

LYNGSTAD T.M., HJORTAAS M.J., KRISTOFFERSEN A.B., MARKUSSEN T., KARLSEN E.T., JONASSEN C.M. & JANSEN P.A. (2011). Use of molecular epidemiology to trace transmission pathways for infectious salmon anaemia virus (ISAV) in Norwegian salmon farming. *Epidemics*, **3**, 1–11.

LYNGSTAD T.M., KRISTOFFERSEN A.B., HJORTAAS M.J., DEVOLD M., ASPEHAUG, V., LARSSSEN, R. B. & JANSEN, P. A. (2012). Low virulent infectious salmon anaemia virus (ISAV-HPR0) is prevalent and geographically structured in Norwegian salmon farming. *Dis. Aquat. Org.*, **101**, 197–206.

LYNGSTAD T.M., JANSEN P.A., SINDRE H., JONASSEN C.M., HJORTAAS M.J., JOHNSEN S. & BRUN E. (2008). Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003–2005. *Prev. Vet. Med.*, **84**, 213–227.

Annex 12 (contd)

MARDONES F.O., PEREZ A.M., VALDES-DONOSO P. & CARPENTER T.E. (2011). Farm-level reproduction number during an epidemic of infectious salmon anaemia virus in southern Chile in 2007–2009. *Prev. Vet. Med.*, **102** (3), 175–184.

~~MAGWILLIAMS C., JOHNSON G., GROMAN D. & KIBENGE F.S. (2007). Morphologic description of infectious salmon anaemia virus (ISAV) induced lesions in rainbow trout *Oncorhynchus mykiss* compared to Atlantic salmon *Salmo salar*. *Dis. Aquat. Org.*, **78**, 1–12.~~

MARKUSSEN T., JONASSEN C.M., NUMANOVIC S., BRAAEN S., HJORTAAS M., NILSEN H. & MJAALAND S. (2008). Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. *Virology*, **374**, 515–527.

MCBEATH A. J., BAIN N. & SNOW M. (2009). Surveillance for infectious salmon anaemia virus HPR0 in marine Atlantic salmon farms across Scotland. *Dis. Aquat. Org.*, **87**, 161–169.

MJAALAND S., HUNGNES O., TEIG A., DANNEVIG B.H., THORUD K. & RIMSTAD E. (2002). Polymorphism in the infectious salmon anemia virus hemagglutinin gene; importance and possible implications for evolution and ecology of infectious salmon anemia disease. *Virology*, **302**, 379–391.

MJAALAND S., MARKUSSEN T., SINDRE H., KJOGLUM S., DANNEVIG B.H., LARSEN S. & GRIMHOLT U. (2005). Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates. *Arch. Virol.*, **150**, 2195–2216.

~~MJAALAND S., RIMSTAD E. & CUNNINGHAM C.O. (2002b). Molecular diagnosis of infectious salmon anaemia. In: Molecular Diagnosis of Salmonid Diseases, Cunningham C.O., ed. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1–22.~~

MJAALAND S., RIMSTAD E., FALK K. & DANNEVIG B.H. (1997). Genomic characterisation of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L): an orthomyxo-like virus in a teleost. *J. Virol.*, **71**, 7681–7686.

MULLINS J.E, GROMAN D.B & WADOWSKA D (1998) Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. *Bull. Eur. Assoc. Fish Pathol.*, **18**, 110–114.

NYLUND A., PLARRE H., KARLSEN M., FRIDELL F., OTTEM K.F., BRATLAND A., & SAETHER P.A. (2007). Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). *Arch. Virol.*, **152**, 151–179.

PLARRE H., DEVOLD M., SNOW M. & NYLUND A. (2005). Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Dis. Aquat. Org.*, **66**, 71–79.

RIMSTAD E., DALE O.B., DANNEVIG B.H. & FALK K. (2011). Infectious Salmon Anaemia. In: Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, Woo P.T.K. & Bruno D., eds. CAB International, Oxfordshire, UK, 143–165.

~~RIMSTAD E. & MJAALAND S. (2002). Infectious salmon anaemia virus. An orthomyxovirus causing an emerging infection in Atlantic salmon. *APMIS*, **110**, 273–282.~~

RIVAS-Aravena A., VALLEJOS-VIDAL E., MARTIN M.C., REYES-LOPEZ F., TELLO M., MORA P., SANDINO A.M., SPENCER E. (2011). Inhibitory effect of a nucleotide analog on ISAV infection. *J. Virol.*, **85**, 8037–8045.

SNOW M., MCKAY P., MCBEATH A. J. A., BLACK J., DOIG F., KERR R., CUNNINGHAM C. O., NYLUND A. & DEVOLD M. (2006). Development, application and validation of a taqman® real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*), Vannier P. & Espeseth D., eds. New Diagnostic Technology: Applications in Animal Health and Biologics Controls. *Dev. Biol.*, Basel, Karger. **126**, 133–145.

~~SNOW M., MCKAY P. & MATEJUSOVA I. (2009). Development of a widely applicable positive control strategy to support detection of infectious salmon anaemia virus (ISAV) using Taqman real-time PCR. *J. Fish Dis.*, **32**, 151–156.~~

Annex 12 (contd)



THORUD K.E. & DJUPVIK H.O. (1988). Infectious salmon anaemia in Atlantic salmon (*Salmo salar* L). *Bull. Eur. Assoc. Fish Pathol.*, **8**, 109–111.

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\* \*

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

UNOFFICIAL VERSION



## CHAPTER 2.3.11.

**VIRAL ENCEPHALOPATHY AND RETINOPATHY****EU position**

**The EU supports the adoption of this modified chapter.**

**1. Scope**

For the purpose of this chapter, viral encephalopathy and retinopathy (VER) otherwise known as viral nervous necrosis (VNN) is considered to be a serious disease of several marine fish species, characterised by significant losses associated to vacuolating lesions of the central nervous system and the retina.

**2. Disease information****2.1. Agent factors****2.1.1. Aetiological agent, agent strains**

The causative agent of VER or VNN was first identified as a new member of the family Nodaviridae following purification from affected larval striped jack *Pseudocaranx dentex*, and the name striped jack nervous necrosis virus (SJNNV) was adopted (Mori *et al.*, 1992). Subsequently other agents of VER/VNN were purified from some diseased fish species (Chi *et al.*, 2001; Comps *et al.*, 1994). The current taxonomy classifies the viruses into the genus *Betanodavirus* within the family Nodaviridae (Thiéry *et al.*, 2011). Betanodaviruses are non-enveloped, spherical and approximately 25 nm in diameter. The genome consists of two molecules of positive-sense ssRNA: RNA1 (3.1 kb) encodes the replicase (110 kDa) and RNA2 (1.4 kb) encodes the coat protein (42 kDa). Complete nucleotide sequences of RNA1 and RNA2 were reported for SJNNV and others (Iwamoto *et al.*, 2001; 2004; Sommerset & Nerland, 2004; Tan *et al.*, 2001). On the basis of the phylogenetic analysis of the T4 variable region of RNA2 encoding the virus capsid protein, betanodaviruses have been preliminarily clustered into four major genotypes, designated: striped jack nervous necrosis virus (SJNNV)-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type, and red-spotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa *et al.*, 1997). The identified genotypes partially correlate with three different serotypes that have been identified by virus neutralisation with polyclonal antibodies, different host species and the *in-vitro* optimum growth temperature (Iwamoto *et al.*, 2000; Mori *et al.*, 2003). Furthermore, an additional genotype including a turbot betanodavirus strain (TNV) has been proposed (Johansen *et al.*, 2004). To avoid confusion regarding the taxonomy, a numerical nomenclature (cluster I, II, III and IV), independent from the host species origin, has been proposed by Thiéry *et al.* (2004). In Table 2.1., the official genotypes, the target host species and the optimal *in vitro* growth temperature are reported (Iwamoto *et al.*, 2000).

**Table 2.1. Betanodavirus genotypic and phenotypic variants**

Genotype	Serotype	Target host fish	Optimum growth temperature
SJNNV	A	Striped jack	20–25°C
TPNNV	B	Tiger puffer	20°C
BFNNV	C	Cold-water fish: Atlantic halibut, Atlantic cod, flounders, etc.	15–20°C
RGNNV	C	Warm-water fish: Asian sea bass, European sea bass, groupers, etc.	25–30°C

## Annex 13 (contd)

### 2.1.2. Survival outside the host

Betanodaviruses are highly resistant in the aquatic environment and can survive for a long time in sea water at low temperatures (Frerichs *et al.*, 2000) while at 25°C or higher, the survival rate is significantly affected. Contamination of the aquatic environment following the appearance of an outbreak is likely to persist during long periods and represent a source of infection for wild susceptible species. In frozen fish the virus may persist for long periods and it may represent a potential risk if raw fish is used for feeding (Mori *et al.*, 2005). Outside the aquatic environment, betanodaviruses seem to lose their cytopathogenicity very easily. In drying conditions, >99% inactivation has been observed following a 7-day period at 21°C (Maltese *et al.*, 2007).

### 2.1.3. Stability of the agent (effective inactivation methods)

Common disinfectants such as sodium hypochlorite, iodine, hydrogen peroxide, and benzalkonium chloride are very useful for inactivating betanodaviruses while formalin shows a poor activity (Frerichs *et al.* 2000). Ozone has also been used to avoid or reduce virus contamination on egg shell surface (Grotmol & Totland, 2000) and virus contaminated water may be effectively sterilised by UV exposure (Frerichs *et al.*, 2000).

### 2.1.4. Life cycle

The presence of reservoirs in the wild is very reasonably the original source of infection of farmed populations while the trade of infected juveniles represents the most common way to spread large amounts of virus particles in the environment. Little is known about the life cycle of betanodaviruses. Considering the results obtained from experimental infections performed by different authors, the virus most likely invades the host through the intestinal epithelium and peripheral nervous system, very soon reaching the central nervous tissues where it may induce the death of the host or remain for several years in survivors (Johansen *et al.*, 2004). Dead decomposed fish may spread the virus in the environment reaching different biological vectors. Furthermore diseased fish may easily be cannibalised by predators who, besides the possibility of becoming infected, may spread the virus through contaminated faeces. Vertical transmission has been highly suspected in some species (Arimoto *et al.*, 1992; Comps *et al.*, 1994; Grotmol & Totland, 2000; Mushiake *et al.*, 1994; Watanabe *et al.*, 2000); in this case virus may reach developing gonads where it has been frequently detected (Dalla Valle *et al.*, 2000; Mushiake *et al.*, 1994; Nishizawa *et al.*, 1996) and infect the eggs and seminal fluids (Nishizawa *et al.*, 1994).

## 2.2. Host factors

### 2.2.1. Susceptible host species

To date, the disease has been reported in more than ~~50~~ 40 fish species, mainly marine with the greatest impact being in striped jack, European sea bass *Dicentrarchus labrax*, groupers, and flatfishes (Munday *et al.* 2002; Sano *et al.* 2011). A few outbreaks have also been documented in freshwater farms (Bovo *et al.*, 2011; Chi *et al.*, 2003); ~~furthermore betanodaviruses have been detected in association with serious mortalities in crustaceans (Pantoja *et al.*, 2007).~~ Fish species naturally affected by VER are listed in Table 2.2; some ornamental freshwater fish species developed clinical signs following experimental infection (Furusawa *et al.*, 2007) suggesting the possibility of new hosts, particularly in the future when new species will be selected for aquaculture.

Table 2.2. Fish species affected by VER/VNN

Order	Family	Common name	Latin name
Acipenseriformes	Acipenseridae	Russian sturgeon	<i>Acipenser gueldenstaedti</i>
Anguilliformes	Anguillidae	European eel	<i>Anguilla anguilla</i>
<u>Gonorynchiformes</u>	<u>Chanidae</u>	<u>milkfish</u>	<u><i>Chanos chanos</i></u>
Siluriformes	Siluridae	Chinese catfish	<i>Parasilurus asotus</i>
	Siluridae	Australian catfish	<i>Tandanus tandanus</i>
Gadiformes	Gadidae	Atlantic cod	<i>Gadus morhua</i>
	Gadidae	haddock	<i>Melanogrammus aeglefinus</i>

Order	Family	Common name	Latin name
Cyprinodontiformes	Poeciliidae	guppy	<i>Poecilia reticulata</i>
Perciformes	<u>Acanthuridae</u>	<u>convict surgeonfish</u>	<u><i>Acanthurus triostegus</i></u>
	<u>Apogonidae</u>	<u>narrowstripe cardinalfish</u>	<u><i>Apogon exostigma</i></u>
	<u>Anarchichadidae</u>	<u>wolf fish</u>	<u><i>Anarchichas minor</i></u>
	Carangidae	striped jack	<i>Pseudocaranx dentex</i>
Perciformes		<u>greater amberjack</u>	<u><i>Seriola dumerili</i></u>
Perciformes		yellow-wax pompano	<i>Trachinotus falcatus</i>
		snub nose pompano	<i>Trachinotus blochii</i>
	Centropomatidae	Asian sea bass or <u>barramundi</u>	<i>Lates calcarifer</i>
		Japanese sea bass	<i>Lateolabrax japonicus</i>
	<u>Cichlidae</u>	<u>tilapia</u>	<u><i>Oreochromis niloticus</i></u>
	Eleotridae	sleepy cod	<i>Oxyeleotris lineolata</i>
	Ephippidae	orbicular batfish	<i>Platax orbicularis</i>
	Latridae	striped trumpeter	<i>Latris lineata</i>
	Lutjanidae	crimson snapper	<i>Lutjanus erythropterus</i>
		<u>mangrove red snapper</u>	<u><i>Lutjanus argentimaculatus</i></u>
	Malacanthidae	<del>Japanese</del> or red tilefish	<i>Branchiostegus japonicus</i>
	Mugilidae	grey mullet	<i>Mugil cephalus</i>
		golden grey mullet	<i>Liza aurata</i>
		<u>red mullet</u>	<u><i>Mullus barbatus</i></u>
	Oplegnathidae	<u>striped beakfish or barred knifejaw or Reek-Bream</u>	<i>Oplegnathus fasciatus</i>
		spotted knifejaw	<i>Oplegnathus punctatus</i>
	Percichthyidae	European sea bass	<i>Dicentrarchus labrax</i>
	Rachicentridae	cobia	<i>Rachycentron canadum</i>
	Sciaenidae	red drum	<i>Sciaenops ocellatus</i>
	Sciaenidae	shi drum	<i>Umbrina cirrosa</i>
	Sciaenidae	white weakfish	<i>Atractoscion nobilis</i>
	Scombridae	Pacific bluefin tuna	<i>Thunnus orientalis</i>
	Serranidae	red spotted grouper	<i>Epinephelus akaara</i>
		yellow grouper	<i>E. awoara</i>
		sevenband grouper	<i>E. septemfasciatus</i>
		blackspotted grouper	<i>E. fuscoguttatus</i>
		brown spotted grouper	<i>E. malabaricus</i>
		dusky grouper	<i>E. marginatus</i>
		kelp grouper	<i>E. moara</i>
		greasy grouper	<i>E. tauvina</i>
		dragon grouper	<i>E. lanceolatus</i>
		humpback grouper	<i>Chromileptes altivelis</i>

Order	Family	Common name	Latin name
		white grouper	<i>E. aeneus</i>
		orange-spotted grouper	<i>E. coioides</i>
	Sparidae	gilthead sea bream	<i>Sparus aurata</i>
	<u>Cichlidae</u>	<u>Nile tilapia</u>	<u><i>Oreochromis niloticus niloticus</i></u>
Tetraodontiformes	Monacantidae	thread-sale filefish	<i>Stephanolepis cirrifer</i>
Tetraodontiformes	Tetraodontidae	Japanese puffer fish	<i>Takifugu rubripes</i>
Pleuronectiformes	Pleuronectidae	barfin flounder	<i>Verasper moseri</i>
	Soleidae	common sole	<i>Solea solea</i>
	<u>Scophthalmidae</u>	turbot	<i>Psetta maxima</i>
	Scophthalmidae	Japanese flounder	<i>Paralichthys olivaceus</i>
	Scophthalmidae	Atlantic halibut	<i>Hippoglossus hippoglossus</i>
Scorpaeniformes	Sebastidae	white weakfish	<i>Sebastes oblongus</i>

[For the source references, please contact the OIE Reference Laboratory.](#)

#### 2.2.2. Susceptible stages of the host

Although the disease mainly affects the larval and juvenile stages, serious mortalities have been also reported in market-size and adult fish, such as ~~*Pseudocaranx dentex*~~ Atlantic halibut *Hippoglossus hippoglossus*, sevenband grouper *Epinephelus septemfasciatus*, and European sea bass *Dicentrarchus labrax*.

#### 2.2.3. Species or subpopulation predilection (probability of detection)

In infected farms the probability of detecting the causal agent is normally higher in juveniles than in older fish, while during spawning season the virus may be found in the gonads of broodstock (Dalla Valle *et al.*, 2000; Mushiake *et al.*, 1994). For this reason, surveillance programmes should include young fish as well as gonadal tissues, ovarian fluids and milt.

#### 2.2.4. Target organs and infected tissue

Brain, spinal cord and retina are considered the target organs in which the virus actively replicates causing extensive tissue vacuolation. Intracytoplasmic inclusions have been described in the brain cells of European sea bass, Asian sea bass (*Lates calcarifer*), Japanese parrotfish (*Oplegnathus fasciatus*), and brown-spotted grouper (*Epinephelus malabaricus*) (Munday *et al.*, 2002). The virus has been also detected in broodstock gonads (Dalla Valle *et al.*, 2000; Mushiake *et al.*, 1994; Nishizawa *et al.*, 1996). In some species such as striped jack, European sea bass, barfin flounder (*Verasper moseri*), sevenband grouper and Atlantic halibut, broodfish are likely to be the most consistent virus reservoir and the most important source of infection for larvae and juvenile fish (Mushiake *et al.*, 1994; Watanabe *et al.*, 2000). Viruses may not replicate and reside in the reproductive organs at all times and are more likely to be found there after stressful conditions (Mushiake *et al.*, 1994).

#### 2.2.5. Persistent infection with lifelong carriers

In the wolfish (*Anarhichas lupus-minor*), experimentally infected by immersion, the virus has been detected in the brain at least up to 16 weeks post-exposure (Johansen *et al.*, 2003). Following a natural outbreak in Atlantic halibut, the progression of the infection has been studied in survivors throughout a 1-year observation period (Johansen *et al.*, 2002). The percentage of fish positive by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) remained high throughout the whole period and the virus was re-isolated from a subclinically infected fish at the end of the year, suggesting that fish surviving the infection may harbour the virus for a long period and may potentially transmit the infection to other fish. A cell line (BB) derived from the brain tissue of barramundi (*Lates calcarifer*) has been recently established and will offer a valid model to study viral infection and replication mechanisms both *in vivo* and *in vitro* (Chi *et al.*, 2005).

#### 2.2.6. Vectors

Considering that water is the most important abiotic vector, betanodaviruses can be easily spread, during a clinical outbreak, by one section of the farm to another directly through the water and by contaminating personnel, nets, boots and other equipment. ~~For this reason strong hygienic barriers~~ Adequate biosecurity measures should be established, particularly inside the hatcheries (Mori *et al.*, 1998). In open sea the transmission of the infection from one site to another is caused by tide, dominant currents, boats visiting different farms and wild migrating fish. Owing to the high resistance of the virus to acid conditions and at 37°C (Frerichs *et al.*, 2000) ichthyophagous birds should be regarded as potential vectors. Furthermore because of the large volume of trade, particular attention should be addressed to molluscs originating from contaminated areas. ~~Bite worms also may act as mechanical vectors and transmit viruses from one region to another. The virus has been detected from sand worms belonging to the family Nereidae, genus Nereis collected close to an infected farm (Bovo *et al.*, unpublished observations). The large international market existing as bait worms should be regarded as a further risk for spreading betanodaviruses from one region to another.~~

#### 2.2.7. Known or suspected wild aquatic animal carriers

Though the role of wild carriers is still to be completely understood, data have been published on the detection of betanodaviruses from wild species in different regions (Barker *et al.*, 2002; Ciulli *et al.*, 2006; Gomez *et al.*, 2004). It is still unclear if infected specimens should be considered just as a viral reservoir in which the pathogen may replicate without causing any mortality or if they should be considered susceptible animals. For this reason experimental infection trials are needed to better understand the significance of betanodavirus infection in wild fish.

### 2.3. Disease pattern

#### 2.3.1. Transmission mechanisms

The disease may be reproduced in healthy fish by co-habitation, immersion, and injection (Munday *et al.*, 2002). Horizontal transmission, frequently observed in the field, should be considered the most common way of disease transmission through contaminated water. Furthermore some evidence exists for vertical transmission from broodstock to offspring in striped jack, European sea bass, Asian sea bass, barfin flounder, Atlantic halibut, and sevenband grouper, as mentioned earlier (Comps *et al.* 1994; Mori *et al.*, 1998; Mushiake *et al.*, 1994; Watanabe *et al.*, 2000). This fact is mainly reflected by the early occurrence of clinical disease and the detection of viral genetic material from mature gonads (Dalla Valle *et al.*, 2000; Mushiake *et al.*, 1994; Nishizawa *et al.*, 1996); whether the virus is located inside or outside the eggs as a shell contaminant remains to be definitively proved. An additional possibility for transmission of the disease is represented by feeding broodstock with raw fish (Mori *et al.*, 2005). There is also very strong evidence that ozonation of fertilised eggs from infected broodstock eliminates or reduces the infection rate in offspring (Mori *et al.* 1998), indicating vertical transmission may occur as egg-shell contamination, at least for some species.

#### 2.3.2. Prevalence

Very few data are available on the prevalence of the disease. In Canada, certain populations of wild fish are suspected of acting as authentic natural reservoirs, in fact, the virus has been shown by PCR to be present in 0.23% of wild winter flounder (*Pleuronectes americanus*) (Barker *et al.*, 2002). In Japan a representative sample of 30 species collected in two different bays confirmed that most farmed and wild fish tested positive (Gomez *et al.*, 2004).

#### 2.3.3. Geographical distribution

The disease has been officially reported from many regions. These include countries in South and East Asia (China [People's Rep. of], Chinese Taipei, India, Indonesia, Iran, Japan, Korea, Malaysia, Philippines, Thailand, Vietnam), Oceania (Australia, Tahiti), Mediterranean (France, Greece, Israel, Italy, Malta, Portugal, Spain, Tunisia), UK, Norway, Caribbean and North America (Canada, USA) (Munday *et al.*, 2002). Furthermore suspicion of mortalities caused by betanodaviruses has been unofficially reported in wild groupers living along the Senegalese and Libyan coasts.

#### 2.3.4. Mortality and morbidity

There are considerable variations in the age at which disease is first noted and the period over which mortality occurs (Munday *et al.*, 2002). In general, the earlier the disease signs occur, the greater is the rate of mortality. In striped jack, mortalities are most frequently observed within 10 days after hatching while the earliest occurrence of disease is at 2 day post-hatching, resulting in almost complete loss of the larvae. In European sea bass mortality is usually not seen until about 30 days post-hatching but outbreaks may occur even in market-size fish. The mortality rate is age-dependent. When larval stages are affected, highest mortality, often reaching 100%, are observed while in juveniles and older fish lower losses have been generally reported.

### 2.3.5. Environmental factors

Water temperature is an important factor and the appearance of clinical signs can be significantly influenced by temperature (~~Yuasa et al., 2007~~). The effect of temperature is particularly well known in sea bass farming and the disease was preliminary identified as *Summer Disease*. The correlation between appearance of clinical signs and water temperature is partly supported by *in-vitro* studies (Iwamoto et al., 2000). Host specificity seems to exist in the RGNNV genotype to warm-water fish and in the BFNNV genotype to cold-water fish. In striped jack larvae infected by SJNNV (*in-vitro* optimum growth temperature: 20–25°C) no difference in mortality was noticed among different groups reared at different water temperatures ranging from 20°C to 26°C (Arimoto et al., 1994), while in sevenband grouper infected with RGNNV (*in-vitro* optimum growth temperature: 25–30°C) rearing water temperature (16–28°C) may influence the development of the disease. Higher mortality and earlier appearance of the disease were observed at higher temperatures (Tanaka et al., 1998), while water temperatures higher than 31°C inhibited the proliferation of RGNNV in humpback grouper (*Chromileptes altivelis*) (Yuasa et al., 2007). On the other hand, the infection of AHNV (BFNNV genotype, optimum: 15–20°C) in Atlantic halibut occurs at 6°C (Grotmol et al., 1999). Salinity does not seem to have any influence on the occurrence of the disease as outbreaks have been reported in freshwater species.

## 2.4. Control and prevention

Prevention of the disease may only be obtained avoiding the exposure of farmed population to the causative agents. Unfortunately the method is very difficult to apply in on-growing facilities while may be very useful in hatcheries provided they are using virus-free water and introducing juveniles obtained from virus-free broodstock.

### 2.4.1. Vaccination

Different studies have shown that immunisation using recombinant viral coat protein expressed in *Escherichia coli* or virus-like particles expressed in a baculovirus expression system or ~~formalin~~ chemically-inactivated virus may be effective in controlling the disease (Kai & Chi, 2008; Tanaka et al., 2001; Thiéry et al., 2006; Yamashita et al., 2009a). Nevertheless there are no commercially available vaccines at present. One study showed that primary infection with an avirulent aquabirnavirus effectively suppressed secondary betanodavirus infection, suggesting the use of the aquabirnavirus as a potential immunomodulator (Yamashita et al., 2009b).

### 2.4.2. Chemotherapy

No chemotherapy is available.

### 2.4.3. Immunostimulation

No data available.

### 2.4.4. Resistance breeding

No data available.

### 2.4.5. Restocking with resistant species

No data exist concerning the selection of resistant lineages within susceptible species.

### 2.4.6. Blocking agents

No data available.

### 2.4.7. Disinfection of eggs and larvae

Washing fertilised eggs in ozone-treated sea water or treatment of rearing water with ozone or chlorination seems to be effective in the control of the disease in larval production of striped jack, seven-band grouper, barfin flounder, and Atlantic halibut (Arimoto et al., 1996; Grotmol & Totland, 2000; Mori et al., 1998; Watanabe et al., 2000).



#### 2.4.8. General husbandry practices

In addition to the general hygiene practices, such the UV treatment of water entering the hatchery, the adoption of sanitary barriers, regular fallowing and disinfection of tanks and biological filters, disinfection of facilities, utensils, avoidance of raw fish for feeding, it is important to reduce stress factors by improving the spawning-induction method, which includes providing adequate food for broodstock and decreasing the stocking density of larvae and juveniles (Mushiake *et al.*, 1994). To avoid vertical transmission in hatcheries it has been proposed to test each brood fish by PCR methods carried out on gonadal biopsies and discard all positive specimens (Mori *et al.*, 1998; Mushiake *et al.*, 1994, Nishizawa *et al.*, 1994); nevertheless on some occasions failure of the PCR method to detect the virus infection in selected spawners have been reported (Nishizawa *et al.*, 1996).

An integrated control strategy, including the use of ELISA to test the level of specific antibody activity in each single brood fish, PCR carried out on sexual products and disinfection of embryonated eggs with ozonated water has been also proposed to control VER in barfin flounder (Watanabe *et al.*, 2000).

Unfortunately the detection of specific antibodies by ELISA or neutralisation tests has not been sufficiently investigated and very little is known about the interpretation of serological results.

In the sea bass industry, it has been suggested that restocking of on-growing facilities located in infected areas should be performed during autumn when the number of clinical outbreaks is decreasing.

### 3. Sampling

#### 3.1. Selection of individual specimens

Fish showing abnormal swimming behaviour, associated with loss of appetite and a progressive change in pigmentation, should be regarded as potentially infected and those specimens, in addition to moribund and freshly dead specimens, should be sampled for diagnostic purposes to confirm the suspicion. Whole fish are normally sent to the laboratory except in the case of very large specimen for which only head may be provided. In surveillance programmes the sampling of apparently healthy fish according to significant statistical sampling must be adopted.

#### 3.2. Preservation of samples for submission

Waiting for submission to the laboratory, samples may be safely maintained at 4°C (2–3 days) or frozen at –20°C or –80°C (2–3 weeks).

#### 3.3. Pooling of samples

Collective samples, representing 5–10 fish with clinical signs, may be accepted for diagnostic purposes. When looking for potential carriers single fish should be tested ~~by highly sensitive methods.~~

#### 3.4. Best organs or tissues

Brain and eyes are the target tissues for diagnostic purposes. When larval or very young stages (4–2 <u>1</u> cm) are suspected the whole body may be processed. When fish length is between 2 1 and 6 cm the whole head including brain and eyes should be separated from the body and included in the sample. For larger fish only the brain and eyes should be collected.

Other organs than brain and eyes should be considered not suitable for diagnostic purposes. Nevertheless when brood fish has to be tested by non-invasive methods, eggs, ovary and seminal fluids and gonadal biopsies should be regarded as suitable samples, ~~although only positive results should be viewed as conclusive.~~

#### 3.5. Samples/tissues that are not suitable

Kidney, spleen, and heart, which are normally recommended for detection of several fish viral agents are not suitable for diagnosis of VER or VNN ~~and should never be used for this purpose.~~

## Annex 13 (contd)

### 4. Diagnostic methods

For several years the "Gold Standard" method to detect VER or VNN was isolation of viral agents in cell culture followed by immunological or molecular identification. Now several molecular tools characterised by high sensitivity have been described but their definitive use needs further validation through inter-laboratory proficiency tests, or by equivalence testing with the Gold Standard.

#### 4.1. Field diagnostic methods

##### 4.1.1. Clinical signs

There are no external signs on the body surface and gills of infected fish except a progressive change in pigmentation described in a few species by different authors. In European sea bass cutaneous erosions in the mandibular and cranial areas, possibly due to traumatic origin caused by visual disturbance, have been occasionally reported.

##### 4.1.2. Behavioural changes

Infected fish show a variety of erratic swimming behaviour patterns, such as spiralling, whirling or belly-up at rest (sometimes with inflation of the swim bladder), or lying down at the tank bottom or swim rapidly in circles or straight-ahead. Flatfish usually show less evident signs. Subjects may remain at length on the bottom bending their body with the head and tail raised. Loss of appetite has been frequently reported.

#### 4.2. Clinical methods

##### 4.2.1. Gross pathology

No macroscopic lesions have been associated to ~~VER~~the infection except hyperinflation of the swim bladder, which has been frequently observed in different species, ~~particularly during larval stages~~.

##### 4.2.2. Clinical chemistry

No data applicable.

##### 4.2.3. Microscopic pathology

The most common microscopical findings detected in different species consist of vacuolation and necrosis of nervous cells of the spinal cord, brain and/or retina. These lesions are by far more prominent in larvae and juveniles while in older symptomatic fish they are sometimes very rare and difficult to detect. The inflammatory process is usually very discreet and the presence of macrophages is possibly due to secondary infection.

##### 4.2.4. Wet mounts

Not applicable.

##### 4.2.5. Smears

Not applicable.

##### 4.2.6. Fixed sections

Not applicable.

##### 4.2.6. Electron microscopy/cytopathology

Subspherical viral particles, about 25 nm in diameter, can be visualised in brain, spinal cord and retina of heavily infected animals. The virions may appear either free in the cytoplasm or associated to the endoplasmic reticulum membranes, primarily in nerve cells astrocytes, oligodendrocytes, and microgliaocytes. Nevertheless because of the limited analytical sensitivity the method is not a reliable diagnostic tool.

### 4.3. Agent detection and identification methods

#### 4.3.1. Direct detection methods

~~Reverse transcription PCR (RT-PCR) is the most rapid and convenient method for diagnosing clinically infected fish while nested PCR or real time PCR are useful tools for diagnosing subclinically infected fish, as carrier fish. In general, PCR-based methods applied to the diagnosis of viral diseases have as advantages short processing time, rapidity of reporting and high sensitivity and specificity, and are therefore suitable tools for the rapid detection of betanodavirus in both clinically and subclinically infected fish.~~ Nevertheless these methods need further validation through inter-laboratory proficiency tests. For this reason cell culture isolation followed by immunostaining or molecular identification still represents the “Gold Standard” for diagnostic purposes.

Furthermore the virus has been identified by immunofluorescence (IF) performed on brain imprints or frozen sections and by immunohistochemistry (IHC) on brain and retina. IHC is the most appropriate test for the detection of virus in material fixed for histological analysis.

ELISA-based methods detecting betanodavirus antigens from target tissues have been published (Arimoto *et al.*, 1992)

#### 4.3.1.1. Microscopic methods

##### 4.3.1.1.1. Wet mounts

No data.

##### 4.3.1.1.2. Smears

Brain imprints stained according to classical IF test have been reported as a suitable method for detection of betanodavirus infection. Because of its unknown sensitivity the method is recommended only in clinically affected fish.

##### 4.3.1.1.2.1. Indirect fluorescent antibody test on brain imprints

This protocol has been advantageously applied for long time in the OIE Reference Laboratory; nevertheless the procedure has not been properly validated in terms of sensitivity and reproducibility. The method is recommended only in clinically affected fish.

- i) Collect the brain from clinically affected fish (at least three specimens).
- ii) Use blotting paper to remove any liquid excess.
- iii) Make light imprints on the slide surface (five from each of the three specimens).
- iv) Allow the imprints to air-dry.
- v) Fix the imprints in absolute ethanol or cold acetone ( $-20^{\circ}\text{C}$ ) for 10 minutes.
- vi) Rinse three times with 0.05% PBS-Tween 80 (PBST).
- vii) Cover the imprints with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) and incubate for 30 minutes at  $37^{\circ}\text{C}$  in a humid chamber.
- viii) Rinse three times with 0.05% PBST.
- ix) Cover the imprints with a commercially available fluorescein isothiocyanate-conjugated secondary antibody (i.e. anti-rabbit Ig antibody) and incubate at  $37^{\circ}\text{C}$  for 30 minutes.
- x) Rinse three times with PBST.
- xi) Mount with a coverslip using glycerol saline, pH 8.5.
- xii) Observe under IF microscope at 100–250 $\times$ .

#### *Interpretation of the results:*

*Positive samples:* fluorescent cells containing green brilliant granules with a stardust appearance are easily detectable.

*Negative samples:* no fluorescent signal should be present.

## Annex 13 (contd)

### *4.3.1.1.3. Fixed sections*

Specific detection of the causative agent may be achieved by indirect fluorescent antibody test (IFA) carried out on paraffin embedded or cryostatic tissues or by IHC carried out on paraffin embedded tissues as reported from different authors (Johansen *et al.*, 2003; Nguyen *et al.*, 1997) using polyclonal or monoclonal antibodies.

#### *4.3.1.1.3.1. Indirect fluorescent antibody test on paraffin sections imprints*

The following protocol is reported as an example but other IFA validated protocols may be used.

- i) Cut 5 µm-thick- sections and transfer to polylysine-coated slides.
- ii) Dry section overnight at 37°C.
- iii) Deparaffinise with xylene 2 × 10 minutes with absolute ethanol 2 × 5 minutes.
- iv) Rehydrate sections: 95°, 70°, 50° and distilled water.
- v) Rinse with PBST.
- vi) Cover the sections with 0.1% trypsin in PBS and incubate at 37°C for 30 minutes.
- vii) Rinse three times with PBST.
- viii) Cover the sections with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) for 30 minutes at 37°C in a humid chamber.
- ix) Rinse three times with PBST.
- x) Cover the sections for 30 minutes at 37°C with a commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody).
- xi) Rinse three times with PBST.
- xii) Mount with coverslip using glycerol saline, pH 8.5.
- xiii) Observe under IF microscope and compare with positive and negative control.

#### *Interpretation of the results:*

*Positive samples:* specific brilliant fluorescence is observed in the cytoplasm of affected cells in brain and retina.

*Negative samples:* no fluorescent signal should be present in negative control.

#### *4.3.1.1.3.1. Indirect fluorescent antibody test on cryostatic sections*

The following protocol is reported as an example but other IFA validated protocols may be used. Because of the limited sensitivity the method is applicable only to clinically affected fish.

- i) Collect brains or eyes from 2–3 clinically affected fish.
- ii) Place the organs together in the same cryostatic sample support.
- iii) Move the support to the cryostatic chamber at –20°C.
- iv) When completely frozen, cut 5 µm-thick sections and transfer them to polylysine-coated slides.
- v) Allow the sections to air-dry.
- vi) Fix the sections with absolute ethanol or cold acetone (–20°C).
- vii) Cover the sections with the primary antibody (i.e. rabbit anti-betanodavirus serum) for 30 minutes at 37°C in a humid chamber.
- viii) Rinse three times with PBST.
- ix) Cover the sections for 30 minutes at 37°C with a commercially available fluorescein isothiocyanate (i.e. conjugated anti-rabbit Ig antibody).
- x) Rinse as before with PBST.
- xi) Mount with a coverslip using glycerol saline, pH 8.5.

- xii) Observe under IF microscope and compare with negative and positive controls.

Annex 13 (contd)

*Interpretation of results:*

*Positive samples:* specific fluorescence is observed in the cytoplasm of the affected cells in brain or retina.

*Negative samples:* no fluorescent signal should be present in negative control.

*4.3.1.1.3.3. Immunohistochemistry (avidin-biotin-peroxidase technique)*

This protocol has been advantageously applied for a long time in the OIE Reference Laboratory and is given as an example; different IHC validated protocols may be adopted.

- i) Dewax sections with xylene (2 × 10 minutes) and dehydrate with ethanol (2 × 5 minutes).
- ii) Incubate in 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes at room temperature (RT), to block endogenous peroxidase.
- iii) Hydrate tissue sections in a decreasing alcohol series 95°, 70°, 50°, distilled water (1 minute).
- iv) Rinse with PBS (pH 7.3) at 37°C.
- v) Incubate sections with 0.1% trypsin and 0.1% CaCl<sub>2</sub> + Tris buffer, for 30 minutes at 37°C.
- vi) Rinse three times with PBS.
- vii) Incubate sections with 5% BSA in PBS for 20 minutes at RT.
- viii) Tap off BSA and wipe away the excess.
- ix) Incubate sections with the primary antibody (i.e. rabbit anti-betanodavirus) diluted in 2.5% BSA for 60 minutes.
- x) Rinse three times in Tris-buffer.
- xi) Incubate with the secondary biotinylated serum (i.e. goat anti-rabbit immunoglobulins in 2.5% BSA for 20 minutes.
- xii) Rinse three times in Tris-buffer.
- xiii) Incubate with ABCComplex/HRP (prepared just before use) for 20 minutes at RT.
- xiv) Tap off ABCComplex/HRP.
- xv) Rinse three times with Tris-buffer.
- xvi) Incubate with chromogen substrate AEC (3-amin-9-ethylcarbazole; prepared just before use) for 20 minutes at RT.
- xvii) Rinse with distilled water for 5 minutes.
- xviii) Counterstain with Harris haematoxylin for 30 seconds.
- xix) Mount sections in glycerol gelatin.
- xx) All immunohistochemical runs should include one positive and one negative control section

*Interpretation of results:*

*Positive samples:* granular red deposits are detectable in tissues. Pale diffuse red stain of tissues is not considered as specific immunolabelling (background)

*Negative samples:* no immunolabelling detected

**4.3.1.2. Agent isolation and identification**

*4.3.1.2.1. Cell culture*

The isolation of betanodaviruses in a small number of established fish cell lines is well documented. Two fish cell lines for isolating and propagating betanodaviruses are available for diagnostic and research purposes through the European Collection of Cell Cultures (ECACC): the SSN-1 cell line derived from striped snakehead (Frerichs et al., 1996) and a cloned cell line (E-11) derived from SSN-1 itself (Iwamoto et al., 2000). Both these cells are useful for qualitative and quantitative analyses of all betanodaviruses. Further susceptible cell cultures (GF-1) have been developed and described (Chi et al., 1999) and may be used for research and diagnostic purposes provided sensitivity is regularly

monitored. To verify the sensitivity of the cell cultures in use, titration of frozen reference virus must be performed at least every 6 months or whenever decreased cell susceptibility is suspected.

#### *Inoculation of cell monolayers*

For details on transportation, antibiotic treatment and virus extraction refers to the General Information chapter.

- i) Homogenise samples with 1:5–1:10 volumes of Hanks' balanced salt solution (HBSS) or other equivalent medium, containing antibiotics to avoid bacterial contaminations.

Note: Gentamycin (1000 µg/ml) or penicillin (800 International Units [IU]/ml) and streptomycin (800 µg/ml) are suggested but different effective antibiotics may be used. The antifungal compounds Mycostatin or Fungizone may also be incorporated into the medium at a final concentration of 400 IU/ml. In addition 5% serum or albumen may be added.

The antibiotic treatment may be performed for 4 hours at 15°C or overnight at 4°C. The alternative of filtration through 0.22 µm membrane filter may be used instead.

- ii) Inoculate the antibiotic treated tissue suspension at two different dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in a final dilutions of tissue material in cell culture medium of 1:50–100 and 1:500–1000 respectively.

Note: each of 100 µl dilution should be inoculated into at least 2 cm<sup>2</sup> actively replicating cell cultures monolayers. Both the normal or the adsorption method may be used.

- iii) In case the adsorption method is adopted, allow the inoculum to adsorb on the drained monolayers, for 1 hour at 20–25°C. After the adsorption period add the new medium supplemented with 5% FBS.
- iv) If the normal method is adopted, the culture medium may be changed with a new one supplemented with 5% FBS, before to add the antibiotic treated suspension.
- v) Incubate at 20–25°C according to the genotype expected.

Note: Optimal viral growth temperatures are different among the four genotypic variants: 25–30°C for RGNNV genotype, 20–25°C for SJNNV genotype, 20°C for TPNNV genotype, and 15–20°C for BFNNV genotype (Table 2.1). For this reason the incubation temperature should be selected according to the genotypes present in the sampled area.

#### *Monitoring incubation*

- i) Follow the course of infection in positive controls and other inoculated cell cultures by regular microscopic examination at ×40–100 magnification for 10 days.
- ii) If cytopathic effect (CPE) appears, identification procedures must be undertaken (see below).

Note: CPE in SSN-1 or E-11 cells is characterised by thin or rounded, refractile, granular cells with vacuoles, and partial or complete disintegration of the monolayer.

- iii) If no CPE occurs after the primary incubation period (10 days), subcultivation must be performed on fresh cultures, using a similar cell growing area to that of the primary culture.

#### *Subcultivation procedures*

- i) Collect aliquots (10%) of cell culture medium from all monolayers inoculated.
- ii) Inoculate those aliquots constituting the primary culture into wells with the new cell monolayers, as described above (well-to-well subcultivation).
- iii) Incubate and monitor as described above for further 10 days
- iv) If no CPE occurs during this period the test may be considered negative
- v) If CPE appears identification procedures must be undertaken (see below).

#### 4.3.1.2.2. Antibody-based antigen detection methods

##### 4.3.1.2.2.1. Indirect fluorescent antibody test

- i) Prepare monolayers of susceptible cells directly in 2 cm<sup>2</sup> wells of cell culture plastic plates or on cover-slips or chamber slides in order to achieve around 70–80% confluency, which is usually reached within 24 hours of incubation at 25°C.
- ii) Inoculate the virus suspensions to be identified using at least two tenfold dilutions.
- iii) Incubate at 20°C or 25°C for 48–72 hours (See Section 4.3.1.2.1 Cell culture).
- iv) Remove the culture medium and fix with absolute ethanol or 80% cold acetone (–20°C) for 10–30 minutes.
- v) Rinse three times with PBST.
- vi) Allow the cell monolayers to air-dry.
- vii) Treat the cell monolayers with the primary antibody (i.e. rabbit anti-betanodavirus immune serum) for 30 minutes at 37°C in a humid chamber.
- viii) Rinse three times with PBST.
- ix) Cover the cell monolayers for 30 minutes at 37°C with commercially available fluorescein isothiocyanate-conjugated (i.e. anti-rabbit Ig antibody).
- x) Rinse with PBST.

Examine the treated cell monolayers directly on plates, or mount the cover-slips using glycerol saline, pH 8.5, prior to microscopic observation.

##### *Interpretation of the results:*

*Positive samples:* brilliant fluorescent cells scattered on the monolayer.

*Negative samples:* No fluorescent signal should be detected.

#### 4.3.1.2.3. Molecular techniques

##### 4.3.1.2.3.1. Conventional PCR

The increasing number of available sequence data allowed the development of PCR-based diagnostic methods for betanodavirus detection, which can be optimised according to individual specificity needs has greatly improved VET diagnostics, increasing test sensitivity and shortening processing times. The F2-R3 primer set, targeting the T4 variable region of the RNA2 segment (Nishizawa *et al.*, 1994), has been extensively used in several studies for both diagnostic and research purposes. ~~These primers, capable of recognising the four established betanodavirus genotypes, failed in some occasions because of sensitivity and specificity limits.~~ Therefore, However, these primers failed in some occasions because of sensitivity and specificity limits. Nishizawa *et al.* (1996) observed that a very small amount of virus in spawners can escape PCR detection, yielding false negative results. On the other hand, Thiéry *et al.* (1999a) reported that the F2-R3 primer set was not capable of recognising the genome of a betanodavirus strain of Atlantic origin because of the genetic diversity of this virus. In agreement with this observation, Grotmol *et al.* (2000) hypothesised that sequence variation among betanodaviruses might result in mismatches between oligonucleotides and their target region, therefore impairing the diagnostic power of the PCR. Since the publication of the F2-R3 primer set, several other PCR-based protocols have been developed to ~~enhance~~ increase sensitivity and specificity of betanodavirus recognition diagnostics, and some are reported in Table 4.1. Assays designed by Dalla Valle *et al.* (2000) and Thiéry *et al.* (1999b) were tested on betanodavirus isolates of Mediterranean origin, while the protocol developed by Grotmol *et al.* (2000) is suitable for detecting cold water strains (Dalla Valle *et al.*, 2000; Grotmol *et al.*, 2000; Thiéry *et al.*, 1999a; 1999b). All primer sets related to the cited literature are summarised in Table 4.1.

Table 4.1. Primer sets used for betanodavirus detection by conventional PCR

Primer	Target	Sequence 5' → 3'	Amplicon size (bp)	Reference
VNNV1	RNA2	ACA-CTG-GAG-TTT-GAA-ATT-CA	605	Dalla Valle <i>et al.</i> , 2000
VNNV2		GTC-TTG-TTG-AAG-TTG-TCC-CA		
VNNV3		ATT-GTG-CCC-CGC-AAA-CAC	255	
VNNV4		GAC-ACG-TTG-ACC-ACA-TCA-GT		
AH95-F1 AH95-R1	RNA2	AGT-GCT-GTG-TCC-CTG-GAG-TG CGC-CCT-GTG-TGA-ATG-TTT-TG	341	Grotmol <i>et al.</i> , 2000
F2 R3	RNA2	CGT-GTC-AGT-CAT-GTG-TCG-CT CGA-GTC-AAC-ACG-GGT-GAA-GA	about 430	Nishizawa <i>et al.</i> , 1994
F2 R3 F'2 R'3	RNA2	CGT-GTC-AGT-CAT-GTG-TCG-CT CGA-GTC-AAC-ACG-GGT-GAA-GA GTT-CCC-TGT-ACA-ACG-ATT-CC GGA-TTT-GAC-GGG-GCT-GCT-CA	420 294	Thiéry <i>et al.</i> , 1999b

#### 4.3.1.2.3.2. Real-time PCR

Generally speaking, real-time PCR methods can improve analytical performance sensitivity and specificity in comparison with conventional PCR assays, providing reliable results with reduced sample handling. Grove *et al.* (2006) developed and validated a real-time RT-PCR method for specifically detecting the Atlantic halibut nervous necrosis virus. The assay appeared to be optimal for the recognition of cold-water viruses but ineffective in targeting warm-water strains (e.g. RGNNV). Dalla Valle *et al.* (2005) standardised a sensitive SYBR Green-based real-time PCR for fish nodaviruses based on two molecular targets (RNA1 and RNA2). The method was capable of detecting the four known genotypes and has been partially validated by using a RGNNV-type strain. Although the use of nonspecific double-stranded DNA dyes results in a considerable improvement of test sensitivity, melting analysis occasionally yields dubious results. It is known that probe-based chemistry is faster and provides higher specificity. Hick *et al.* (2010) optimised a TaqMan-based RT-qPCR (qR2T assay) for betanodavirus detection, which was extensively validated. Analytical and diagnostic sensitivity, repeatability and reproducibility were assessed, and the inhibition of betanodavirus detection due to tissue origin inhibitors was evaluated. Assay specificity was also determined, but the protocol has not been tested on cold-water viral strains. To date, the TaqMan-based method developed by the OIE Reference Laboratory for VER or VNN, validated according to OIE Standards, appears to be suitable for detecting the four established genotypes as well as the Atlantic cod (*Gadus morhua*) and Atlantic halibut betanodaviruses (Panzarin *et al.*, 2010). All primers and probe sets related to the cited literature are reported in Table 4.1. Below is the detailed protocol by Panzarin *et al.* (2010) although other methods may be used.

#### Sample preparation and RNA purification

- i) Homogenise approximately 1 g of Total RNA can be extracted directly from tissue, tissue in a mortar with sterile sand and homogenates diluted it 1:5 in Eagle minimum essential medium (E-MEM) containing 5% fetal calf serum (the same homogenate can be used for virus isolation in cell cultures; for sample preparation see above) or infected cell monolayers, by using the NucleoSpin RNAII (Macherey-Nagel GmbH & Co.) according to the manufacturer's recommendations. Alternative RNA isolation kits of proven performance can be used.
- ii) Centrifuge homogenised tissue for 15 minutes at 4000 g at +4°C.
- iii) Add an antibiotic antimycotic solution (10% v/v) to the clarified supernatant and incubate at +4°C overnight.
- iv) Purify total RNA from 100 µl of clarified supernatant using the NucleoSpin RNAII (Macherey-Nagel GmbH & Co.) following the manufacturer's recommendations. Keep purified RNA at -80°C until use. Alternative RNA isolation kits of proven performance can be used.

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



*Reverse-transcription (RT) and cDNA synthesis*

- i) Prepare a reaction mix for the number of samples to be analysed. The 30 µl master mix for one reaction is made as follows (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems): 6.3 µl PCR-grade water; 3 µl 10× RT buffer; 1.2 µl 25× dNTP mix (100 mM); 3 µl 10× RT random primers; 1.5 µl MultiScribe™ reverse transcriptase (50 U/µl); 15 µl purified RNA.
- ii) Place the tubes in the thermal cycler and apply the following conditions: 10 minutes pre-incubation at 25°C, 120 minutes reverse transcription at 37°C.
- iii) Keep cDNA at –20°C until use.

*Real-time TaqMan PCR*

- i) Prepare a reaction mix for the number of samples to be analysed. The 20 µl master mix for one reaction is made as follows (LightCycler® TaqMan® Master, Roche Diagnostics GmbH): 7.7 µl PCR-grade water; 0.9 µl primer RNA2 FOR (20 µM); 0.9 µl primer RNA2 REV (20 µM); 1.5 µl RNA2 probe (10 µM); 4 µl 5× Master Mix; 5 µl cDNA.
- ii) Place the samples in the real-time platform and start the following thermal profile: 10-minutes incubation at 95°C followed by 45 cycles of 10 seconds denaturation at 95°C, 35 seconds annealing at 58°C and 1 second elongation at 72°C. Cycling conditions refer to the LightCycler 2.0 platform.
- iii) Analyse data with the instrument-related software. The diagnostic cutoff limit is set at 36 CP (crossing point). In case of doubtful results (e.g. CP ≥ 36), repeat the analysis.

NOTE: Assay performances can vary depending on the conditions under which the protocol is carried out (for example, the thermal profile might need optimisation, depending on the platform in use). Of note, it is highly recommended to perform the protocol by Panzarin *et al.* (2010) in two-steps, otherwise test sensitivity might be dramatically affected.

Table 4.2. Primers/probe sets used for betanodavirus detection by Real-time PCR

Primer	Target	Sequence 5' → 3'	Amplicon size (bp)	Reference
Q-RdRP-1 Q-RdRP-2	RNA1	GTG-TCC-GGA-GAG-GTT-AAG-GAT-G CTT-GAA-TTG-ATC-AAC-GGT-GAA-CA	273	Dalla Valle <i>et al.</i> , 2005
Q-CP-1 Q-CP-2	RNA2	CAA-CTG-ACA-ACG-ATC-ACA-CCT-TC CAA-TCG-AAC-ACT-CCA-GCG-ACA	230	
P1 P2 Probe	RNA2	GGT-ATG-TCG-AGA-ATC-GCC-C TAA-CCA-CCG-CCC-GTG-TT TTA-TCC-CAG-CTG-GCA-CCG-GC*	194	Grove <i>et al.</i> , 2006
qR2TF qR2TR R2probe2	RNA2	CTT-CCT-GCC-TGA-TCC-AAC-TG GTT-CTG-CTT-TCC-CAC-CAT-TTG CAA-CGA-CTG-CAC-CAC-GAG-TTG*	93	Hick <i>et al.</i> , 2010
RNA2 FOR RNA2 REV RNA2 probe	RNA2	CAA-CTG-ACA-RCG-AHC-ACA-C CCC-ACC-AYT-TGG-CVA-C TYC-ARG-CRA-CTC-GTG-GTG-CVG*	69	Panzarin <i>et al.</i> , 2010

\* Reporter dye, FAM; quencher, BHQ1

NOTE: with the exception of the above method, none of the protocols cited in paragraphs 4.3.1.2.3.1. and 4.3.1.2.3.2. has been officially validated through documented inter-laboratory proficiency tests. The protocol by Panzarin *et al.* (2012) has been subjected to a ring trial involving five European laboratories.

#### 4.3.1.2.3.3. Sequencing

Genome sequencing, besides being valuable for diagnosis confirmation, is also fundamental useful for epidemiological investigations. Phylogenetic studies. Sequence analysis of the T4 variable region (Nishizawa *et al.*, 1997) allows the correct assignment of genotype. However, the phylogenetic analysis based on the sequence of both genomic segments, appears to be fundamental more informative, and it is recommended in order to highlight possible reassortment events occurring between different betanodavirus genotypes and within genotypes (Oliveira *et al.*, 2009; Panzarin *et al.*, 2012; Toffolo *et al.*, 2007).

## Annex 13 (contd)

### 4.3.1.2.4. Agent purification

Betanodaviruses may be easily purified by ultracentrifugation through cesium chloride gradients (Chi *et al.*, 2001; Comps *et al.*, 1994; Mori *et al.*, 1992).

### 4.3.2. Serological methods

As there has been insufficient research, detection of specific antibodies has not been so far considered as a routine screening method for assessing the viral status of fish populations. Nevertheless evidence of specific antibodies has been reported by different authors. According to field observations an ELISA titre  $\geq 1:40$  is indicative of viral infection while values  $\leq 1:10$  is indicative of freedom from viral infection (Watanabe *et al.*, 2000).

## 5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of VER/VNN 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, but cost and availability limit its application; 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. ~~n.a = not applicable~~. 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. Betanodavirus surveillance, detection and diagnostic methods**

Method	Targeted surveillance	Presumptive diagnosis	Confirmatory diagnosis
	Larvae	Juveniles	Adults
Histopathology	d	c	d
Histopathology followed by immunostaining	c	b	a
Transmission EM	d	c	d
Isolation in cell culture followed by immunostaining, real-time PCR or PCR and sequencing	b	a	a
RT-PCR	c	a	c
RT-PCR followed by sequencing	b	a	a
Real-time PCR	a	a	a*

EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction.

## 6. Test(s) recommended for targeted surveillance to declare freedom from viral encephalopathy and retinopathy

Targeted surveillance should rely on regular monitoring of farms rearing susceptible species. If available, larval stages and juveniles should preferably be sampled during the most suitable season taking in account the optimum temperature of the genotypes expected.

NOTE: At present no official or recommended procedures to test healthy populations have been issued to demonstrate freedom from the disease. Real-time PCR followed by virus isolation ~~or conventional RT-PCR and sequence analysis in case of positive results~~ should be considered the most suitable method for targeted betanodaviruses surveillance.

## 7. Corroborative diagnostic criteria

### 7.1. Definition of suspect case

VER or VNN shall be suspected if at least one of the following criteria is met:

- i) Appearance of abnormal swimming behaviour in susceptible species;
- ii) Typical histopathological lesions detected in a population of susceptible species;
- iii) Typical CPE observed in cell cultures without identification of the causative agent;
- iv) A single positive result from one of the diagnostic assays ranked as 'a' or 'b' in "Presumptive diagnosis" of Table 5.1.;
- v) Transfer of live fish from an infected farm to another site;
- vi) Existing of different epidemiological links between one infected farm and a second farm;
- vii) Detection of specific antibody activity.

### 7.2. Definition of confirmed case

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

- i) ~~—A confirmed case of VER or VNN is defined as a suspect case which has produced a typical CPE in cell culture with subsequent identification of the causative agent by either an antibody-based and/or molecular test or a~~
- ii) ~~—A second positive result from a different diagnostic assay ranked as 'a' in "Confirmatory diagnosis" of Table 5.1. (except the combination of PCR + real-time PCR).~~

## 8. References

- ARIMOTO M., MARUYAMA K. & FURUSAWA I. (1994). Epizootiology of viral nervous necrosis (VNN) in striped jack. *Fish Pathol.*, **29**, 19–24.
- ARIMOTO M., MUSHIAKE K., MIZUTA Y., NAKAI T., MUROGA K. & FURUSAWA I. (1992). Detection of striped jack nervous necrosis virus (SJNNV) by enzyme-linked immunosorbent assay (ELISA). *Fish Pathol.*, **27**, 191–195.
- ARIMOTO M., SATO J. MARUYAMA K., MIMURA G. & FURUSAWA I. (1996). Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV). *Aquaculture* **143**, 15–22.
- BARKER D.E., MACKINNON A.M., BOSTON L, BURT M.D.B., CONE D.K., SPEARE D.J., GRIFFITHS S., COOK M., RITCHIE R. & OLIVIER G. (2002). First report of piscine nodavirus infecting wild winter flounder *Pleuronectes americanus* in Passamaquoddy Bay, New Brunswick, Canada. *Dis. Aquat. Org.*, **49**, 99–105.
- BOVO G, GUSTINELLI A., QUAGLIO F., GOBBO F., PANZARIN V., FUSARO A., MUTINELLI F., CAFFARA M., FIORAVANTI M. L. (2011). Viral encephalopathy and retinopathy outbreak in freshwater fish farmed in Italy. *Dis. Aquat. Org.*, **96**, 45–54.
- CHI S.C., HU W.W. & LO B.L. (1999). Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coioides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV). *J. Fish Dis.*, **22**, 173–182.
- CHI S.C., LO C.F. & LIN S.C. (2001). Characterization of grouper nervous necrosis virus. *J. Fish Dis.*, **24**, 3–13.
- CHI S.C., SHIEH J.R. & LIN S.J. (2003). Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Dis. Aquat. Org.*, **55**, 221–228.

Annex 13 (contd)

CHI S.C., WU Y.C. & CHENG T.M. (2005). Persistent infection of betanodavirus in a novel cell line derived from the brain tissue of barramundi *Lates calcarifer*. *Dis. Aquat. Org.*, **65**, 91–98.

CIULLI S., DI MARCO P., NATALE A., GALLETI E., BATTIMANI M., SCAGLIARINI A., PUR PARI G, CANNELLA V., CASTIGLIONE F. & GUERCIO A. (2006). Detection and characterization of Betanodavirus in wild fish from Sicily., Italy. *Ittiopatologia*, **3**, 101–112.

COMPS M., PEPIN J.F. & BONAMI J.R. (1994). Purification and characterization of two fish encephalitis viruses (FEV) infecting *Lates calcarifer* and *Dicentrarchus labrax*. *Aquaculture*, **123**, 1–10.

DALLA VALLE L., TOFFOLO V., LAMPRECHT M., MALTESE C., BOVO G., BELVEDERE P. & COLOMBO L. (2005). Development of a sensitive and quantitative diagnostic assay for fish nervous necrosis virus based on two-target real-time PCR. *Vet. Microbiol.*, **110**, 167–179.

DALLA VALLE, L., ZANELLA L., PATARNELLO P., PAOLUCCI L., BELVEDERE P & COLOMBO L (2000). Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. *J. Fish Dis.*, **23**, 321–327

FRERICHS G.N., RODGER H.D. & PERIC Z. (1996). Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. *J. Gen. Virol.*, **77**, 2067–2071.

FRERICHS G.N., TWEEDIE A., STARKEY W.G. & RICHARDS R.H. (2000). Temperature, pH, and electrolyte sensitivity, and heat, UV and disinfectant inactivation of sea bass (*Dicentrarchus labrax*) neuropathy nodavirus. *Aquaculture*, **185**, 13–24.

FURUSAWA R., OKINAKA Y., UEMATSU K. & NAKAI T. (2007). Screening of freshwater fish species for their susceptibility to a betanodavirus. *Dis. Aquat. Org.*, **77**, 119–125.

GOMEZ D.K., SATO J., MUSHIAKE K., ISSHIKI T., OKINAKA Y. & NAKAI T. (2004). PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *J. Fish Dis.*, **27**, 603–608.

GROTMOL S., BERGH O & TOTLAND G.K. (1999). Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: occurrence of nodavirus in various organs and a possible route of infection. *Dis. Aquat. Org.*, **36**, 95–106.

GROTMOL S., NERLAND A.H., BIERING E., TOTLAND G.K. & NISHIZAWA T. (2000). Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and design an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. *Dis. Aquat. Org.*, **39**, 79–88.

GROTMOL S. & TOTLAND G.K. (2000). Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. *Dis. Aquat. Org.*, **39**, 89–96.

GROVE S., FALLER R., SOLEIM K.B. & DANNEVIG B.H. (2006). Absolute quantitation of RNA by a competitive real-time RT-PCR method using piscine nodavirus as a model. *J. Virol. Methods*, **132**, 104–112.

HICK P. & WHITTINGTON R.J. (2010). Optimisation and validation of a real-time reverse transcriptase-polymerase chain reaction assay for detection of betanodavirus. *J. Virol. Methods*, **163**, 368–377.

IWAMOTO T., NAKAI T., MORI K., ARIMOTO M. & FURUSAWA I. (2000). Cloning of the fish cell line SSN-1 for piscine nodaviruses. *Dis. Aquat. Org.*, **43**, 81–89.

IWAMOTO T., MISE K., MORI K., ARIMOTO M., NAKAI T. & OKUNO T. (2001). Establishment of an infectious RNA transcription system for *Striped jack nervous necrosis virus*, the type species of the betanodavirus. *J. Gen. Virol.*, **82**, 2653–2662.

IWAMOTO T., OKINAKA Y., MISE K., MORI K., ARIMOTO M., OKUNO T. & NAKAI T. (2004). Identification of host-specificity determinants in betanodaviruses by using reassortants between striped jack nervous necrosis virus and sevenband grouper nervous necrosis virus. *J. Virol.*, **78**, 1256–1262.

JOHANSEN R., RANHEIM T., HANSEN M.K., TAKSDAL T. & TOTLAND G.K. (2002). Pathological changes in juvenile Atlantic halibut *Hippoglossus hippoglossus* persistently infected with nodavirus. *Dis. Aquat. Org.*, **50**, 161–169.

## Annex 13 (contd)

JOHANSEN R., AMUNDSEN M. DANNEVIG B.H. & SOMMER A.I. (2003). Acute and persistent experimental nodavirus infection in spotted wolffish *Anarhichas minor*. *Dis. Aquat. Org.*, **57**, 35–41.

JOHANSEN R., ROVE S., SVENDSEN N.A.K., MODAHL I. & DANNEVIG B. (2004). A sequential study of pathological findings in Atlantic halibut, *Hippoglossus hippoglossus* (L), throughout one year after an acute outbreak of viral encephalopathy and retinopathy. *J. Fish Dis.*, **27**, 327–341.

KAI Y.H. & CHI S.C. (2008). Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. *Vaccine*, **26**, 1450–1457.

MALTESE C. & BOVO G. (2007). Viral encephalopathy and retinopathy. *Ittiopatologia*, **4**, 93–146.

MORI K., NAKAI T., MUROGA K., ARIMOTO M., MUSHIAKE K. & FURUSAWA I. (1992). Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187**, 368–371.

MORI K., MANGYOKU T., IWAMOTO T., ARIMOTO M., TANAKA S. & NAKAI T. (2003). Serological relationships among genotypic variants of betanodavirus. *Dis. Aquat. Org.*, **57**, 19–26.

MORI K., MUSHIAKE K. & ARIMOTO M. (1998). Control measures for viral nervous necrosis in striped jack. *Fish Pathol.*, **33**, 443–444.

MORI K., SUGAYA T., NISHIOCAT., GOMEZ D.K., FUJINAMY Y., OKA M., ARIMOTO M., OKINAKA Y. & NAKAI T. (2005). Detection of Betanodaviruses from feed fish used in marine aquaculture. *In: 12th International Conference Diseases of Fish and Shellfish*, European Association of Fish Pathologists. Copenhagen (Denmark), 11–16 September 2005. Abstract O-142.

MUNDAY B.L., KWANG J. & MOODY N. (2002). Betanodavirus infections of teleost fish: a review. *J. Fish Dis.*, **25**, 127–142.

MUSHIAKE K., NISHIZAWA T., NAKAI T., FURUSAWA I. & MUROGA K. (1994). Control of VNN in striped jack: Selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). *Fish Pathol.*, **29**, 177–182.

NGUYEN H.D., MUSHIAKE K., NAKAI T. & MUROGA K. (1997). Tissue distribution of striped jack nervous necrosis virus (SJNNV) in adult striped jack. *Dis. Aquatic. Org.*, **28**, 87–91

NISHIZAWA T., MORI K., NAKAI T., FURUSAWA I. & MUROGA K. (1994). Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Dis. Aquat. Org.*, **18**, 103–107.

NISHIZAWA T., MUROGA K. & ARIMOTO M. (1996). Failure of the polymerase chain reaction (PCR) method to detect jack nervous necrosis virus (SJNNV) in striped jack *Pseudocaranx dentex* selected as spawners. *J. Aquat. Anim. Health.*, **8**, 332–334.

NISHIZAWA T., FURUHASHI M., NAGAI T., NAKAI T. & MUROGA K. (1997). Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Appl. Environ. Microbiol.*, **63**, 1633–1636.

OLVEIRA J.G., SOUTO S., DOPAZO C.P., THIÉRY R., BARJA J.L. & BANDÍN I. (2009). Comparative analysis of both genomic segments of betanodaviruses isolated from epizootic outbreaks in farmed fish species provides evidence for genetic reassortment. *J. Gen. Virol.*, **90**, 2940–2951.

~~PANTOJA C.R., TANG K.F.J., REDMAN R. & LIGHTNER D.V. (2007). Identification of a nodavirus that causes muscle necrosis in *Litopenaeus vannamei* and the development of an *in situ* hybridization and RT-PCR assay for its detection. Meeting abstract World Aquaculture Society.~~

PANZARIN V., FUSARO A., MONNE I., CAPPELLOZZA E., PATARNELLO P., BOVO G., CAPUA I., HOLMES E.C. & CATTOLI G. (2012). Molecular epidemiology and evolutionary dynamics of betanodavirus in southern Europe. *Infect Genet Evol* **12**, 63–70.

PANZARIN V., PATARNELLO P., MORI A., RAMPAZZO E., CAPPELLOZZA E., BOVO G. & CATTOLI G. (2010). Development and validation of a real-time TaqMan PCR assay for the detection of betanodavirus in clinical specimens. *Arch. Virol.*, **155**, 1193–1203.

Annex 13 (contd)

SANO M., NAKAI T. & FIJAN N. (2011). Viral diseases and agents of warmwater fish. In: Fish Diseases and Disorders, Vol. 3: Viral, Bacterial and Fungal Infections, 2nd edition, Woo P.T.K & Bruno D.W., eds. CABI, London, UK, 166–244.

SCHNEEMANN A., BALL L.A., DELSERT C., JOHNSON J.E. & NISHIZAWA T. (2005). FAMILY NODAVIRIDAE. In: VIRUS TAXONOMY—EIGHTH REPORT OF THE INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES, FAUQUET C.M., MAYO M.A., MANILOFF J., DESSELBERGER U. & BALL L.A., EDS. ELSEVIER ACADEMIC PRESS, LONDON, UK, 865–872.

SOMMERSET I. & NERLAND A.H. (2004). Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). *Dis. Aquat. Org.*, **58**, 117–125.

TAN C., HUANG B., CHANG S.F., NGOH G.H., MUNDAY B.L., CHEN S.C. & KWANG J. (2001). Determination of the complete nucleotide sequence of RNA1 and RNA2 from greasy grouper (*Epinephelus tauvina*) nervous necrosis virus, Singapore strain. *J. Gen. Virol.*, **82**, 647–653.

TANAKA S., MORI K., ARIMOTO M., IWAMOO T & NAKAI T. (2001). Protective immunity of sevenband grouper, *Epinephelus septemfasciatus* Thunberg, against experimental viral nervous necrosis. *J. Fish Dis.*, **24**, 15–22.

TANAKA S., AOKI H. & NAKAI T. (1998). Pathogenicity of the nodavirus detected from diseased sevenband grouper *Epinephelus septemfasciatus*. *Fish Pathol.*, **33**, 31–36.

THIÉRY R., ARNAULD C., DELSERT C. (1999a). Two isolates of sea bass, *Dicentrarchus labrax* L., nervous necrosis virus with distinct genomes. *J. Fish Dis.*, **22**, 201–207.

THIÉRY R., RAIMOND J.C., CASTRIC J. (1999b). Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. *Virus Res.*, **63**, 11–17.

~~THIÉRY THIÉRY R.,~~ COZIEN J., DE BOISSESON C., KERBART-BOSCHER S. & NEVAREZ L. (2004). Genomic classification of new betanodavirus isolates by phylogenetic analysis of the coat protein gene suggest a low host-fish species specificity. *J. Gen. Virol.*, **85**, 3079–3087.

THIÉRY R., COZIEN J., CABON J., LAMOUR F., BAUD M. & SCHNEEMANN A. (2006). Induction of a protective immune response against viral nervous necrosis in the European sea bass *Dicentrarchus labrax* by using betanodavirus virus-like particles. *J. Virol.*, **80**, 10201–10207.

THIÉRY R., JOHNSON K.L., NAKAI T., SCHNEEMANN A., BONAMI J.R., LIGHTNER D.V. (2011). Family Nodaviridae. In: Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses, King A.M.Q., Adams M.J., Carstens E.B. & Lefkowitz E.J., eds. Elsevier Academic Press, London, UK, 1061–1067.

TOFFOLO V., NEGRISOLO E., MALTESE C., BOVO G., BELVEDERE P., COLOMBO L. & DALLA VALLE L. (2007). Phylogeny of betanodaviruses and molecular evolution of their RNA polymerase and coat proteins. *Mol. Phylogenet. Evol.*, **43**, 298–308.

WATANABE K., NISHIZAWA T. & YOSHIMIZU M. (2000). Selection of broodstock candidates of barfin flounder using an ELISA system with recombinant protein of barfin flounder nervous necrosis virus. *Dis. Aquat. Org.*, **41**, 219–223.

YAMASHITA Y., FUJITA Y., KAWAKAMI H. & NAKAI T. (2005). The efficacy of inactivated virus vaccine against viral nervous necrosis (VNN). *Fish Pathol.*, **40**, 15–21.

YAMASHITA H., MORI K., KURODA A. & NAKAI T. (2009a). Neutralizing antibody levels for protection against betanodavirus infection in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg), immunized with an inactivated virus vaccine. *J. Fish Dis.*, **32**, 767–775.

Annex 13 (contd)

YAMASHITA H., MORI K. & NAKAI T. (2009b). Protection against viral nervous necrosis conferred by simultaneous inoculation of aquabirnavirus and inactivated betanodavirus in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg). *J. Fish Dis.*, **32**, 201–210.

YUASA K., ISTI KOESHARYANI & KETUT MAHARDIKA (2007). Effect of high water temperature on betanodavirus infection of fingerling humpback grouper *Cromileptes altivelis*. *Fish Pathol.*, **42**, 219–221.

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**NB:** There is an OIE Reference Laboratory for Viral encephalopathy and retinopathy (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> ).

Please contact the OIE Reference Laboratories for any further information on viral encephalopathy and retinopathy





## CHAPTER 2.4.9

**INFECTION WITH OSTREID  
HERPESVIRUS 1 MICROVARIANT**

**EU position**

**The EU thanks the OIE and in general supports the adoption of this modified chapter.**

**The EU notes that the revised title of the chapter indicates that this chapter deals with OsHV1- microvariant, the strain of OsHV1 which has caused significant mortality in *C. gigas* in both the northern and southern hemisphere since 2007. The text which has been presented however, has references to both the microvariant and the reference strain throughout, making it difficult to determine in places, which characteristics refer to the microvariant, which is the subject of the chapter.**

**Furthermore, the EU notes that the abbreviation "μVar" is still used in some places in the text of this chapter. For reasons of consistency, the EU suggests the word "microvariant" be used throughout the text instead of the abbreviation.**

**Specific comments are inserted in the text below.**

**1. Scope**

For the purpose of this chapter, infection with ostreid herpesvirus 1 microvariant (OsHV-1) is considered to be a viral infection caused by variants of OsHV-1 affecting mainly Pacific cupped oysters, *Crassostrea gigas* 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. Ostreid herpesvirus 1 μVar is strictly defined by a 12 bp deletion in ORF4; however, the scope of this chapter includes related variants with a deletion of around 12 base pairs in this region. The term OsHV-1 microvariant is used in this chapter to refer to OsHV-1 μVar and these related variants. Infection with OsHV-1 microvariant mainly affects the Pacific cupped oyster, *Crassostrea gigas*.

**EU comment**

**The EU suggests rewording the final sentence of the paragraph above as follows:**

**"Mortality associated with OsHV1 microvariant has only been reported in the pacific cupped oyster *Crassostrea gigas*".**

The term OsHV-1 is used throughout the present chapter to define a virus species. The virus reported by Davison *et al.* (2005) corresponds to a particular 'isolate' or strain collected from infected Pacific oyster larvae in France in 1995. As this 'isolate' was the first to be described (through complete genome sequencing), it can be assumed to be a reference type. Other isolates, including OsHV-1 var and OsHV-1 μVar, have also been identified and studied. In this context, OsHV-1 means all virus isolates or strains and the virus reported by Davison *et al.* (2005) defined as the reference type. Differences in virulence may exist among OsHV-1 strains.

**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 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.

#### 2.1.1.1. 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 herpes viruses. 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 (OshV-1var) 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.

More recently, polymerase chain reactions (PCRs) using different primer sets and PCR product sequencing IA1-IA2 and C2-C6 enabled the detection of a variant (OshV-1  $\mu$ Var) called  $\mu$ Var and related variants in association with the high mortality events reported in Europe, Australia and New Zealand since 2008 (Lynch *et al.*, 2012; Martenot *et al.*, 2011; Peeler *et al.*, 2012; Renault *et al.*, 2012; Segarra *et al.*, 2010). The term  $\mu$ Var is used to define a single variant presenting all the mutations reported by Segarra *et al.* (2010) in 2 different virus genome areas.

Although the aetiological agent is represented by all specimens or variants strains of ostreid herpesvirus 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  $\mu$ Var or related viral variants suggested differences in terms of virulence among OshV-1 variants. However, the detection of the variant  $\mu$ Var or related variants have also been reported in absence of mortality events (Dundan *et al.*, 2011; EFSA, 2010; Shimahara *et al.*, 2012) suggesting that viral infection is influenced by both host and environmental factors.

#### 2.1.1.2. 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.

#### 2.1.1.3. 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.

#### 2.1.1.4. Life cycle

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

Ostreid herpesvirus 1 has been reported from the Pacific oyster, *C. gigas*, Portuguese oyster, *C. angulata*, suminoe oyster, *C. ariakensis*, European flat oyster, *O. edulis*, Manila clam, *R. philippinarum*, carpet shell clam, *R. decussates*, and scallops, *P. maximus* (Arzul *et al.*, 2001a; 2001b; Renault *et al.*, 2000). Ostreid herpesvirus 1  $\mu$ Var (Segarra *et al.*, 2010) has been mainly reported as affecting the Pacific oyster, *Crassostrea gigas*.

#### EU comment

**The EU is of the opinion that point 2.2.1. above should only reflect the species which are susceptible to OsHV 1  $\mu$ Var.**

**Furthermore, the final sentence states that the variant strain has "been mainly reported as affecting the Pacific oyster [...]". However, to the best of our knowledge, this strain has only been reported to cause mortality in the Pacific oyster, although it has been detected in other species such as mussels and clams, but without associated mortality. Therefore, the EU suggests adding that mortality attributable to the microvariant has only been observed in *Crassostrea gigas*.**

### 2.2.2. Susceptible stages of the host

OsHV-1 infection causes mortality in larvae and juveniles of several bivalve species. The virus can be found in adult bivalves most often in absence of mortality.

#### EU comment

**The EU notes that point 2.2.2. above deals with OsHV-1 infection, not with the microvariant. The EU suggests adding details with references of where the microvariant has occurred without observable mortalities.**

### 2.2.3. Species or sub-population predilection (probability of detection)

*Crassostrea gigas*, *O. edulis*, *R. philippinarum*, *R. decussates* and *P. maximus* are naturally infected. 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). P  pin *et al.* (2008) showed that DNA copy numbers  $\text{mg}^{-1}$  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 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  $\mu$ V<sub>var</sub> DNA has been recently detected in France 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.

#### EU comment

**The EU would like to inform the OIE that  $\mu$ V<sub>var</sub> DNA has also been detected in blue mussels (*M. edulis*) in Ireland. Furthermore, the final sentence of point 2.2.7. above should be extended to say that mortalities have not been reported in these 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  $\mu$ V<sub>var</sub> can be experimentally transmitted horizontally via the water (Schikorski *et al.*, 2011a), which presumably is the main natural mode of OshV-1 transmission.

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).

#### EU comment

**The EU notes that the paragraph above only deals with OshV-1 infection, not with the microvariant. Given the revised title of the chapter, the OIE should consider deleting that paragraph.**

Experimental transmission of OshV-1 $\mu$ V<sub>var</sub> 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. Using-Based on qPCR detection, results suggest that the virus ~~was shown to~~ may enter the digestive gland and haemolymphatic system, following which the virus was disseminated to other organs.

#### 2.3.2. Prevalence

Reported mortality rates vary considerably between sites and countries and depend on the age of affected stocks. 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.

#### EU comment

**The EU notes that the paragraph above solely deals with OshV-1 infection, not with the microvariant.**

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 emergence detection of OshV-1  $\mu$ V<sub>var</sub> 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). OshV-1  $\mu$ var was detected in most of the investigated samples.

### 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 microvariant has been reported associated with Pacific oyster mass mortalities in Europe, Australia and New Zealand, but is known to occur elsewhere in the absence of oyster mortalities.

#### EU comment

**The first sentence of the paragraph above refers to the countries where the reference strain has been found, including individual countries in the EU. Given the revised title of the chapter, the EU questions whether this detailed information is needed. On the other hand, the second sentence gives details of where the  $\mu$ Var strain is found, but does not name the EU countries which have been affected.**

### 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 are more frequent during summer, which might suggest a link between seawater temperature and OsHV-1 infection. The temperature influence on OsHV-1 detection and virus expression was demonstrated for *C. gigas* larvae (Le Deuff *et al.*, 1996) and strongly suspected for *C. gigas* spat (Burge *et al.*, 2007; Friedman *et al.*, 2005; Renault *et al.*, 1995; Sauvage *et al.*, 2009). A temperature threshold related to enhanced OsHV-1 expression or mortality appears difficult to define precisely. In the literature, according to the site, the temperature threshold was variable: 22°C to 25°C on the west coast of the USA (Friedman *et al.*, 2005; Burge *et al.*, 2007) and 18 to 20°C in France (Samain *et al.*, 2007; Soletchnik *et al.*, 1999). High seawater temperatures appear to be one of the potential factors influencing OsHV-1 infection.

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.

#### EU comment

**The EU notes that point 2.3.5. above solely deals with OsHV-1 infection, not with the microvariant.**

### 2.4. Control and prevention

#### 2.4.1. Vaccination

None Not applicable

#### 2.4.2. Chemotherapy

None

#### 2.4.3. Immunostimulation

None Not applicable

#### 2.4.4. Resistance breeding

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

#### 2.4.5. Restocking with resistant species

In France, a project of restocking with selected Pacific oysters is ongoing. None

#### 2.4.6. Blocking agents

None

#### 2.4.7. Disinfection of eggs and larvae

None

#### 2.4.8. General husbandry practices

Bio-security 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. However, it has been demonstrated that individual herpes virus species may have different levels of stability to inactivation treatment and that inorganic salts such as  $\text{Na}_2\text{SO}_4$  present in seawater may stabilise herpes viruses (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 ( $-80^\circ\text{C}$ ).

#### 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

<b>EU comment</b>
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**It is not clear which is the recommendation of the Manual as to the pooling of samples for PCR analysis.**

**3.4. Best organs or tissues**

For histology, a 25-µm thick section through the visceral mass that includes digestive gland, gill and mantle is 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 may cause an acute disease. Animals are likely to die within a few days of demonstrating clinical signs of the disease. Clinical signs may be dead or gaping bivalves but these clinical signs are not specific to infection with OsHV-1.

**EU comment**

**The EU notes that point 4.1.1. above describes the clinical signs of OsHV-1 infection, whereas no information is given as regards the microvariant.**

**4.1.2. Behavioural changes**

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

**4.2. Clinical methods**

**4.2.1. Gross pathology**

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

**4.2.2. Clinical chemistry**

None

**4.2.3. Microscopic pathology**

See Section 4.2.6. Fixed sections

**4.2.4. Wet mounts**

Not applicable

**4.2.5. Smears**

Not applicable

**4.2.6. Fixed sections**

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

Histological examination of the animal is not sufficient to identify infection with herpes virus. Whilst Cowdry type A inclusions (eosinophilic intranuclear inclusions with perinuclear chromatin) are typical of many herpes virus infections they are not a diagnostic feature of herpes virus 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  $\times 400$ .

*Positive controls:* These are recommended and are available from the Genetics and Pathology Laboratory, Ifremer, La Tremblade, France. Positive controls are tissue sections from 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

#### EU comment

**The EU notes that the recommendation in the point above for positive controls is that they should come from molluscs infected with OsHV1, while there is no mention of obtaining positive controls from animals infected with the  $\mu$ Var strain.**

##### 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 OsHV-4 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 bivalve 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 (Pepin *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 ORF4 area (microsatellite) for the variant  $\mu$ Var (Pepin *et al.*, com pers).

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

*Samples to be taken:* Live or moribund molluscs. Larvae (100–200 mg), spat (100–200 mg) or 2–3 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.*, 2000a). 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 (OsHV-1var) in *C. gigas* and *R. philippinarum* larvae (Arzul *et al.*, 2001c).

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.*, 2000a). An experimental procedure scheme used for the detection of OsHV-1 DNA by conventional PCR has been proposed by Bastista *et al.* (2007).

### EU comment

**It is noted that in point 4.3.1.2.3.1. above, no reference is made to a conventional PCR that has recently been developed by Aranguren *et al.*, 2012 (polymerase chain reaction restriction fragment length polymorphism assay PCR-RFLP), by which it is possible to distinguish between the reference herpesvirus and OsHV-1 microvariant. The EU would like to ask the OIE to consider including this new method in the Manual.**

#### 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®) 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.*, 2001a; ORF99 encoding a BIR protein) and C9/C10 (Barbosa-Solomieu *et al.*, 2004; ORF4) were previously designed for single PCR, whereas the Gp4/Gp7 primer pair (ORF88 encoding a class I membrane protein) was assessed for qPCR. The primer pairs B4/B3, C9/C10 and Gp4/Gp7 yield PCR products of 207, 197 and 85 bp, respectively.

B4:	5'-ACT-GGG-ATC-CGA-CTG-ACA-AC-3'
B3:	5'-GTG-GAG-GTG-GCT-GTT-GAA-AT-3'
C9:	5'-GAG-GGA-AAT-TTG-CGA-GAG-AA-3'
C10:	5'-ATC-ACC-GGC-AGA-CGT-AGG-3'
Gp4:	5'-GGC-GTC-CAA-ACT-CGA-TTA-AA-3'
Gp7:	5'-TTA-CAC-CTT-TGC-CGG-TGA-AT-3'

The C9/C10 primer pair yield reliable parameters for qPCR with OsHV-1 DNA, as well as the B3/B4 primer pair, which show closely similar parameters with a slightly lower *E* value (96.3%). The Gp4/Gp7 primer pair is less efficient (*E* = 91.3%) and less sensitive ( $\geq 50$  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 values obtained with the standard curve, using the Thermocycler software. Each experiment includes a positive DNA control (OsHV-1 genomic DNA for absolute quantitation) and blank controls (NTC, no template control consisting of deionised sterile water). PCR efficiency ( $E$ ) is calculated from standard curves as the percentage of template molecules that is doubled during each cycle ( $[10^{(-1/\text{slope})} - 1] \times 100$ ), with requirements that it fell into the range 95–105% and that the coefficient of determination ( $R^2$ ) is  $>0.98$ . In order to allow detection of non-specific products, a dissociation protocol (melt curve) takes place after the amplification cycles. The temperature ( $T_m$ ) at which SYBR®Green fluorescence is generated by the double-stranded amplicon dissociation is recorded.

The sensitivity was considered to 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).

The reaction mixture contained 12.5  $\mu\text{l}$  premix ExTaq® 2× Takara® (Lonza, Verviers, Belgium), 0.5  $\mu\text{l}$  each primer (20  $\mu\text{M}$ ), 0.5  $\mu\text{l}$  TaqMan® probes (10  $\mu\text{M}$ ) and 9  $\mu\text{l}$  water. Two  $\mu\text{l}$  DNA sample was added to 23  $\mu\text{l}$  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  $\mu\text{m}$  thick tissue sections on silane-prep™ slides are dewaxed in xylene (2 × 5 minutes), treated in absolute ethanol (2 × 5 minutes) and air dried at room temperature (15 minutes). Sections are then permeabilised with proteinase K (100  $\mu\text{g ml}^{-1}$  in distilled water) for 30 minutes at 37°C in a humid chamber. Proteolysis is stopped by one 3-minute wash in 0.1 M Tris, 0.1 M NaCl buffer (pH 7.5) at room temperature. Sections are dehydrated in 95° ethanol for 1 minute, absolute ethanol for 1 minute and air dried (15 minutes).

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

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

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

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

After hybridisation, coverslips were removed carefully and slides were washed for 10 minutes in 1 × 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. herpes virus-like until further investigations are done to provide further evidence of the identity of the virus. As different herpes viruses 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, immuno-histochemistry or *in situ* hybridisation.

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.

## Annex 14 (contd)

Table 5.1. Methods for targeted surveillance and diagnosis

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

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

**EU comment**

**In table 5.1. above, the EU wonders whether the deletion of methods for presumptive and confirmatory diagnosis is intended. It seems inconsistent with text in the section which states that there are no tests recommended for targeted surveillance.**

**Furthermore, sequencing is only used to confirm a positive PCR test so its rating for all other purposes should be N/A (not applicable) or just left blank.**

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

Not applicable. ~~The test recommended for targeted surveillance is qPCR on extracted nucleic acids from bivalve samples.~~

**EU comment**

**In point 6. above, the EU wonders why the PCR is proposed to be removed as attest for targeted surveillance. Is this because the microvariant is listed only as an emerging disease?**

**Furthermore, the EU would like to inform the OIE that it has been operating a targeted surveillance programme for the past 3 years using a sampling scheme and laboratory methodology recommended by the EURL. The EU therefore invites the OIE to consider mentioning this programme under this point.**

**7. Corroborative diagnostic criteria**

**7.1. Definition of suspect case**

A suspect case of infection with OsHV-1 is a positive result microvariant is a case of mortality of susceptible species associated with detection of OsHV-1 by PCR, or qPCR, or *in-situ* hybridisation.

## 7.2. Definition of confirmed case

1. ~~In the case of mortality of susceptible species in a country, zone or compartment in which OsHV-1 was previously detected, a confirmed case of infection with OsHV-1 is a positive result by any one of the following methods: PCR, qPCR or ISH.~~

2. ~~In other cases, A confirmed case is defined as a suspect case followed positive results of PCR or qPCR targeting different viral genome regions confirmed by sequencing of the microsatellite locus upstream the ORF4 several PCR products (Segarra *et al.*, 2010) leading to sequences consistent with the definition of microvariant.~~

## 8. References

- ARZUL I., NICOLAS J.-L., DAVISON A.J. & RENAULT T. (2001a). French scallops: a new host for ostreid herpesvirus-1. *Virology*, **290**, 342–349.
- ARZUL I., RENAULT T., LIPART C. & DAVISON A.J. (2001b) Evidence for inter species transmission of oyster herpesvirus in marines bivalves. *J. Gen. Virol.*, **82**, 865–870.
- ARZUL I., RENAULT T., THÉBAULT A. & GÉRARD A. (2002). Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Res.*, **84**, 151–160.
- BARBOSA-SOLOMIEU V., DÉGREMONT L., VÁZQUEZ-JUÁREZ R., ASCENCIO-VALLE F., BOUDRY P. & RENAULT T. (2005). Ostreid herpesvirus 1 (OsHV-1) detection among three successive generations of Pacific oysters (*Crassostrea gigas*). *Virus Res.*, **107**, 47–56.
- BARBOSA-SOLOMIEU V., MIOSSEC L., VÁZQUEZ-JUÁREZ R., ASCENCIO-VALLE F. & RENAULT T. (2004). Diagnosis of Ostreid herpesvirus 1 in fixed paraffin-embedded archival samples using PCR and *in situ* hybridisation. *J. Virol. Methods*, **119**, 65–72.
- BATISTA F.M., ARZUL I., PEPIN J.F., RUANO F., FREIDMAN C., BOUDRY P. & RENAULT T. (2007) Detection of ostreid herpesvirus-1 DNA in bivalve molluscs: a critical review. *J. Virol. Methods*, **139** (1), 1–11.
- BURGE C.A., JUDAH L.R., CONQUEST L.L., GRIFFIN F.J., CHENEY D.P., SUHRBIER A., VADOPALAS B., OLIN P.G., RENAULT T. & FRIEDMAN C.S. (2007). Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: The influence of oyster stock, planting time, pathogens, and environmental stressors. *J. Shellfish Res.*, **26**, 163–172.
- DAVISON A.J., EBERLE R., EHLERS B., HAYWARD G.S., MCGEOCH D.J., MINSON A.C., PELLETT P.E., ROIZMAN B., STUDDERT M.J. & THIRY E. (2009). The order Herpesvirales. *Arch. Virol.*, **154**, 171–177.
- DAVISON A.J., TRUS B.L., CHENG N., STEVEN A.C., WATSON M.S., CUNNINGHAM C., LE DEUFF R.M. & RENAULT T. (2005). A novel class of herpesvirus with bivalve hosts. *J. Gen. Virol.*, **86**, 41–53.
- DÉGREMONT L. (2011). Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. *Aquaculture*, **317** (1–4), 94–98.
- DUNDON W.G., ARZUL I., OMNES E., ROBERT M., MAGNABOSCO C., ZAMBON M., GENNARI L., TOFFAN A., TERREGINO C., CAPUA I., & ARCANGELI G. (2011). Detection of Type 1 Ostreid Herpes variant (OsHV-1 mu var) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. *Aquaculture*, **314**, (1–4), 49–52.
- EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2010). Scientific opinion on the increased mortality events in Pacific oysters, *Crassostrea gigas*. *EFSA J.*, **8**, 1–59.
- FRIEDMAN C.S., ESTES R.M., STOKES N.A., BURGE C.A., HARGOVE J.S., BARBER B.J., ELSTON R.A., BURRESON E.M. & REECE K.S. (2005). Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. *Dis. Aquat. Org.*, **63** (1), 33–41.
- GARCIA C., THÉBAULT A., DÉGREMONT L., ARZUL I., MIOSSEC L., ROBERT M., CHOLLET B., FRANÇOIS C., JOLY J.-P., FERRAND S., Kerdudou N. & RENAULT T. (2011). OsHV-1 detection and relationship with *C. gigas* spat mortality in France between 1998 and 2006. *Vet. Res.*, **42**, 73–84.
- LE DEUFF R.M., NICOLAS J.L., RENAULT T. & COCHENNEC N. (1994) Experimental transmission of a herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas*. *Bull. Eur. Assoc. Fish Pathol.*, **14**, 69–72.

- LE DEUFF R.-M. & RENAULT T. (1999). Purification and partial genome characterization of a herpes-like virus infecting the Japanese oyster, *Crassostrea gigas*. *J. Gen. Virol.*, **80**, 1317–1322.
- LE DEUFF R.-M., RENAULT T. & GERARD A. (1996). Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*, *Dis. Aquat. Org.*, **24**, 149–157.
- LIPART C. & RENAULT T. (2002). Herpes-like virus detection in *Crassostrea gigas* spat using DIG-labelled probes. *J. Virol. Methods*, **101**, 1–10.
- LYNCH S.A., CARLSON J., REILLY A.O., COTTER E. & CULLOTY S.C. (2012). A previously undescribed ostreid herpes virus 1 (OshV-1) genotype detected in the Pacific oyster, *Crassostrea gigas*, in Ireland. *Parasitol.*, **139**, 1526–1532.
- MARTENOT C., ODEN E., TRAVAILLE E., MALAS J.P. & HOUSSIN M. (2010). Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. *J. Virol. Methods*, **70** (1–2), 86–99.
- MARTENOT C., ODEN E., TRAVAILLE E., MALAS J.P. & HOUSSIN M. (2011). Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. *Virus Res.*, **160**, 25–31.
- MOSS J.A., BURRESON E.M., CORDES J.F., DUNGAN C.F., BROWN G.D., WANG A., WU X. & REECE K.S. (2007). Pathogens in *Crassostrea ariakensis* and other Asian oyster species: implications for non-native oyster introduction to Chesapeake Bay. *Dis. Aquat. Org.*, **77**, 207–223.
- PEELER J.E., REESE R.A., CHESLETT D.L., GEOGHEGAN F., POWER A. & TRUSH M.A. (2012). Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 µVar in the Republic of Ireland in 2009. *Preventive Vet. Med.*, **105**, 136–143.
- PÉPIN J. F., RIOU A. & RENAULT T. (2008). Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *J. Virol. Methods*, **149**, 269–276.
- REN W., RENAULT T., CAI Y. & WANG C. (2010) Development of a loop-mediated isothermal amplification assay for rapid and sensitive detection of ostreid herpesvirus 1 DNA. *J. Virol. Methods*, **170**, 30–36.
- RENAULT T., COCHENNEC N., LE DEUFF R.M. & CHOLLET B. (1994a) Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. *Bull. Eur. Assoc. Fish Pathol.*, **14**, 64–66.
- RENAULT T., LE DEUFF R.M., COCHENNEC N., CHOLLET B. & MAFFART P. (1995). Herpes-like viruses associated with high mortality levels in larvae and spat of Pacific oysters, *Crassostrea gigas*: A comparative study, the thermal effects on virus detection in hatchery-reared larvae, reproduction of the disease in axenic larvae. *Vet. Res.*, **26**, 539–543.
- RENAULT T., LE DEUFF R.-M., COCHENNEC N. & MAFFART P. (1994b). Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France – comparative study. *Revue de Médecine Vétérinaire*, **145**, 735–742.
- RENAULT T., LE DEUFF R.-M., LIPART C. & DELSERT C. (2000). Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. *J. Virol. Methods*, **88**, 41–50.
- RENAULT T., MOREAU P., FAURY N., PEPIN J.-F., SEGARRA A. & WEBB S. (2012). Analysis of Clinical Ostreid Herpesvirus 1 (*Malacoherpesviridae*) Specimens by Sequencing Amplified Fragments from Three Virus Genome Areas. *J. Virol.*, **86**(10), 5942–5947.
- ROQUE A., CARRASCO N., ANDREE K.B., LACUESTA B., ELANDALOUSSI L., GAIRIN I., RODGERS C.J. & FURONES M.D. (2012). First report of OshV-1 microvar in Pacific oyster (*Crassostrea gigas*) cultured in Spain. *Aquaculture*, **324–325**, 303–306.
- SAMAIN J.F., DÉGREMONT L., SOLETCHEK P., HAURE J., BÉDIER E., ROPERT M., MOAL J., HUVET A., BACCA H., VAN WORMHOUDT A., DELAPORTE M., COSTIL K., POUVREAU S., LAMBERT C., BOULO V., SOUDANT P., NICOLAS J.L., LE ROUX F., RENAULT T., GAGNAIRE B., GERTET F., BOUTET I., BURGEOT T. & BOUDRY P. (2007). Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture*, **268** (1–4), 227–243.
- SAUVAGE C., PÉPIN J.F., LAPÈGUE S., BOUDRY P. & RENAULT T. (2009). Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: difference in viral DNA detection and quantification using real-time PCR. *Virus Res.*, **142**, 181–187.
- SCHIKORSKI D., FAURY N., PEPIN J.F., SAULNIER D., TOURBIEZ D. & RENAULT T. (2011a). Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. *Virus Res.*, **155** (1), 28–34.
- SCHIKORSKI D., RENAULT T., SAULNIER D., FAURY N., MOREAU P. & PEPIN J.F. (2011b). Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. *Vet. Res.*, **42**, 1–13.

SEGARRA A., PEPIN J.F., ARZUL I., MORGA B., FAURY N. & RENAULT T. (2010). Detection and description of a particular *Ostreid herpesvirus 1* genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.*, **153**, 92–95.

SHIMATA Y., KURITA J., KIRYU I., NISHIOKA T., YUASA K., KAWANA M., KAMAISHI T. & OSEKO N. (2012). Surveillance of Type 1 Ostreid Herpesvirus (OsHV-1) variants in Japan. *Fish Pathol.*, **47** (4), 129–136.

SOLETCHNIK P., LE MOINE O., FAURY N., RAZET D., GEAIRON P. & GOULLETQUER P. (1999). Mortalité de l'huître *Crassostrea gigas* dans le bassin de Marennes-Oléon : étude de la variabilité spatiale de son environnement et de sa biologie par un système d'informations géographiques (SIG), *Aquatic Living Resources.*, **12**, 131–143.

VÁSQUE YEOMANS R., GARCÍA-ORTEGA M. & CÁCERES-MARTÍNEZ J. (2010). Gill erosion and herpesvirus in *Crassostrea gigas* cultured in Baja California, Mexico. *Dis. Aquat. Org.*, **89**, 137–144.

VIGNERON V., SOLLIEC G., MONTANIE H. & RENAULT T. (2004). Detection of ostreid herpes virus 1 (OsHV-1) DNA in seawater by PCR: influence of water parameters in bioassays. *Dis. Aquat. Org.*, **62**, 35–44.

WALLIS C. & MELNICK J. (1965). Thermostabilization and thermosensitization of herpesvirus. *J. Bacteriol.*, **90**, 1632–1637.

WEBB S.C., FIDLER A. & RENAULT T. (2007). Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): Application in a survey of New Zealand molluscs. *Aquaculture*, **272**, 126–139.

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## CHAPTER X . X

**CRITERIA FOR DETERMINING SUSCEPTIBILITY  
OF AQUATIC ANIMALS TO  
SPECIFIC PATHOGENIC AGENTS**

**EU comments**

**The EU commends the OIE Aquatic Animals Commission and in general supports this new chapter. Specific comments are inserted in the text below.**

## Article X.X.1

The purpose of this chapter is to provide criteria for determining which *susceptible species* are listed 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 does not provide criteria for identifying mechanical vectors (i.e. species that may carry the pathogen 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 outlined in this chapter to assessing susceptibility of a species to *infection* with a specified aetiological agent:

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

## Article X.X.4

**Stage 1: criteria for transmission of infection**

The evidence should be classified as transmission through: i) natural occurrence, ii) non-invasive experimental procedure, or iii) invasive experimental procedure.

Consideration needs to be given to whether experimental procedures (e.g. inoculation, infectivity load, host stress) mimic natural pathways for *disease* transmission.

Annex 15 (contd)

## Article X.X.5

**Stage 2: criteria 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 infection**

The following criteria should be used to determine *infection*:

- 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 demonstrated via 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.

**EU comment**

**Four criteria are currently proposed. For reasons of transparency, the EU would suggest splitting criteria B above so that i) isolation of a viable agent is distinguished from ii) evidence of transmission, giving the following five criteria:**

- A. Evidence that the agent is multiplying in or on the host, or that developing or latent stages of the agent are present in or on the host;**
- B. Demonstrated transmission from infected individuals of the proposed susceptible species to other aquatic animals;**
- C. Viable infectious agent isolated from the proposed susceptible species, or viability demonstrated via transmission to naive individuals (by natural routes);**
- D. Clinical and/or pathological changes associated with the infection;**
- E. Specific location of the pathogen (e.g. in one or more tissue types).**

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**

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

1. 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.

2. Definite susceptible species:

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

AND

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

AND

- c) there is evidence of infection with the aetiological agent in the suspect host species in accordance with 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.

**EU comment**

**In the above article, it is suggested that a species be recognised as a definite susceptible species only if transmission is demonstrated (point 2a above), which in the opinion of the EU is too rigorous. Indeed, given the definition of infection in the glossary of the Aquatic Code [*means the presence of a multiplying or otherwise developing or latent pathogenic agent in a host*], criteria A or B of Article X.X.6. should be sufficient evidence to conclude that a species was a definite susceptible species. One problem with the currently proposed point 2 of Article X.X.7. is that for many species / pathogen combinations transmission studies have not been done, hence many species which clearly are susceptible based on other evidence would be classed as "possible" hosts. Furthermore, so called "dead end hosts" would be excluded from being definite susceptible species, even if it can be demonstrated that they are susceptible for infection.**

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.



## Report of the Joint FAO/OIE Aquatic Animal Health Coordination Group

10-11 January 2013, FAO, Rome

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### Background

During July 2012 meetings were held between the OIE Director General, Bernard Vallat, and the FAO Director General Dr Jose Graziano da Silva and the FAO Assistant Director General, Fisheries and Aquaculture Department, Dr Arni Mathiesen, in which it was agreed that the FAO/OIE Chart and Vade Mecum be completed with the same framework to include aquatic animals. In addition it was also agreed to convene a Joint FAO/OIE Coordination Group to undertake this work and to renew institutional commitments to jointly address mutual issues on aquatic animal health and aquaculture. This proposal was also endorsed by the OIE Aquatic Animals Health Standards Commission when they met in September 2012. It was also agreed that Group meet annually and that the outcomes be reported through the FAO/OIE/WHO Tripartite Coordination Meetings mechanism.

The members of the Group are presented at Annex I.

The participants of the Joint FAO/OIE Aquatic Animal Health Coordination Group (hereafter referred to as the JAAHCG) commenced the meeting by developing an agenda for the meeting (presented at Annex II).

### 1. Review and amended the FAO/OIE Chart and Vade Mecum

The JAAHCG reviewed the FAO /OIE Chart and Vade Mecum (presented at Annex III).

The JAAHCG noted the global recognition on the increasing demand for aquatic food and the growing contribution of aquaculture for meeting this demand. It also recognized the importance of better management of aquatic animal health and the complementary roles that FAO and OIE could play in sustaining this contribution.

The JAAHCG also agreed that the complementarities and synergies in the FAO/OIE Chart similarly apply to aquatic animal health.

The JAAHCG proposed that the Chart be amended to include aquatic animal health.

The suggested amendments would be:

1. to amend Footnote 1 of the Cover Note as “to be understood its broader extent, i.e., through the livestock and aquaculture supply chains”

The JAAHCG also noted that, although the Chart, as its present form, adequately reflects in a general way, the aquatic animal health interests, it might be opportune to review if any explicit mention on aquatic animal health in the Chart would add value to the cooperation between FAO and OIE on aquatic animal health activities. For example, replace the word ‘*animal*’ with ‘*aquatic and terrestrial animals*’.

### 2. Develop the Terms of Reference.

The JAAHCG developed a Terms of Reference for this group (presented at Annex IV).

### 3. Review on-going and new FAO/OIE activities for potential cooperation.

In view of establishing a common work plan the JAAHCG reviewed on-going and possible new FAO/OIE activities for future inclusion in the JAAHCG work plan. Below is a list of relevant activities that were discussed:

Annex 16 (contd)**1. FAO Shrimp health in Mozambique/Madagascar/Tanzania**

Activity/issue: White Spot Disease (WSD) outbreak in Mozambique (2010) followed by detection in Madagascar (2012). This is a trans-boundary disease requiring a regional approach. Both FAO and OIE have been involved in assisting Mozambique and FAO in Madagascar. FAO is currently working with a World Bank-led consortium to assist the two countries (and possibly Tanzania) in developing a sub-regional biosecurity strategy and also to identify the compelling future needs of the sub-regional shrimp industry in sourcing quality seeds.

FAO and OIE actions: FAO will work closely with OIE to ensure broader engagement in this activity in particular in jointly developing capacities on aquatic animal health for relevant fisheries/aquaculture and veterinary personnel in the sub-region.

**2. Concept Note for a regional project on aquatic biosecurity in eastern and southern Africa**

Activity/issue: OIE and FAO have provided assistance to NEPAD in the development of a Concept Note for a regional project on aquatic biosecurity. The project is aimed at building capacity on fish health and aquatic biosecurity to sustain and develop aquaculture and fisheries in eastern and southern Africa. Such activities are required to prevent future disease outbreaks such as the extensive EUS outbreak in the Zambezi Basin, a trans-boundary disease requiring a regional approach.

FAO and OIE actions: Personnel from the OIE Sub-Regional Office, Gaborone, assisted Rhodes University and NEPAD in developing a Concept Note for this project. FAO is providing support in obtaining funds for this project.

Towards cooperation: FAO and OIE to combine efforts to assist in providing technical support and funding for this project.

Actions: Update to be provided at March, 2013 teleconference.

**3. OIE Focal Point seminars**

Activity/issue: How to maximize FAOs input in these seminars recognizing the relevance of some existing FAO activities (e.g. Technical Cooperation Programme (TCP) assistance for developing national capacities for responsible trade in aquatic animals and animal products, through efficient implementation of relevant OIE standards and FAO guidelines, at the regional and national level).

FAO and OIE actions: OIE have been conducting a series of regional seminars for OIE National Focal Points for Aquatic Animals since 2009. These seminars have included some FAO contribution.

Towards cooperation: Identify activities relevant to OIE Focal Point seminars to optimize FAO's input.

Actions: Proposed a presentation by the Bosnian Focal Point on the outcomes of the FAO Regional TCP in Western Balkans during OIE seminar for Aquatic Animal Focal Points for Europe, Portugal 9-11 April 2013; OIE to provide update on any upcoming Focal Point seminars for 2013.

**4. FAO Technical Cooperation Programmes (TCPs) on implementation of OIE aquatic animal health standards**

Activity/issue: Some FAO TCPs request support for technical assistance in the implementation of some aspects of OIE aquatic animal health standards, e.g. legislation, risk analysis, surveillance.

FAO and OIE actions: OIE has the mandate for the development of standards for aquatic animal health. FAO has the mandate to provide technical assistance to implement standards at the regional and national level.

Annex 16 (contd)

Towards cooperation: OIE to provide support to TCPs where relevant. FAO to provide feedback to OIE on TCPS implementation.

Actions: At March teleconference, FAO to provide update on any upcoming TCP projects (both approved and under development).

**5. OIE PVS Tool: Aquatic and FAO Aquatic animal health performance and capacity survey questionnaire**

Activity/ issue: OIE and FAO have each developed evaluation tools for aquatic animal health.

FAO and OIE actions: OIE has developed a *PVS Tool: Aquatic* for the on field evaluation by independent accredited experts of aquatic animal health services. FAO has developed an aquatic animal health performance and capacity survey questionnaire which is used as the basis for FAO Technical Assistance Development Projects at regional and national levels.

Towards cooperation: The JAAHCG to review both tools to determine the aspects in common and identify possible synergies.

Actions: Share documents and information for discussion at the next physical meeting (January 2014).

**6. Education of veterinarians and aquatic animal health professionals (AAHP)**

Activity/ issue: In aquatic animal health both veterinarians and aquatic animal health professionals (AAHP) undertake some similar activities and responsibilities. The competency of both veterinarians and AAHP involved in aquatic animal health needs to be defined and harmonized.

FAO and OIE actions: OIE has developed recommendations on the Competencies of graduating veterinarians which includes competencies that cover both terrestrial and aquatic animals. The OIE is also developing a definition for AAHP. FAO has run short term courses for veterinarians and AAHP aimed at improving competency on an *ad hoc* basis.

Towards cooperation: Review OIE work on veterinary education relevant to aquatic animals and the course work developed by FAO. Identify the minimum competencies required to meet defined tasks of an AAHP. Propose that the OIE consider the inclusion of the topic of education of veterinarians and AAHPs in aquatic animal health in the OIE Global Conference on Veterinary Education to be held in Brazil in 4-6 Dec 2013.

Actions: OIE to table this proposal at the March 2013 meeting of the Aquatic Animals Commission.

**7. Co-ordination of emergency response activities, e.g. WSD outbreak in Mozambique and EMS.**

Activity/issue: FAO and OIE receive parallel requests from a Member country for expert assistance in response to disease outbreaks. This often results in duplication and overlap in resource mobilization by the two organizations.

FAO and OIE actions: There have been several occasions when FAO and OIE responded to a parallel Member request and initiated expert missions to the same country, e.g. WSD in shrimp in Mozambique, Early Mortality Syndrome in shrimp in Vietnam.

Towards cooperation: Improved cooperation between FAO and OIE regarding emergency response to disease outbreaks to avoid duplication and to improve efficiency;

Actions: initiate a teleconference call for JAAHCG members in the event of a request for emergency assistance to ensure coordination.

Annex 16 (contd)**8. Coordination and communication between Veterinary Services (VS) and Aquatic Animal Health Service (AAHS)**

Activity/issue: AAHS may reside in the VS or another Competent Authority. Coordination and communication between relevant departments is often difficult or lacking. FAO has direct links and networking in aquaculture and fisheries departments and ministries in Member countries. Similarly OIE has direct links and networking in Veterinary Services which may be the Competent Authority for AAHS in many countries.

FAO and OIE actions: OIE undertakes training of OIE Focal Points in Aquatic Animals who may work in the VS or AAHS. FAO undertakes seminars, courses and projects that involve both the VS and AAHS.

Towards cooperation: FAO to map the organizational structure dealing with aquatic animal health in selected Member countries to enable a functional analysis and identify critical contact points for better coordination and communication. This information would be derived from OIE PVS evaluations of AAHS and FAO aquatic animal health performance and capacity surveys. Consider the output of this functional analysis and identify ways to enhance communication at the national level regarding aquatic animal health.

Actions: FAO to initiate a mapping exercise using FAO aquatic animal health performance and capacity surveys and OIE Aquatic PVS evaluations, if countries agree. FAO to provide an update at the March teleconference.

**9. FAO Global Aquaculture Advancement Participation**

Activity/ issue: FAO is developing a concept for a global programme on aquaculture, “Global Aquaculture Advancement Programme” with activities and partners all around the world. This initiative is based on the assessment of aquaculture and its contribution to food security worldwide and reflects the FAO/FI’s vision on aquaculture.

FAO and OIE actions: FAO/FI will keep OIE fully informed of the development of the programme. It is considered that OIE should be a partner in addressing aquatic biosecurity and SPS standards issues in the programme.

Towards cooperation: FAO, in due course, will specify the expected OIE contribution in this project.

**4. Next meeting**

The JAAHCG agreed to meet via teleconference twice prior to the next physical meeting. The teleconference meetings are proposed for the week of 18-21 March 2013 and at the last week of October 2013. The next physical meeting is proposed for the second week of January 2014.

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.../... Annexes



**Joint FAO/OIE Aquatic Animal Health Coordination Group****10-11 January 2013, FAO, Rome**

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**List of participants****OIE REPRESENTATIVES**

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**Dr Gillian Mylrea**  
Deputy Head  
International Trade Department  
OIE  
12, rue de Prony  
75017 Paris  
FRANCE  
Tel.: 33 (0)1 44 15 16 67  
g.mylrea@oie.int

**Dr Franck Berthe**  
President of the OIE Aquatic Animal Health Standards  
Commission  
Head of the Animal Health and Welfare Unit  
European Food Safety Authority - EFSA  
Animal Health and Animal Welfare unit  
Via Carlo Magno 1, 43100 Parma  
ITALY  
Tel.: + 39 0521 036 870  
Fax: + 39 0521 036 0870  
Franck.Berthe@efsa.europa.eu

**FAO REPRESENTATIVES**

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**Dr Rohana P. Subasinghe**  
Senior Fishery Resources Officer  
Fisheries and Aquaculture Department  
Food and Agriculture Organization of the  
United Nations (FAO)  
Viale Terme di Caracalla, 00153  
Rome, ITALY  
Tel.: 39 06 570 56473  
Fax: 39 06 570 53020  
Rohana.Subasinghe@fao.org

**Dr Melba B. Reantaso**  
Aquaculture Officer  
Fisheries and Aquaculture Department  
Food and Agriculture Organization of the United Nations (FAO)  
Viale Terme di Caracalla, 00153  
Rome, ITALY  
Tel: + 39 06 570 54843  
Fax: + 39 06 570 53020  
Melba.Reantaso@fao.org



Annex 16 (contd)

Annex II

**Joint FAO/OIE Aquatic Animal Health Coordination Group**

**10-11 January 2013, FAO, Rome**

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**Adopted agenda**

1. Review and amend the FAO / OIE Chart and *Vade Mecum* to include aquatic animals
  2. Review on-going and new FAO/ OIE activities for cooperation
  3. Next Meeting.
-





**Chart on FAO and OIE competencies and complementarities in the field of animal health and companion Vade Mecum**

**COVER NOTE**

The mandates of OIE and FAO converge in the field of animal health<sup>1</sup>. For some tasks in this field, OIE, the leading Organisation entirely devoted to animal health issues and in charge of animal health and welfare standards, guidelines and recommendations setting which have a global scope, and FAO, the United Nation's Specialized Agency for Food and Agriculture, have primary responsibility; for others, the two organizations join forces and work synergistically.

FAO and OIE have collaborated for over fifty years. In the last decade, due to the international sanitary context with major crises such as Foot and Mouth disease in the UK and Europe in 2001, and more recently, the worldwide avian influenza epidemic, this collaboration has increased substantially and strategies, meetings, and capacity building initiatives have been designed and implemented jointly at the national, regional and global level.

Such collaborative efforts have been guided by an inter-institutional agreement signed on 24 May, 2004 (see [Agreement](#)), which details matters of common interest pertaining to the two organizations' mandates and competencies. It has also been technically and operationally structured within the GF-TADs initiative<sup>2</sup> and several specific tools have been established such as OFFLU, GLEWS, CMC-AH<sup>3</sup> and Regional Animal Health Centres.

To optimize the collaboration, avoid overlaps and provide clear and coherent messages to all FAO and OIE teams as well as to partners including donors, complementarities and synergies in the mandates of the two organizations have been assessed and agreed in detail and are hereby communicated.

<sup>1</sup> to be understood in its broader extent, i.e. throughout the livestock supply chains

<sup>2</sup> Global Framework for the Progressive Control of Transboundary Animal Diseases


<sup>3</sup> OFFLU = OIE / FAO network of expertise on Avian Influenza; GLEWS = FAO / OIE / WHO Global Early Warning System on Transboundary Animal Diseases; CMC-AH = FAO / OIE Crisis Management Center for Animal Health

The attached Chart on the competencies and complementarities of FAO and OIE delineates, through a set of histogram bars, the agreed responsibilities and synergies for seven areas – standards, guidelines and recommendations, strategies and best practices, sanitary information and epidemiological intelligence, expertise, scientific and technical publications, training; and development programmes - and several cross-cutting issues - awareness, research, communication, and coordination. The *Vade Mecum* complements the Chart, explaining in short paragraphs the significance of each of the histogram bars.

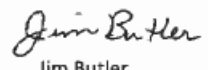
The Chart was initially drafted in February 2007, revised in March 2008 and completed by the *Vade Mecum* in May 2008. The two documents were endorsed by the OIE during the 76<sup>th</sup> *General Session of the OIE* in May 2008 and approved on the same time by the FAO Deputy Director General.

The Chart is a living document and it will be reviewed and revised regularly according to the evolution of the sanitary context and of the two organizations' priorities.

The Chart and its companion *Vade Mecum* are available in English, in French and in Spanish.



Bernard Vallat  
Director General  
World Organisation for Animal Health  
(OIE)



Jim Butler  
Deputy Director-General  
Food & Agriculture Organization  
of the United Nations  
(FAO)

- 6 OCT. 2008

Final version signed on 6th October 2008



**CHART ON FAO AND OIE COMPETENCIES AND COMPLEMENTARITIES IN THE FIELD OF ANIMAL HEALTH**

**1. Introduction**

-The Chart of mandates, competences and complementarities between FAO and OIE is set up in the context of the FAO-OIE initiative named GF-TADs: Global Framework for the Progressive Control of Transboundary Animal Diseases initiative signed in May 2004. The chart, which has been endorsed by the FAO and OIE DGs in February 2007, details the competences, complementarities and synergies as sketched in the last page.

-The fields taken into account include animal health and animal welfare, as well as food safety of products of animal origin at the production until slaughter house stages (the transformation and distribution stages are not envisaged here while they are also part of the collaboration among FAO, OIE and WHO, notably via the Codex Alimentarius).

-The types of activities (topics) are sorted from the strategical to the technical and then operational levels.

-Each bar of the chart represents the activity of the organisations for a given topic. When a bar is over the 2 organisations, it indicates the respective role of each organisation towards the other one, for the given topic.

-For each topic, there are three possible collaborations: support, complementarity and synergy.

**2. List of specific fields to be considered**

**2.1. Standards and guidelines (elaboration and endorsement)**

-This field is a core mandate of the OIE, as the leading Organisation entirely devoted to animal health issues and in charge of animal health and welfare standards and guidelines setting (for both preventing, controlling and eradicating diseases, as well as for ensuring safe trading of animals and products) which have a global scope. FAO provides its support by participating in several OIE working groups or commissions and assistance to countries in the implementation of these standards.

**2.2. Strategies and best practices guides for developing and in transition countries**

-This field is a core activity of FAO (as the leading technical Organisation devoted to food and agricultural issues in developing countries and in charge of designing and implementing strategies and development programs in such countries) which mandate is to assist developing and in transition countries. The OIE brings a valuable guarantee and expertise from its Reference Laboratories and Collaborating Centres. FAO and OIE work synergistically in this field.

Final version signed on 6th October 2008

### **2.3. Sanitary information and epidemiological intelligence**

-Official information on the sanitary status of the countries is OIE's exclusive competence (as part of the obligations of OIE Members to notify the OIE on their sanitary status). FAO brings its support by communicating information from non-OIE member countries as well as by encouraging all countries to notify the OIE.

-Verifying diseases outbreaks, rumours and suspicions of outbreaks are performed both by FAO and OIE. Since the sources of information are often different (for OIE, CVOs network; for FAO, FAO national representations network, field operations, etc...) and the sharing of information made possible via the FAO-OIE-WHO common platform GLEWS (Global Early Warning System), the FAO-OIE collaboration is synergistic.

-Disease analysis and epidemiological intelligence are carried out in a synergistic manner notably via the FAO-OIE-WHO called GLEWS. FAO, supported by its multi-disciplinary teams and multiple sources of information on agriculture, socio-economy and climate changes and its link with crises and population movements, trade, etc..., operates in favour of a holistic approach to animal production and health, which targets in priority developing countries where major sanitary crises originate. Therefore, it was agreed that FAO hosts the GLEWS joint core-team in charge of the epidemiological analysis.

-A warning on animal disease events is officially provided by OIE. FAO provides a more synthetic warning, based on analysis and prediction. The platform GLEWS will also display warning messages by links to OIE, FAO and WHO websites.

### **2.4. Expertise**

-Global expertise required to fulfil the OIE mandate including standard setting activities is provided through its network of experts from worldwide network of OIE Reference Laboratories and Collaborating Centres. FAO also provides expertise from its reference centres, in a complementary manner.

-Expertise to provide assistance to developing and in transition countries is within the core mandate of FAO; for that purpose FAO has teams in headquarters and in regional and national Representations, adding to the experts from its reference centres. OIE provides strong support, in a complementary manner, by offering experts from OIE Headquarters, OIE Reference Laboratories and Collaborating Centres, as well as through strategic advice from OIE Regional and Sub-Regional Representations.

-OIE/FAO Regional Animal Health Centres staff main activities are addressed to provide technical assistance and expertise (in a complementary and synergistic collaboration) to developing countries as necessities arise.

### **2.5. Scientific and technical publications**

-Of global scope: OIE and its experts from its Reference Laboratories and Collaborating Centres play a key role in this field. FAO provides support in a complementary manner including as invited author of OIE scientific publications.

-For developing and in transition countries: with its own experts or in close collaboration with experts from its reference centres, FAO publishes bulletins, manuals, monographies and publications in scientific and technical reviews. When relevant, OIE collaborates in this field with FAO.

-Publications on specific topics are issued jointly between OIE and FAO as the need arise, through a complementary and synergistic collaboration.



Final version signed on 6th October 2008

## **2.6. Training**

-At the global level, for the OIE Delegates (most of them Chief Veterinary Officers -CVOs) and OIE Focal Points: OIE contributes to the training and information of staff that have worldwide responsibilities, on official standard setting process, good veterinary governance and disease prevention and control methods. FAO is likely to provide support where the need is identified

-At the regional level, in developing and in transition countries, for the OIE Delegate (most of them Chief Veterinary Officers CVOs) and OIE Focal Points, as well as the private sector: OIE has important training activities, to which FAO synergistically contributes.

-At the national level, in developing and in transition countries, for animal health system operational teams (public veterinary services and private sector actors), FAO performs one of its core mandate to its member countries, with the support of the OIE. The OIE can organise national seminars on good governance with key policy makers with the support of FAO.

## **2.7. Development programs in animal health in developing countries**

-This is the core mandate of FAO. OIE brings its support to this activity through its experts and the use by countries of its standards and guidelines, at the global, regional and national levels.

-The OIE/FAO global programme "Ensuring Good Governance to Address Emerging and Re-Emerging Animal Disease Threats – Supporting the Veterinary Services of Developing Countries to Meet OIE International Standards on Quality" is implemented in a complementary collaboration. The PVS evaluation of Veterinary Services is the prerogative of the OIE as well as the follow up of good governance achievements by countries in the framework of PVS use. The subsequent gap analysis of PVS evaluations is managed in common by the OIE and FAO. FAO is responsible of national programmes directed to the prevention and control of animal diseases and emergency responses to sanitary crises in developing and in transition countries.

## **3. Cross-cutting topics and common tools**

FAO and OIE have concerted activities in the following intervention fields:

- Awareness / lobbying, for an improved governance and enhanced support and investments in the prevention and control of animal diseases.
- Priority identification and support for research in animal health.
- Communication towards and awareness of public and private sectors for the best practices for the prevention and control of animal diseases.
- Several coordination and support activities are jointly implemented at the international and regional levels, notably during international conferences and thematic meetings.

The tools for the implementation of the FAO and OIE activities cannot all be listed in this note. However, some can be mentioned such as GF-TADs, GLEWS platform (jointly with WHO), OFFLU and the Crises Management Centre for animal health (CMC-AH) or Regional Animal Health Centres (RAHC).

OIE / FAO - COMPLEMENTARITIES and SYNERGIES



<p>Animal Health Standards and Guidelines setting and Adoption</p>	<p>Standards and Guidelines Setting</p>
<p>Good Farming Practices Guidelines and Strategies</p>	<p>Good Farming Practices &amp; Strategies</p>
<p>Animal Disease Information and Intelligence</p>	<p>Official Disease Information</p> <p>Disease Tracking</p> <p>Disease Intelligence</p>
<p>Expertise on Animal Health worldwide</p>	<p>Expertise</p>
<p>Expertise for Development Programs on Animal Health</p>	<p>Expertise for Development Programs on Animal Health</p>
<p>Animal Health Publications</p>	<p>Technical and Scientific Publications</p>
<p>Capacity Building Programs on Animal Health on standards and guidelines implementation</p>	<p>Global</p> <p>Regional</p> <p>National</p>
<p>Development programs on Animal Health</p>	<p>Global</p> <p>Regional</p> <p>National</p>

**Joint FAO/OIE Aquatic Animal Health Coordination Group****10-11 January 2013, FAO, Rome**

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**Background and Terms of Reference for the Joint FAO/OIE  
Aquatic Animal Health Coordination Group (JAAHCG)****Background**

Noting the increasing demand and production in aquatic animals worldwide and recognizing the growing contribution of aquaculture to food security;

Considering that the OIE and FAO's mandate also converge in the field of aquatic animal health;

Noting that FAO is an Observer to the OIE Aquatic Animal Health Standards Commission and that the OIE is an Observer to FAO's Committee on Fisheries (COFI) and the Sub-Committee on Aquaculture (COFI-SCA);

Noting that during meetings held in July 2012 between the OIE Director General, Bernard Vallat, and the FAO Director General Dr Jose Graziano da Silva and the FAO Assistant Director General, Fisheries and Aquaculture Department, Dr Arni Mathiesen, it was agreed that relevant personnel from the FAO and OIE should meet annually to enhance cooperation between the two organizations regarding aquatic animal health.

**Terms of Reference****1. Objective**

To jointly address mutual issues on aquatic animal health and aquaculture within the framework of the OIE/FAO Chart and Vade Mecum.

**2. Composition**

Permanent membership would include representatives from FAO Fisheries and Aquaculture Department and representatives from OIE International Trade Department and the OIE Aquatic Animals Commission. Additional participants would be invited depending on the specific agenda items.

**3. Reporting**

Outcomes to be reported through the FAO/OIE/WHO Tripartite coordination meeting mechanism.

**4. Meeting frequency**

An annual 2 day meeting and two teleconferences between annual meetings.

**5. Tasks**

1. Share information on on-going and new activities.
  2. Identify areas for cooperation, including mechanisms for resource mobilisation.
  3. Develop a common work plan.
-



## AQUATIC ANIMALS COMMISSION WORK PLAN 2013–2014

### *Aquatic Code*

Tasks	Sept 2012 – Feb 2013	March 2013	May GS 2013	Sept 2013	March 2014	May GS 2014
<b>Chapter 10.5. Infectious salmon anaemia</b> – revise text based on the guiding principles established by the AHG on pathogen differentiation and comments from Member Countries		Consider Members comments	Propose for adoption			
<b>OsHV-1 <math>\mu</math>var</b> - propose for listing as an emerging disease		Consider Members comments	Propose for adoption		Review status as emerging disease	
<b>Infection with salmon pancreas disease virus</b>		Consider Members comments	Propose for adoption	If adopted for listing develop Code chapter		
<b>Chapter 6.1. Chapter on control of hazards in feed</b>		Develop a Concept Note on revised scope, purpose and content.		Consider the structure of a revised chapter. Convene an AHG to undertake this work	Review AHG draft chapter and circulate for Members' comments	

Annex 17 (contd)

<b>Chapter 1.2. Import risk analysis</b>	OIE Trade Dept. to revise text to harmonise with <i>Terrestrial Code</i> chapter	Review revised text	Propose for adoption			
<b>Develop Chapter 6.X. Risk analysis for antimicrobial resistance in aquaculture</b>				Review report of AHG and circulate draft chapter for comment	Consider Members comments	Propose for adoption
<b>Develop a new chapter with the criteria for listing susceptible species</b>		Develop draft chapter and circulate for comment		Review Member comments		
<b>Chapter on Evaluation of AAHS</b>				Consider 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		
<b>Chapter 1.2. criteria for listing a disease and an ED</b>				Review outcomes of TCC and SCAD discussions on ED		

*Aquatic Manual*

Manual Tasks	Sept 2012 – Feb 2013	March 2013	May 2013	Sept. 2013	Mar 2014	May GS 2014
<b>Revise Chapter 2.3.5. infection with ISAV</b>	Author to revise chapter	Consider revised chapter and align with the <i>Code</i> chapter	Propose for adoption			
<b>Revise Chapter 2.4.9 to focus information on OshV-1 µvar</b>	Author to revise chapter	Consider revised chapter and align with proposed disease for listing	Propose for adoption			
<b>Chapter on SPD</b>	Request expert to develop a Disease card	Develop a disease card				
<b>Revise Chapter 2.3.1 EUS, based AHG recommendations</b>	Author to revise chapter	Revised chapter and align with proposed name for listing	Propose for adoption			

Annex 17 (contd)**Other Items**

<b>Tasks</b>	<b>Mar 2013</b>	<b>May 2013</b>	<b>Sept. 2013</b>	<b>Mar. 2014</b>	<b>May 2013</b>	<b>late 2014</b>	<b>early 2015</b>
<b>OIE Conference on Implementation of OIE standards, including compartmentalisation</b>	Provide input to development of concept note, programme once date confirmed					Possible date for Conference	Possible date for Conference
<b>OIE Ref Lab Conf (7-9 Oct 2014)</b>	AAC to provide input into the programme and Scientific Committee					Conference (7-9 October)	



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