WORLD ORGANISATION FOR ANIMAL HEALTH
*Protecting animals, preserving our future*

# ANNEX 2

*Original: English*
February 2021

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

**Paris, 17–24 February 2021**

**PART A – Texts to be proposed for adoption at the OIE 88th General Session in May 2021**

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**EU position**

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

**A number of general comments on this part A of the report of the February 2021 meeting of the Aquatic Animals Commission as well as the intended positions of the EU on the draft Aquatic Code and Manual chapters proposed for adoption at the 88th OIE General Session are inserted in the text below, while specific comments are inserted in the text of the respective annexes to the report.**

**The EU would like to stress once 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 (the Aquatic Animals Commission) held its meeting electronically from 17 to 24 February 2021. The list of participants is attached as Annex 1.

Considering the ongoing COVID-19 pandemic, the 88th Annual General Session will be held virtually from Monday 24 to Friday 28 May 2021. During the 88th General Session new and revised chapters of the OIE International standards (the *Aquatic Animal Health Code*, the *Terrestrial Animal Health Code*, the *Manual of Diagnostic Tests for Aquatic Animals* and the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*) will be proposed for adoption.

To facilitate this process, the February 2021 meeting report of the Aquatic Animals Commission will be distributed in two parts: Part A (herewith) provides information about the new and revised texts for the *Aquatic Code* and the *Aquatic Manual* that will be proposed for adoption at the 88th General Session; and Part B (to be published in April 2021) will provide information about other topics discussed at the Commission's February 2021 meeting including texts circulated for comments and information.

In preparation for the 88th General Session, the OIE will organise a series of information webinars to ensure that Members are aware of the background and key aspects of the standards being presented for adoption. Attendance to these webinars will be by invitation only. Please note that Delegates will soon receive detailed information about the virtual 88th General Session, and in particular the process for the adoption of standards.

The Aquatic Animals Commission thanked the following Members for providing written comments on draft texts for the OIE *Aquatic Animal Health Code* (hereinafter referred to as the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter referred to as the *Aquatic Manual*) circulated in the

Commission's September 2020 meeting report: Armenia, Australia, Canada, Chile, China (People's Rep of), Chinese Taipei, Cuba, Japan, Korea (Rep. of), Liberia, Mexico, New Caledonia, New Zealand, Singapore, Switzerland, Thailand, the United Kingdom (the UK), the United States of America (the USA), Members of the OIE Americas region, the Member States of European Union (the EU), and the African Union Inter-African Bureau for Animal Resources (AU-IBAR) on behalf of African Members. The Commission also wished to acknowledge the valuable advice and contributions from numerous experts of the OIE scientific network.

The Commission reviewed all comments that were submitted on time and were supported by a rationale. The Commission made amendments to draft texts, where relevant, in the usual manner by 'double underline' and '~~strikethrough~~'. In the Annexes, amendments proposed at this meeting are highlighted with a coloured background in order to distinguish them from those made previously. The Commission could not consider some comments where no rationale was provided or that were unclear. Due to the large volume of work, the Commission was not able to draft a detailed explanation for the reasons for accepting or not each of the comments received, and focused its explanations on the most significant issues. Where amendments were of an editorial nature, no explanatory text has been provided. The Commission wished to note that not all texts proposed by Members to improve clarity were accepted; in these cases, it considered the text clear as currently written.

The Commission encourages Members to consider relevant information in previous Commission and *ad hoc* Group reports when preparing comments, especially on longstanding issues. These reports are available on the OIE Website.

The table below lists the agenda items and provides links to these items within this report. Members should note that texts in **Annexes 2 to 16** will be proposed for adoption at the 88th General Session in May 2021.

**Agenda**

## 1. Welcome from the Deputy Director General

Dr Gillian Mylrea, Head of the Standards Department, on behalf of the OIE Deputy Director General, International Standards and Science, Dr Matthew Stone, welcomed members of the Aquatic Animals Commission, noting that this was the last meeting of the three-year term. The Commission had maintained excellent productive output despite significant challenges. The OIE recognized that it has drawn heavily on the Specialist Commissions when responding to the COVID-19 pandemic, and the response has always been in the spirit of goodwill, innovation and scientific excellence. Dr Mylrea thanked all Commission members for their contributions during the term, including the forthcoming meeting, and extended this appreciation to the members' employing institutions and national governments. Dr Mylrea briefed the members on the ongoing design process for a fully virtual OIE General Session. She summarized the ongoing work on the OIE standards development and review system, including Standard Operating Procedures development and planning for digital tools. Finally, she provided an overview of the OIE's continuing support to the COVID-19 pandemic response, including ad hoc groups, the development and implementation of the OIE Wildlife Health Management Framework and the compilation of services under the OIE Supporting Veterinary Services Resilience paper.

The members of the Commission thanked Dr Mylrea for this update and appreciated being informed of some of the new areas of work being undertaken across the organisation.

## 2. Meeting with the Director General

Dr Monique Eloit, the OIE Director General, met the Commission on 23 February 2021. She commended the Commission's work during this three-year term and thanked its members for their support and commitment to achieving OIE objectives. She recognised the Commission's efforts and adaptability to develop new ways of working to sustain the OIE standard setting process despite the challenges imposed by the COVID-19 pandemic. Dr Eloit provided an update on progress in the implementation of the 7th OIE Strategic Plan and noted that it includes promoting priorities such as aquatic animal health and wildlife health, as well as major structural changes towards digital transformation and data management. The Director General recognised that these transformations demand significant resources and will also impact the way the Commission and its Secretariat undertake some of their work.

Dr Eloit recalled that the idea to develop an OIE Aquatic Animal Health Strategy had emerged from the Global Conference on Aquatic Animal Health held in April 2019 in Chile. She acknowledged the significant work that the Commission and the OIE Secretariat had invested in developing the Strategy and how delighted she was that it would be launched at the 88th General Session in May 2021. She reiterated her support for this strategy and to its implementation.

The members of the Commission thanked Dr Eloit for making time to meet with its members and commended the excellent work of the Secretariat for meeting preparations and its work during the meeting especially given the challenges of a virtual meeting.

## 3. The *AQUATIC CODE*

The Aquatic Animals Commission considered comments received on the following new and revised texts of the *Aquatic Code*, which had been previously circulated for Member comments. The Commission's responses are presented below.

### 3.1. Texts that will proposed for adoption in May 2021

The Aquatic Animals Commission thanked Members for highlighting some translation issues in some of the Annexes circulated for comment in French and Spanish versions, and reported that these have been reviewed and corrected.

#### 3.1.1. Glossary Definitions

##### 3.1.1.1. 'Aquatic animal waste' and 'Aquatic animal products'

Comments were received from Armenia, Canada, Cuba, Switzerland and the EU.

*Background*

At its September 2019 meeting, the Aquatic Animals Commission proposed a new Glossary definition for 'aquatic animal waste' given that the term is used extensively in the new draft Chapter 4.X, Biosecurity for aquaculture establishments, as well as in Chapter 4.7, Handling, disposal and treatment of aquatic animal waste.

At its February 2020 meeting, the Commission agreed to amend the glossary definition for 'aquatic animal products' and to align it with the new definition of 'aquatic animal waste'.

The Commission noted that once the new Glossary definition for 'aquatic animal waste' is adopted, the definition for 'aquatic animal waste' in Article 4.7.3 will be deleted and the term 'waste' will be amended to the italicised term 'aquatic animal waste' where appropriate throughout relevant chapters of the *Aquatic Code*, to reflect the new defined term.

**Previous Commission meeting reports where this item was discussed:**

September 2019 report (Item 6.7, page 9); February 2020 (Item 7.1.6, page 14); September 2020 (Item 4.5.1, page 8).

**February 2021 meeting**

The Commission considered comments received and agreed not to make any additional amendments to the definitions of 'aquatic animal waste' and 'aquatic animal products'.

In response to a comment to change 'liquids' to 'biological products', the Commission explained that waste generated from processing often includes water, which is captured by 'liquid'. However, the term 'biological products' is inconsistent with the definition's reference to 'intended for disposal'. The Commission also agreed that the suggested addition of 'sludge' would not be appropriate, as it considered that this would extend the scope of the definition to include fomites.

Proposed consequential amendments to chapters of the *Aquatic Code*, following adoption of the new definition for 'aquatic animal waste', are presented in **Annex 2**.

No further comments were provided on the definition 'aquatic animal products'.

The new Glossary definitions for 'Aquatic animal waste' and the revised Glossary definition 'Aquatic animal products' are presented in **Annex 2** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of these revised Glossary definitions.**

#### 3.1.1.2. 'Vector'

Comments were received from Armenia, Canada, Cuba, Switzerland, the USA and the EU.

*Background*

At its meetings in February and September 2020, the Aquatic Animals Commission amended the Glossary definition for 'vector' to make it clear that vectors for a specified infectious agent cannot be listed as a susceptible species for the same pathogenic agent. The Commission also clarified that for an organism to be classified as a vector, there must be evidence that it can transfer the specific pathogenic agent to susceptible species.

**Previous Commission meeting reports where this item was discussed:**

February 2020 report (Item 7.2.1, page 15); September 2020 report (Item 4.5.2, page 8).

**February 2021 meeting**

The Commission agreed with comments to delete 'a population of' from the definition and to edit the reference to a pathogenic agent in the second sentence, for improved readability. It also agreed to replace 'transfer' with 'transmit' as it considered this to be consistent with other *Aquatic Code* texts and to distinguish between vectors and fomites.

The Commission did not agree with comments to differentiate non-susceptible species from vectors as it considered vectors to be a subcategory of non-susceptible species.

The revised Glossary definition for 'Vector' is presented as clean and track change versions in **Annex 2** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of this revised Glossary definition.**

### 3.1.2. New Draft Chapter on Biosecurity for Aquaculture establishments (Chapter 4.X)

Comments were received from Armenia, Australia, Canada, Chile, China (People's rep of), Cuba, Korea (Rep. of), Liberia, Mexico, New Caledonia, New Zealand, Switzerland, the UK, the USA, the EU, Members of the OIE Americas region, and AU-IBAR.

*Background*

The new draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) is the second new chapter to be developed as part of the ongoing revision of Section 4, Disease Prevention and Control.

**Previous Commission meeting reports where this item was discussed:**

September 2018 report (Item 2.9, page 11); February 2019 report (Item 2.1, page 10); September 2019 report (Item 6.1, page 4); February 2020 report (Item 7.1.1, page 6); September 2020 report (Item 4.1, page 3).

**February 2021 meeting**

**General comments**

In response to a comment regarding the scope of this chapter, the Aquatic Animals Commission reiterated that it is intended to provide recommendations on biosecurity at the level of aquaculture establishments, as already agreed by Members. Broader national biosecurity planning is not within the scope; however, the Commission noted the importance of aquaculture establishment biosecurity to support national biosecurity arrangements.

The Commission agreed with a comment that more guidance to Members on implementation of biosecurity measures would be beneficial but noted that it would not be appropriate to include such information in this chapter which will be a standard of the *Aquatic Code*. The Commission noted that the development of any such additional guidance would need to be considered within the context of workplan priorities of the next Commission. The Commission also noted the work of the Food and Agriculture Organization of the United Nations (FAO) on aquatic animal biosecurity and that the FAO has invited input from the OIE on this initiative.

**Article 4.X.1. Purpose**

The Commission did not agree with a comment to add 'or already present' after, '...if *pathogenic agents* are introduced...', noting that the text as written was clear and that any *pathogenic agents* already present would also be subject to the mitigation measures.

**Article 4.X.2. Scope**

The Commission agreed to add 'area management' in the last sentence, to align with the previous addition of Article 4.X.5 bis.

**Article 4.X.3. Introduction**

The Commission did not agree with a comment to add a sentence to describe the relationship between biosecurity and husbandry measures as it considered that the suggested additional text does not align with the *Aquatic Code* definition of biosecurity and is more relevant for broader health management activities.

**Article 4.X.4. General principles**

In the first paragraph, the Commission did not agree with a comment to include 'biosecurity measures to mitigate the risks associated with the release from aquaculture establishments' because it considered that the focus of this article is to reduce the risk of introducing a pathogenic agent to aquatic animal populations, not the release from the establishment.

In the first sentence, the Commission did not agree to add 'and the release from' an aquaculture establishment as it considered that it was redundant in the context of this sentence. The broader concept of biosecurity addressing pathogen entry into, spread within and release from and aquaculture establishment is highlighted throughout the chapter.

In the second sentence of the first paragraph, the Commission did not agree to add new text to emphasise the importance of biosecurity measures to minimise the identified risks to an acceptable level for achieving overall biosecurity objectives, as the Commission considered this to be implicit in the current wording.

In the second sentence of the first paragraph, the Commission did not agree to replace 'Planning' with 'Formation' because it considered that the intent of the sentence was to indicate that planning was required prior to implementation of biosecurity.

In the third sentence of the first paragraph, the Commission did not agree to add 'characteristics of the pathogenic agents' after '…likelihood of exposure to pathogenic agents …' as the Commission considered it was already addressed by likelihood of exposure to pathogenic agents.

In point 6, the Commission did not agree to add 'poisoning', 'malnutrition' and 'other abnormal mortalities' as examples of triggers for *ad hoc* review as they did not consider these

to be associated with infectious pathogenic agents and are also outside of the scope of the *Aquatic Code*.

**Article 4.X.5. Categories of aquaculture production systems**

In the semi-open systems section, the Commission agreed to add 'cages' as an additional example to 'net pens' to reflect the different types of aquaculture infrastructure in semi-open systems and the terminology used globally. It agreed to make this edit throughout the chapter, where relevant. The Commission also agreed to add 'rope systems' as an example of infrastructure in mollusc production systems.

In the semi-closed systems section, the Commission agreed with a comment to amend 'enclosed floating pens' to 'floating enclosures' as it agreed it more accurately described this type of production system.

**Article 4.X.6. Transmission pathways, and mitigation measures**

1. Aquatic animals

   In the second paragraph, in response to several comments, the Commission agreed to add 'larvae' as another example of a life-stage of aquatic animals, and considered that this term also addressed the other proposed examples of life-stages. The Commission reminded Members that the type of aquatic animal would not need to be identified by origin as the glossary definition for 'aquatic animals' includes farmed and wild aquatic animals.

   In a), the Commission did not agree to add a sentence concerning the introduction of naïve populations into areas where specific disease agents are present as it considered that this would be addressed in the risk assessment.

   In f), the Commission agreed to replace 'Isolating' with 'Where possible, isolate' to add flexibility because isolating aquatic animal populations is not possible for every farming system, particularly in open and semi-open systems.

   In g), the Commission did not agree with a comment to replace 'moribund' with 'sick' as it considered that the principle of removing moribund and dead animals is to decrease the source of disease contamination in the population. Sick animals could potentially be treated to resolve infection and would not need to be removed from the population.

   In h), the Commission did not agree with a request to insert 'all' before 'notifiable disease' because only one disease would be notified per event. The Commission also added 'emerging' to emphasise the importance of notifying emerging diseases.

   In i), the Commission agreed to replace 'totally' and 'depopulate' with 'completely remove aquatic animals from all or parts of' as it considered that depopulation is a term more generally used in response to a disease event but is not the only way to remove animals from an establishment, for example, the animals may be sold or moved to another section of the establishment.

2. Aquatic animal products and aquatic animal waste

   In the second paragraph, the Commission did not agree with comments to add a new first sentence to describe that aquatic animal waste should be stored, transported, disposed of, and treated following the guidance in Chapter 4.7, Handling, disposal and treatment of aquatic animal waste, as the Commission noted this was addressed later in the chapter.

   In the second paragraph, the Commission did also not agree to amend the second sentence to prohibit the movement of aquatic animal waste, or the movement of high risk aquatic animal waste into aquaculture establishments. It noted the complexity of aquaculture systems globally, that some aquaculture establishments may not have their own facilities for disposal and that they may need to use secure facilities in other establishments. The

Commission did however, agree to delete 'if possible' to provide strength and clarity to the statement that movement of aquatic animal waste into establishments should be avoided.

3. Water

In the first paragraph, the Commission agreed with a comment to simplify the wording by deleting 'is an important asset that supports productivity and aquatic animal health but'.

In the second paragraph, the Commission did not agree with a comment that the concept of semi-closed systems are not used in other literature and thus should be removed. The Commission reminded Members that these categories have been presented for Member comments several times and there has been general agreement to this approach based on comments received.

In b), the Commission did not agree to add wording to reflect the on-site water treatment, as the Commission considered this was already addressed by the current wording.

4. Feed

In the first paragraph, the Commission amended the wording to clarify that feed may contain pathogenic agents rather than being infected with pathogenic agents.

In the second paragraph, the Commission agreed with a comment to add a new sentence at the end of the paragraph to ensure that risks of pathogen transmission to the environment are also considered.

5. Fomites

In the first paragraph, the Commission did not agree with a comment to add 'vessels' to the list of fomites, because the Glossary definition of 'vehicles' include vessels.

The Commission reiterated that it did not agree to include a Glossary definition for 'fomites' as it considered that the common dictionary definition is sufficient. The Commission reminded Members that the Glossary does not include common dictionary definitions that are deemed to be adequate and 'fomite' is one such example.

The Commission did not agree with a comment that 'risk' should be replaced by 'likelihood' in the article as it considered that where 'risk' is used it is the appropriate term to reflect the likelihood and consequences of introduction of pathogenic agents.

In b), the Commission did not agree to include additional information on disinfection as it considered that this is addressed in Chapter 4.3 which is referenced.

In (c), the Commission agreed with a comment to include 'or areas within an establishment' as it noted that this concept is also important for areas of different disease status within an aquaculture establishment, e.g. production units for different life stages.

6. Vectors

The Commission agreed with a comment to replace 'transfer' with 'transmit' for consistency with the Glossary definition of 'vector' and because 'transmit' is also used elsewhere in this chapter. This change was made throughout the chapter, where relevant.

In a ii), the Commission agreed to add 'for authorized personnel and visitors to improve clarity.

In a iii), the Commission did not agree to replace 'wild aquatic animals and other animals' with 'vectors' as it considered that aquatic animals outside a production system can also be susceptible species. These animals would not be addressed if only vectors were included.

In a iv), the Commission agreed to add 'outdoor or" to include all types of aquaculture infrastructure systems where covers are necessary to prevent access of birds.

In b), the Commission did not agree to include considerations of food safety standards and country wildlife regulations as it considered this to be outside the scope of the chapter.

The Commission did not agree with a comment to add an additional point about the potential for live feed to act as a vector as it considered this is addressed in point 4. Feed.

**Article 4.X.7 Risk analysis**

The Commission did not agree with a comment that this article would restrict the use of different methods to evaluate risks. It noted the text in the second paragraph reflected that different approaches may be applied based on a number of factors specific to an aquaculture establishment.

In Step 2 'Risk assessment', paragraph two, the Commission agreed with a comment to add a cross-reference to Table 4. It also decided to remove 'here' in Table 1 for consistency within the table.

In response to a request to harmonise the definitions in this article with the ones in the 'Handbook on Import Risk Analysis for Animals and Animal Products Vol. 1 – Introduction and Qualitative Risk Analysis', the Commission did not agree as it considered that the definitions in this chapter have been developed for aquaculture establishments, which may be different from those provided in the handbook.

**Article 4.X.8. Biosecurity plan development**

2. Key components of a biosecurity plan

The Commission agreed with a comment that the new point f) on training of personnel should be moved to b), and that the last paragraph of a) on the training of staff personnel in the application of the SOPs should be moved to the new b). This was considered to be a more logical order.

In 2 d), the Commission agreed with a comment that different measures for morbidity and mortality should be calculated within a farm, and included wording to reflect that all monitoring should be completed at both production unit and establishment levels.

In 2 e), the Commission did not agree with a comment to add 'Early detection' at the beginning of the second sentence as it considered and that monitoring is not restricted to early detection.

The new draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) is presented as clean and track change versions in **Annex 3**, and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

### 3.1.3. Article 1.3.3. of Chapter 1.3, Diseases of crustaceans listed by the OIE

Listing of infection with decapod iridescent virus 1 (DIV1)

Comments were received from Armenia, Australia, China (People's Rep of), Chinese Taipei, Cuba, Korea (Rep. of), New Caledonia, Switzerland, the UK, the USA, the EU and Members of the OIE Americas region.

*Background*

The Aquatic Animals Commission, at its February 2019 meeting, assessed infection with shrimp haemocyte iridescent virus (SHIV) against the criteria for listing aquatic animal diseases in accordance with Article 1.2.2, and agreed that infection with SHIV meets the OIE criteria for listing and should be added to Article 1.3.3, Diseases of crustaceans listed by the OIE. The Commission also agreed to change the name to 'Infection with decapod iridescent virus 1 (DIV1)' in accordance with the classification by the International Committee of Taxonomy of Viruses (ICTV).

**Previous Commission meeting reports where this item was discussed:**

February 2019 report (Item 3.1.1, page 13); September 2019 report (Item 6.2, page 6); February 2020 report (Item 7.1.2, page 11); September 2020 (Item 4.2, page 6).

**February 2021 meeting**

The Commission expressed its appreciation to Members for providing additional information regarding DIV1 and made minor changes to the assessment of DIV1 to reflect recently published scientific information.

The Commission noted the general support by Members for the listing of infection with DIV1. The Commission emphasised that the objective of listing is support Member Countries to take appropriate action to prevent the trans-boundary spread of DIV1 through transparent, timely and consistent notification.

In response to several comments regarding the limited availability of diagnostic testing for DIV1, the Commission agreed to explore with experts the possibility of sharing positive control materials with Members.

The revised 'Assessment of infection with decapod iridescent virus 1 (DIV1) for listing in Chapter 1.3 of the *Aquatic Code'*, is presented as **Annex 4** for Member information.

The revised Article 1.3.3, Diseases of crustaceans listed by the OIE, is presented as **Annex 4** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of this revised article.**

### 3.1.4. Model Article 10.X.13. for Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 of Chapter 10.4.)

Comments were received from Armenia, Cuba, Switzerland, the UK and the EU.

*Background*

Revision of the model Article 10.X.13, Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with pathogenic agent X to be applied in Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 of Chapter 10.4), was initiated by the Aquatic Animals Commission in September 2019 in response to requests to clarify the intended purpose of this article.

**Previous Commission meeting reports where this item was discussed:**

February 2019 report (Item 3.2, page 13); September 2019 report (Item 6.3, page 7); February 2020 meeting (Item 7.1.3, page 11); September 2020 (Item 4.3, page 6).

**February 2021 meeting**

In 1 a), the Commission agreed with a comment that the recommendations in Chapter 4.4, Recommendations for Surface Disinfection of Salmonid Eggs, were best applied only to point

2 a), which discusses measures to mitigate the risk of pathogen transfer with the importation of disinfected eggs. It was therefore agreed to remove the reference to Chapter 4.4 in 1 a). In addition, the Commission agreed with a comment to delete point 1 c) as it considered that this duplicated information already provided in point 2.

The Commission reviewed a scientific evaluation of Atlantic halibut (*Hippoglossus hippoglossus*) egg disinfection protocols for inactivation of infectious pancreatic necrosis virus and viral haemorrhagic septicaemia virus (VHSV) submitted by a Member. After discussing this evaluation, the Commission agreed that there was insufficient evidence at this time to propose a new disinfection protocol for halibut eggs to Chapter 4.4 of the *Aquatic Code*. The Commission reminded Members that there is information on egg disinfection for some species in *Aquatic Manual* chapters. The Commission invited Members to submit any additional information on disinfection protocols for other non-salmonid species for consideration.

The Commission reminded Members that once this model article is adopted, the proposed changes will be made in Articles 10.5.13, 10.6.13, 10.10.13 and 10.4.17.

The revised Model Article 10.X.13 for Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 of Chapter 10.4) is presented as clean and track change versions in **Annex 5**, and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU thanks the OIE and supports the adoption of this revised model article.**

### 3.1.5. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus

*Background*

The revised list of susceptible species in Article 10.9.2 of Chapter 10.9, Infection with spring viraemia of carp virus (SVCV), was adopted at the 87th General Session in May 2019. However, in light of new scientific evidence on the susceptibility of zebrafish (*Danio rerio*) to infection with SVCV, the Aquatic Animals Commission reviewed the previous assessment for this species and agreed that zebrafish did meet the criteria for listing as a susceptible species and should, therefore, be included in Article 10.9.2. This proposal was circulated for comment in the Commission's September 2019 report, and it was noted in the Commission's February 2020 report that Members supported zebrafish being included in the list of susceptible species in Article 10.9.2. At its September 2020 meeting, the Commission agreed to defer its discussion on this article until its February 2021 meeting.

**Previous Commission meeting reports where this item was discussed:**

September 2019 (Item 6.5, page 8); February 2020 (Item 7.14, page 12).

**February 2021 meeting**

The Commission noted that no comments were received from Members on the annex circulated in its February 2020 report and agreed that no further amendments were needed.

The revised Article 10.9.2 of Chapter 10.9 Infection with spring viraemia of carp virus presented as **Annex 6** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of this revised article.**

### 3.1.6. Article 10.10.2. of Chapter 10.10 Infection with viral haemorrhagic septicaemia virus

Comments were received from Armenia, Canada, Cuba, Korea (Rep. of), Switzerland, the UK, the EU and AU-IBAR.

*Background*

At its September 2019 meeting, the Aquatic Animals Commission reviewed the September 2019 report of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases. The *ad hoc* Group had applied the criteria for listing species as susceptible to infection with a specific pathogenic agent, in accordance with Chapter 1.5 of the *Aquatic Code*, to infection with viral haemorrhagic septicaemia virus (VHSV). The Commission had agreed to amend the list of susceptible species in Article 10.10.2 in line with recommendations made by the *ad hoc* Group. (report available at https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/).

At its September 2020 meeting, the Commission confirmed its decision that genotypes should not be included in Chapter 10.10.2 as the Commission had not assessed whether VHSV genotypes can be differentiated for the purpose of distinguishing risk management measures for traded commodities.

**Previous Commission meeting reports where this item was discussed:**

September 2019 report (Item 6.4, page 8); February 2020 report (Item 7.1.5, page 13), September 2020 (Item 4.4, page 7).

**February 2021 meeting**

Several requests were made to initiate an assessment of VHSV genotypes, as had been conducted for ISAV, with respect to strain differentiation. The Commission agreed to initially seek the advice of Reference Laboratory experts on this issue so that it can be further considered at the next Commission meeting in September 2021. The Commission noted that strain differentiation would require significant work and would need to provide tangible benefits to Members to be prioritised against other items in the Commission's workplan.

The Commission did not agree with a comment to include a statement in the chapter that a Member may, based on a risk assessment and a claim of freedom from a specified VHSV genotype, take appropriate measures to protect its declared free status. The Commission did, however, agree that further guidance on the principal of applying risk assessments to justify mitigation measures directed at specific genotypes would be useful. It noted that this issue could be addressed through the possible restructuring of articles of disease-specific chapters and that approaches in the *Terrestrial Code* should be considered. The next Commission would need to prioritise this activity against other items in its workplan.

The Commission did not agree with a request to make additional amendments to the list of species in Article 10.10.2. The Commission explained that some of the proposed species had previously been considered by the Commission and given that no new scientific evidence was provided, a re-assessment was not justified. The Commission undertook assessments for the other proposed species against the criteria in Chapter 1.5 and none were found to meet the criteria for susceptibility.

The revised Article 10.10.2 of Chapter 10.10 Infection with viral haemorrhagic septicaemia virus is presented as **Annex 7** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of this revised article.**

### 3.1.7. Articles 11.3.1. and 11.3.2. of Chapter 11.3 Infection with *Bonamia ostreae*

Comments were received from Armenia, Canada, China (People's Rep. of), Cuba, Mexico, Switzerland, the UK, and the EU.

*Background*

At its September 2020 meeting, the Aquatic Animals Commission reviewed the July 2020 report of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases. The *ad hoc* Group had applied the criteria for listing species as susceptible to infection with a specific pathogenic agent in accordance with Chapter 1.5 of the *Aquatic Code* for infection with *B. ostreae.* The Commission had agreed to amend the list of susceptible species in Article 11.3.2 in line with recommendations made by the *ad hoc* Group. (report available at: https://www.oie.int/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/).

**Previous Commission meeting reports where this item was discussed:**

September 2020 report (Item 4.8, page 11).

**February 2021 meeting**

The Commission noted that all comments were supportive of the proposed amendments.

The Commission noted that some comments were received on the *ad hoc* Group report. These were reviewed by the Commission in consultation with the *ad hoc* Group, and the Commission agreed that the comments did not have any impact on the outcome of the assessments.

The Commission made no additional changes to Article 11.3.2 in line with its decision from the Commission's September 2020 meeting. It noted that of the six species currently listed in Article 11.3.2 as susceptible to infection with *B. ostreae*, three species met the criteria for listing as a susceptible species: European flat oyster (Ostrea edulis), Chilean flat oyster (*Ostrea chilensis*) and Suminoe oyster (*Crassostrea ariakensis*). The remaining three currently listed species, Australian mud oyster (*Ostrea angasi*), Argentinean flat oyster (*Ostrea puelchana*) and Asiatic oyster (*Ostrea denselammellosa*), did not meet the criteria for listing as a susceptible species and are therefore proposed to be deleted from Article 11.3.2. It also noted that no new species were found to meet the criteria for listing as susceptible to infection with *B. ostreae*.

Relevant sections of Chapter 2.4.3, Infection with *Bonamia ostreae*, in the *Aquatic Manual* were amended in line with the conclusions from the September 2020 meeting (also see Item 4.1.5).

The amended Articles 11.3.1 and 11.3.2 of Chapter 11.3, Infection with *Bonamia ostreae* are presented as **Annex 8** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU supports the adoption of these revised articles.**

## 4. THE *AQUATIC MANUAL*

Members were reminded that the Aquatic Animals Commission has commenced the process of progressively reformatting the disease-specific chapters of the *Aquatic Manual* into a new template. As the reformatted and updated chapters have substantial changes, the Commission agreed that only clean versions of the chapters would be provided in the report. Subsequent changes made to these initial revisions following Member comments are indicated in the usual style (i.e. strikethrough for deletions and double

underline for additions for first-round comment, and strikethrough for deletions and double underline for additions and all changes highlighted in yellow for second-round comment).

The Commission wished to acknowledge the substantial contributions of Reference Laboratory experts and the technical editor, Dr Mark Crane, in assisting with the comprehensive revision of *Aquatic Manual* chapters.

## 4.1. Texts proposed for adoption in May 2021

*Horizontal amendments*

The Aquatic Animals Commission agreed with a comment to delete Section 3.5.4 *Samples for electron microscopy* from all the disease-specific chapters as electron microscopy is not a recommended diagnostic method for aquatic animal diseases.

In Section 4.3 (Section 4.4 in Chapter 2.3.3), the Commission agreed with a comment to delete 'or artificial media' from the title so that the title is now 'Cell culture for isolation'. A corresponding amendment would be made to the tests listed in Table 4.1.

The Commission agreed to maintain the word 'conventional' before PCR and RT-PCR (to distinguish from real-time PCR and real-time RT-PCR) as it considered that this terminology provides additional clarity, is included in the chapter template and has been widely used in the disease-specific chapters throughout the *Aquatic Manual*. Additionally, the existing convention in the *Aquatic Manual* is to use 'real-time PCR' or 'real-time RT-PCR' rather than 'qPCR' or 'RT-qPCR'. The Commission noted that this convention is consistent with the *Terrestrial Manual*.

In reply to a comment to recommend more tests from Table 4.1 in Section 5, Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations, the Commission reiterated that the purpose of Section 5 is to highlight the assays that are considered the most suitable. Two or more assays may be recommended if they are considered equally suitable.

The Commission considered a comment on Section 6, Corroborative diagnostic criteria, requesting that the case definitions for apparently healthy animals not be separated from clinically affected animals. The Commission considered that the approach remains sound. The Commission also noted that the new *Aquatic Manual* disease chapter template, where this approach is presented, had been developed by an *ad hoc* Group and first appended to the report of the February 2018 meeting. The first chapters to be updated using the new template were appended to the report of the February 2019 meeting. Newly updated chapters had been appended to each report since then.

### 4.1.1. Review of non-susceptible species

**February 2021 meeting**

The Aquatic Animals Commission reviewed the inclusion of the new Section 2.2.3, Non-susceptible species, in the template for disease-specific chapters of the *Aquatic Manual*. A number of Members questioned the inclusion of non-susceptible species in the *Aquatic Manual*, and noted the purpose of the criteria for non-susceptibility in *Aquatic Code* Chapter 1.5, Criteria for listing species susceptible to infection with a specific pathogenic agent. The Commission agreed that the identification of non-susceptible host species was only relevant for pathogenic agents with a wide host range that are assessed against Article 1.5.9, Listing susceptible species at a taxonomic ranking of Genus or higher. The Commission agreed that Section 2.2.3 would be removed from the *Aquatic Manual* disease-specific chapter template and from the revised *Aquatic Manual* chapters.

The Commission noted that assessments against Article 1.5.9 would continue to be made where relevant and any assessment of evidence of non-susceptibility would be provided in the *ad hoc* Group reports.

### 4.1.2. Chapter 2.3.3. Infection with *Gyrodactylus salaris*

Comments were received from Armenia, Canada, China (People's Rep. of) Cuba, Switzerland, UK, USA, the EU and AU-IBAR.

**Previous Commission reports where this item was discussed**

February 2020 (Item 8.3.1, page 21), September 2020 (Item 5.2, page 13)

**February 2021 meeting**

The Aquatic Animals Commission thanked Members for their valuable comments on this chapter.

In the Scope, the Commission agreed with a comment to add 'freshwater' as it considered it a worthwhile change to emphasis a key characteristic of the parasite.

In the last sentence of Section 2.1.1, Aetiological agent, the Commission agreed with a comment to change the wording to make it clear that for the purposes of the chapter *G. salaris* and *G. thymalli* are being treated as separate species.

For Section 2.2.2, Species with incomplete evidence for susceptibility, in response to a question why only replication had been used to identify susceptible hosts, the Commission referred Members to the report of the *ad hoc* Group on Susceptible fish species (Annex 30, the Commission's February 2018 meeting report) where an explanation for the application of Article 1.5.6 to *G. salaris* was provided (Aquatic Animals Commission/A_AAC_Sept_2017.pdf [oie.int]). The *ad hoc* Group assessing host species for *G. salaris* concluded that as attachment of the parasite occurs transiently on many species, clinical signs and location of infection could not constitute a true infection. Therefore, viability/infectivity (B), pathology/clinical signs (C) and location (D) were not used to determine host susceptibility, leaving evidence of replication (A) as the sole criterion.

Section 2.2.3, Non-susceptible species was deleted (see Item 4.1.1).

In regard to Section 2.2.7, Vectors, the Commission appreciated a Member providing a risk assessment for *G. salaris* vectors. The Commission noted that the proposed definition of vector requires that a vector has been demonstrated to transmit the pathogenic agent to susceptible species (see Item 3.1.1.2). It is noted in Section 2.2.7 that *G. salaris* can attach for brief periods of time to fish species not considered susceptible. However, as there is no evidence of transmission other than by susceptible species, no species are mentioned in Section 2.2.7. The Commission considered that these circumstances are presented appropriately in Section 2.2.7. The Commission agreed that species could be named as vectors for *G. salaris* if there was empirical evidence of transmission.

In Section 2.3.5, Environmental factors, the Commission added text on survival at different temperatures and salinities.

In Section 2.4.5, Inactivation methods, the Commission added details on methods to kill the parasite or eliminate its transfer.

In Section 2.4.7, General husbandry, the Commission clarified that the parasite can be killed by bath treatments, e.g. with formaldehyde or chlorine.

In Section 3.1, Selection of populations and individual specimens, the Commission removed a statement that grayling should not be samples as there is no category for 'not highly susceptible' in the chapter.

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

In Section 3.6, Pooling of samples, the Commission clarified that pooling of fish or fins for examination for parasites was acceptable. However, pooling of parasites for molecular

diagnosis could not be recommended as there is a lack of information on which to estimate the impact on test performance.

In reply to a comment to recommend more tests from Table 4.1 in Section 5, Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations, see Item 4.1.

The Commission noted that sample size calculations are outside the scope of the disease-specific *Aquatic Manual* chapters, and that guidance can be found in *Aquatic Code* Chapter 1.4 (currently under review).

In response to a question on Section 4.5, Nucleic acid amplification, the Commission noted that pooling of subsamples of digest prior to extraction cannot be recommended.

In Section 4.6.2, CO1 sequencing and sequence analysis, the Commission agreed with a comment to add a definition of a clade as 'a group of haplotypes with a common ancestor' to provide greater clarity.

In reply to a comment on Section 6, Corroborative diagnostic criteria, that the case definitions for apparently healthy animals should not be separated from clinically affected animals, the Commission referred to Item 4.1.

The revised Chapter 2.3.3, Infection with *Gyrodactylus salaris*, is presented as **Annex 9** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU thanks the OIE and in general supports the adoption of this revised chapter. A comment is inserted in the text of Annex 9.**

### 4.1.3. Chapter 2.3.6 Infection with salmonid alphavirus

Comments were received from Armenia, Canada, China (People's Rep. of), Cuba, Switzerland, Thailand, the USA, the UK and the EU.

**Previous Commission reports where this item was discussed**

February 2020 (Item 8.3.2, page 21) and September 2020 (Item 5.3, page 14)

**February 2021 meeting**

In Section 2.1.3, Survival and stability outside the host, the Aquatic Animals Commission did not agree to a proposal to refer to the 'SAV viral genome' rather than "SAV" because the cited study used cell culture to detect SAV. The Commission agreed to delete the statement that survival times are reduced in the presence of organic matter because there have been reports that some types of organic material such as fat may increase the survival and spread of the virus.

Section 2.2.3, Non-susceptible species, was deleted (see Item 4.1.1).

In Section 2.2.4 (renumbered as 2.2.3), Likelihood of infection by species, host life stage, population or sub-populations, the Commission did not agree with a comment to add an updated reference on the susceptibility of species at different life stages as the scientific paper had not yet been published. The Commission clarified that SAV 1 has been detected in rainbow trout.

In Section 2.3.1, Mortality, morbidity and prevalence, the Commission did not agree that it is necessary to specify that the RT-PCR detects viral genome, believing that it is implicit and such a change would need to be made throughout the *Aquatic Manual*.

In Section 3.2, Selection of organs or tissues, the Commission did not agree with a comment to add 'q' to real-time RT-PCR because the agreed style of the *Aquatic Manual* (see Item 4.1). Although the Commission noted that fish have a coelomic cavity, they agreed to retain 'abdominal cavity' as it is widely understood.

In Section 3.5.1, Samples for pathogen isolation, the Commission removed the generic information and added it to Chapter 2.3.0, General information, (diseases of fish) and inserted a cross reference to that chapter.

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

In Section 3.6, Pooling of samples, the Commission did not agree to add a description of the diagnostic performance of assays on pooled samples. The Commission clarified that although there has been some evaluation of pooled samples, testing of individual samples is more appropriate for testing to demonstrate freedom.

In Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals, the Commission had received a comment that the real-time PCR is rated '+++' for the purpose 'Surveillance of apparently healthy animals' and 'Presumptive diagnosis of clinically affected animals' and that amplicon sequencing is rated '+++' for the purpose 'Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis' yet the level of validation of these tests is 1 or 2. The Commission explained that the level of validation is not the only factor that determines an assays recommendation rating. The methods have been used routinely for diagnosis of infection with SAV and are recommended based on available information. However, validation is a continual process and their level of validation will likely change in the future.

In Section 4.4.1, Real-time RT-PCR, the Commission did not agree with a comment to replace 'sequencing' with 'sequence analysis' in a sentence referring to 'RT-PCR and sequencing' as it considered that the term 'sequencing' is used consistently in this context in all the disease-specific chapters of the *Aquatic Manual* and is well understood. However, in Section 4.5, Amplicon sequencing, the Commission did agree with a comment to replace 'nucleotide sequencing' with 'nucleotide sequence analysis' as in this context it was considered to add accuracy to the recommendation.

In reply to a comment to recommend more tests from Table 4.1 in Section 5, Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations, see Item 4.1.

In reply to a comment on Section 6, Corroborative diagnostic criteria, that the case definitions for apparently healthy animals should not be separated from clinically affected animals, see Item 4.1.

In Section 6.1.1, Definition of suspect case in apparently healthy animals, the Commission did not agree with a request to reinstate two of the criteria that previously had been proposed for deletion as the criteria were not consistent with Table 4.1: neither conventional RT-PCR nor SAV-typical CPE in cell culture is recommended for surveillance in Table 4.1.

The Commission thanked the expert reviewers who had completed Table 6.3, Diagnostic performance of tests recommended for surveillance or diagnosis.

The revised Chapter 2.3.3, Infection with salmonid alphavirus, is presented as **Annex 10** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

#### 4.1.4. Chapter 2.3.0 General information (diseases of fish)

Comments were received from Armenia, Canada, China (People's Rep. of), Chinese Taipei, Cuba, Japan, Korea (Rep. of), New Zealand, Switzerland, UK, USA and the EU.

**Previous Commission reports where this item was discussed**

February 2020 (Item 8.4.1, page 22); September 2020 (Item 5.6, Page 15)

**February 2021 meeting**

The Aquatic Animals Commission removed trade names and replaced them with generic terms when possible throughout the chapter. As an international standard-setting organisation, the OIE does not endorse or recommend chemical and biological reagents, diagnostic kits or vaccines, from specific commercial suppliers or manufacturers in OIE international standards.

The Commission agreed with a comment to include general information on fish sampling in Section A.1.2, Specifications according to fish populations, rather than the disease-specific chapters where only pathogen-specific information would be included.

In Section A.1.3, Specifications according to clinical status, the Commission agreed to recommend a sampling range of five to ten clinically diseased fish consistent with the disease of interest. A sentence on disease-specific information was deleted from this section and replaced with a cross reference to Section 3.2, Selection of organs or tissues, of the disease-specific chapters of the *Aquatic Manual*.

In Section A.1.4, Specifications according to fish size, the Commission did not agree with a request to add a reference for the rationale for the removal of the yolk sac. The Commission noted that yolk sac removal is a common, long standing practice of laboratories as it can be toxic to some cell lines and it did not see the value of adding a reference. Also, the Commission did not agree with a request to delete Section A.1.4, as it considered it was important to include it here, even if pathogen specific texts are usually found in relevant disease-specific chapters.

In Section A.2.2, Preservation of samples for subsequent virological examination, the Commission revised the concentration range of the formalin fixative from '4–10%' to '10%' as this is the most commonly recommended concentration.

In Section A.2.3.2, Virus isolation, the Commission corrected the title and replaced 'pre-screening' by 'other tests' in point 6. The Commission also added a new point 7 on the recommendation to aliquot homogenised sample material to avoid repeated freeze–thawing.

In Section B.1.1, Fish cell lines, the Commission agreed with a comment to add 'rainbow trout gonad (RTG-2)' as this cell line is considered important for the diagnosis of infection with viral haemorrhagic septicaemia (VHSV) and also to include it in the VHSV chapter. The Commission added grass carp cell lines (GCO) to the list as it is now widely available and important for diagnosis of infection with spring viraemia of carp virus.

The Commission added VHSV at 15°C to the table in Section B.1.3.1, Virus Production, on the preferred temperatures for virus propagation.

The Commission agreed to move a sentence on the frequency at which titration of reference isolates should be performed to verify cell line susceptibility to infection from Section B.2.4.1, Virus isolation, to the end of Section B.1.3.2, Preservation and storage of virus stock cultures.

In Section B.2.2.1, Preparation of slides for histological examination, the Commission clarified that fish should be examined 'after humane euthanasia' in compliance with animal welfare standards. The Commission also agreed to add a range of thickness for cut sections starting at 3 µm.

In Section B.2.4, Virus isolation, the Commission agreed with a comment to include substantial additional text on general information pertaining to virus isolation rather than to

repeat it in the disease-specific chapters where only pathogen-specific information would be included.

In Section B.2.4.5, Sub-Cultivation, the Commission deleted text on increasing confidence of a negative result by testing for the presence of virus using antibody-based or nucleic acid-based (PCR) methods because the recommendation does not take account of false positive PCR results. For virological examinations, the Commission clarified that repeat freeze–thaw cycles will reduce virus titres and recommended aliquoting the homogenised sample material.

In Section B.2.4.6, Virus identification, clarified that infected cell cultures are used for virus identification by IFAT and that supernatant from cultures demonstrating CPE is used for virus identification by nucleic acid-based techniques.

In Section B.2.5.1, Sample preparation and types, the Commission did not agree to delete the statement that for *in-situ* hybridisation fixation for over 24–48 hours should be avoided as it considered that it is accurate reflection of best practice.

The revised Chapter 2.3.0, General information (diseases of fish), is presented as **Annex 11** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

**4.1.5. Sections 2.2.1 and 2.2.2 (on susceptible species) of Chapter 2.4.3 Infection with *Bonamia ostreae***

Comments were received from Armenia, China (People's Rep. of), Cuba, Switzerland, the USA and the EU.

**Previous Commission reports where this item was discussed**

September 2020 (Item 5.7, Page 16)

**February 2021 meeting**

The Aquatic Animals Commission did not agree with a Member comment to add references to these sections and noted that references can be found in the relevant *ad hoc* Group reports. Consistent with its decision at item 4.1.1, the Commission agreed to delete Section 2.2.3, Non-susceptible species.

The revised Sections 2.2.1 and 2.2.2 of Chapter 2.4.3, Infection with *Bonamia ostreae,* are presented as **Annex 12** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

**4.1.6. New draft Chapter 2.1.X. Infection with *Batrachochytrium salamandrivorans***

Comments were received from New Zealand, Thailand, UK and the EU.

**Previous Commission reports where this item was discussed**

February 2019 (Item 6.1.2, Page 17), September 2019 (Item 6.8.2, Page 12), February 2020 (Item 8.2.2, Page 19)

**February 2021 meeting**

In Section 1 Scope, the Aquatic Animals Commission replaced the categories 'Genus' and 'Family' with 'Division' and 'Order' of the pathogenic agent to be consistent with the *Aquatic Code* chapter.

In Section 2.2.1, Susceptible host species, and Section 2.2.2, Species with incomplete evidence for susceptibility, the Commission added '[under study]' to the titles as the disease has not yet been reviewed by an *ad hoc* Group on Susceptibility of amphibian species to OIE listed diseases.

Section 2.2.3, Non-susceptible species, was deleted (see Item 4.1.1).

In Section 2.3.5, Environmental factors, the Commission decided to move a paragraph on density independent disease dynamics to Section 2.3.1, Mortality, mortality and prevalence, and a paragraph on the role of barriers to pathogen dispersal in the prevention of transmission to Section 2.4.7, General husbandry, as the paragraphs fit better in these sections.

In Section 3.4, Non-lethal sampling, the Commission agreed with a comment to replace 'cotton-tipped swabs' with 'medical swabs' as it is more appropriate and added a sentence on guidance on how to submit the swabs to the diagnostic laboratory.

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

For section 3.6, Pooling of samples, the Commission added a reference on the reliability of test procedures using pooled samples.

A Member had asked if the rating of the real-time PCR given in Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals, should be changed from '+++' to '++' as the Table and the text in Section 4.4.1 *Real-time PCR* indicates that the test is 'partially validated to level 2'. The Commission reviewed the relevant publications and noted that the validation included assessment of reliability and reproducibility. The test could therefore be considered to be validated to level 3, and the text in Section 4.4.1 was amended accordingly. As validation levels are given both in Table 4.1 and Table 6.3 Diagnostic performance of tests recommended for surveillance or diagnosis, the Commission agreed to remove mention of validation levels in the text.

The Commission did not agree with a Member comment to include information on sequencing of the real-time TaqMan PCR product in Section 4.5, Amplicon sequencing, because there is no published information on sequencing of real-time PCR products.

The new draft Chapter 2.1.X, Infection with *Batrachochytrium salamandrivorans*, is presented as **Annex 13** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU thanks the OIE and in general supports the adoption of this revised chapter. A comment is inserted in the text of Annex 13.**

### 4.1.7. Chapter 2.3.9. Infection with spring viraemia of carp virus

Comments were received from Australia, Canada, Singapore, Thailand, the UK and the EU.

**Previous Commission reports where this item was discussed**

February 2019 (Item 6.1.1, Page 16), September 2019 (Item 6.8.1, Page 10), February 2020 (Item 8.2.1, Page 18)

**February 2021 meeting**

Section 2.2.3, Non-susceptible species, was deleted (see Item 4.1.1).

In Section 2.2.6, Aquatic animal reservoirs of infection, the Aquatic Animals Commission agreed to describe fish that are potential reservoirs of infection as fish 'with long term subclinical infections' rather than fish 'surviving infection'.

In Section 2.3.4, Modes of transmission and life cycle, the Commission agreed to delete a statement on the difficulty of eradicating SVCV once it is established in populations because such difficulties apply to other pathogens.

In Section 3.1, Selection of populations and individual specimens, the Commission agreed to remove crucian carp and silver carp from the list of species to be targeted as they are listed as species with incomplete evidence of susceptibility. These species were replaced with bream and roach as examples of other cyprinid species. The Commission also clarified that water sources should be evaluated to determine the risk of disease and amended the text to specify that the highest risk water source should be targeted, and all water sources of equal risk should be included in the sample.

In Section 3.5.2, Preservation of samples for molecular detection, the Commission did not agree with a comment to change the percentage of ethanol for preservation of diagnostic material as the percentage given in the SVCV chapter corresponds to the general guidance given in Chapter 2.3.0.

Section 3.5.3, Samples for histopathology, the Commission deleted the text in immunohistochemistry or *in-situ* hybridisation, and replaced it with a cross reference to Chapter 2.3.0.

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

The Commission considered a request to include a recommended validation protocol for pooled samples in Section 3.6 and agreed that such guidance could usefully be included in Chapter 1.1.2, Principles and methods of validation of diagnostic assays for infectious diseases. The Commission agreed that this issue should be considered for inclusion in its work programme. In the meantime, the Commission suggested a recent publication on pooling of aquatic animal diagnostic samples as guidance:

> LAURIN E., THAKUR K., MOHR P.G., HICK P., CRANE M.S.J., GARDNER I.A., MOODY N.J.G., COLLING A. & ERNST I. (2019). To pool or not to pool? Guidelines for pooling samples for use in surveillance testing of infectious diseases in aquatic animals. *J. Fish Dis*., **42**, 1471–1491.

In Table 4.1 *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals* virus isolation and amplicon sequencing are given a level 3 validation rating. A comment had been received requesting the inclusion of references to support these ratings. The Commission stated that there are currently no data published on level 3 or level 2 validation because of the lack of diagnostic sensitivity and specificity and analytical sensitivity and specificity. Validation studies are being undertaken, which will address this issue. In the meantime, the Commission recommended to change the validation to level 1 for cell culture, conventional PCR and amplicon sequencing.

The Commission amended Section 4.3.1, Cell lines, to align it with the information on cell lines in the other chapters in viral diseases of fish (e.g. Infection with IHNV and VHS).

In Section 4.4.2, the Commission did not agree with a request for clarification on inclusion of sequencing information, as it considered that amplicon sequencing should not be considered as a separate test for confirmation of infection, but rather the confirmatory step that follows PCR amplification. The information on sequencing will thus be maintained in Section 4.4.2.

In Section 5, the Commission did not agree with a comment to include conventional nested RT-PCR assay as one of the methods for surveillance in healthy animals because cell culture is considered the most suitable method despite the lack of validation data for diagnostic methods for SVCV. A statement was included in the section to reflect this.

The Commission did not accept a request to add 'under study' to Table 6.3 *Diagnostic sensitivity and specificity for diagnostic tests:* the Commission recognises that there are currently no performance data for tests for SVCV and also that validation studies are being undertaken. However, adding 'under study' would suggest that the Commission is coordinating these studies.

The revised Chapter 2.3.9, Spring viraemia of carp virus, is presented as **Annex 14** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

**The EU in general supports the adoption of this revised chapter. A comment is inserted in the text of Annex 14.**

### 4.1.8. Chapter 2.3.4. Infection with infectious haematopoietic necrosis virus

Comments were received from Canada, China (People's Rep. of), Japan, New Zealand and the EU.

**Previous Commission reports where this item was discussed**

September 2019 (Item 6.8.3, Page 13), February 2020 (Item 8.2.3, Page 19)

**February 2021 meeting**

Section 2.2.3, Non-susceptible species, was deleted (see Item 4.1.1).

In Section 2.2.5 (renumbered as 2.2.4), Distribution of the pathogen in the host, the Aquatic Animals Commission agreed to include the oral region, pharynx, pancreas and cartilage as target tissues for virus isolation and added the supporting references.

In Section 3.1, Selection of populations and individual specimens, the Commission agreed to delete sentences suggesting rainbow trout are the most susceptible species to infection with IHNV as there is no published evidence of this assumption and it contradicts the statement that rainbow trout and other susceptible species should be sampled proportionally.

Section 3.2, Selection of organs or tissues, the Commission agreed with a comment for a complete revision to better reflect that selection of optimal tissues depends on whether clinically diseased or healthy animals are sampled.

In Section 3.3, Samples or tissues not suitable for pathogen detection, the Commission noted a comment regarding the toxicity of yolk sacs to all cell lines but did not consider that any change was required to the text (see comments on Section A.1.4 in Item 4.1.7).

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

The Commission agreed with a comment that the level of validation for conventional PCR should be the same for the purpose 'presumptive diagnosis of a clinically affected animals' and the purpose 'Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis' and changed it to level 2 in Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals.

In Section 4.3.2, Sample preparation and inoculation, the Commission amended the text to align it with the other chapters on viral diseases of fish by removing the subheading 'Interpretation of results', aligning the text on CPE, and including a section on 'Subcultivation'.

In Section 4.4.1 the Commission did not agree with a suggestion that the one-step real-time RT-PCR should not be included as it was not validated. The two-step real-time PCR has been

validated and showed very good specificity and sensitivity. For reasons of practicality, most laboratories prefer to use a one-step assay, which was adapted from the two-step assay without affecting the test characteristics. The Commission added text and a reference confirming that the performance of the one-step assay does not differ from that of the two-step assay.

The Commission corrected Table 6.3, Diagnostic performance of tests recommended for surveillance or diagnosis, by replacing 'steelhead' with 'rainbow trout'.

The revised Chapter 2.3.4, Infection with infectious haematopoietic necrosis virus, is presented as **Annex 15** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

### 4.1.9. Chapter 2.3.10. Infection with viral haemorrhagic septicaemia virus

Comments were received from Canada, Japan, Korea (Rep. of) and the EU.

**Previous Commission reports where this item was discussed**

September 2019 (Item 6.8.4, Page 13), February 2020 (Item 8.2.4, Page 20)

**February 2021 meeting**

In Section 2.1.2, Survival and stability in processed or stored samples, the Aquatic Animals Commission agreed with a comment to amend text to clarify that the commercial freezing process means storage at a core block temperature of –24°C.

In Section 2.2.2, Species with incomplete evidence for susceptibility, the Commission agreed to add *Petromyzon marinus* (sea lamprey) for genotype IVb following reassessment by the *ad hoc* Group on Susceptibility of fish species to OIE listed diseases.

Section 2.2.3, Non-susceptible species, was deleted (see Item 4.1.1).

In Section 2.2.7, the Commission did not agree with a comment to add the water flea as a possible vector by the oral route of infection because the reference did not show transmission of infection. The Commission agreed to add a sentence clarifying that VHSV has been detected in numerous animal species but they have not been demonstrated to transmit infection.

The Commission agreed to amend the text in Section 2.4.1, Vaccination, to clarify that commercial vaccines are not currently available.

In Section 3.2 the Commission did not agree to include liver or gastrointestinal track for sampling populations with clinical disease because the high level of enzymes in these organs can inactivate the virus. The Commission clarified that in apparently healthy populations, the optimal tissues are anterior kidney and heart and, during the chronic phase of infection, brain, as VHSV can persist in tissues of the nervous system. Supporting references were added.

Section 3.5.4, Samples for electron microscopy, was deleted (see Item 4.1).

In Table 4.1, the Commission agreed to remove the antibody ELISA and serum neutralisation for antibody detection as recommended tests for surveillance of apparently healthy animals for consistency with Section 4.9, Antibody- or antigen-based detection methods.'.

As a result of the amendments made to Table 4.1, the Commission amended Section 6.1.1, Definition of suspect case in apparently healthy animals, to remove detection of antibodies as one of the criteria. Also in this section, the Commission amended the real-time and conventional PCR assays in criteria ii) and iii) to real-time and conventional reverse transcription PCRs.

In Section 4.5, the Commission added a sentence and a reference that the VHSV genotype can be identified by sequencing the amplicon generated by the conventional RT-PCR using the 3F2R primer set.

The revised Chapter 2.3.10, Infection with viral haemorrhagic septicaemia virus, is presented as **Annex 16** and will be proposed for adoption at the 88th General Session in May 2021.

**EU position**

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

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

**Paris, 17–24 February 2021**

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# GLOSSARY

**EU position**

**The EU supports the adoption of these revised Glossary definitions.**

***AQUATIC ANIMAL WASTE*** **(new Glossary definition originally proposed in Commission's September 2019 report)**

means ~~the~~ entire carcasses of ~~an anything generated from *aquatic animals,* *aquatic animals*, its that have died, have been killed for *disease* control purposes, or have been killed and processed for human consumption or other purposes. This may include the entire carcass of *aquatic animals,* their~~ parts of *aquatic animals*, or associated liquids which are intended for disposal.

***AQUATIC ANIMAL WASTE (CLEAN VERSION)***

means entire carcasses of *aquatic animals*, parts of *aquatic animals*, or associated liquids which are intended for disposal.

***AQUATIC ANIMAL PRODUCTS***

means non-viable *aquatic animals*, parts of *aquatic animals,* or manufactured goods containing any material derived ~~from and products~~ from *aquatic animals* that are intended for sale or trade.

***AQUATIC ANIMAL PRODUCTS (CLEAN VERSION)***

means non-viable *aquatic animals*, parts of *aquatic animals,* or manufactured goods containing any material derived from *aquatic animals* that are intended for sale or trade.

***VECTOR***

means any living organism~~, other than *susceptible species,*~~ that has been demonstrated to ~~transports~~ ~~transfer~~ transmit a *pathogenic agent* to ~~a population of~~ *susceptible species* ~~susceptible *aquatic animal* or its food or immediate surroundings. The *pathogenic agent* may or may not pass through a development cycle within the vector~~. *Susceptible species* ~~of a *pathogenic agent*~~ are not considered as *vectors* for ~~that~~ a specific *pathogenic agent*.

***VECTOR (CLEAN VERSION)***

means any living organism that has been demonstrated to transmit a *pathogenic agent* to *susceptible species*. *Susceptible species* are not considered as *vectors* for a specific *pathogenic agent*.

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**Proposed consequential amendments of the term 'waste' following adoption of the revised definition for 'aquatic animal waste'**

| Article | Page number | Proposed change |
|---|---|---|
| User's guide, point 7 of Section C. | | The assessment for inclusion of *aquatic animal products* in these articles is based on the form and presentation of the product, the expected volume of ~~waste~~ *aquatic animal waste* ~~tissues~~ generated by the consumer and the likely presence of viable *pathogenic agent* in the ~~waste~~ *aquatic animal waste*. |
| 2.1.4., 2.c), last point | | – ~~waste~~ *Aquatic animal waste* disposal practices |
| 4.2.3., 2.i) | | i) disposal of ~~waste~~ *aquatic animal waste*; |
| 4.3.6. | 60 | These conditions include a high level of disease risk (due to the significance of the *disease*), high pathogen loading, potential high volumes of infected *aquatic animals* and ~~waste~~ *aquatic animal waste*, large areas requiring disinfection and large volumes of contaminated water. |
| 4.7.1. | 71 | The objective of this chapter is to provide guidance on storage, transport, disposal and treatment of *aquatic animal* ~~wastes~~ *waste* so as to manage risks to *aquatic animal* health. |
| 4.7.2. | 71 | The scope of this chapter covers *aquatic animal* ~~waste~~ *waste* derived from: i) routine *aquaculture* operations; ii) on shore processing, irrespective of origin; iii) mass killing for *disease* control purposes and iv) mass mortality (including in the wild). |
| 4.7.3. | 71 | For the purpose of this chapter:<br>~~*Aquatic animal* waste means the entire body or parts of *aquatic animals* that have died or have been killed for disease control purposes as well as slaughtered *aquatic animals*, and their parts, that are not intended for human consumption.~~<br>High risk waste means *aquatic animal* ~~waste~~ *waste* that constitutes, or is suspected of constituting, a serious health risk to *aquatic animals* or humans.<br>Low risk waste means *aquatic animal* ~~waste~~ *waste* that is not high-risk waste. |
| 4.7.4. | 71 | The *Competent Authority* should oversee the efficient and effective disposal of *aquatic animal* ~~waste~~ *waste*. […]:<br>1) physical, logistical and data access by relevant personnel, in cooperation with stakeholders, including access of the *Competent Authority* to the *aquatic animal* ~~waste~~ *waste*;<br>2) movement controls and the authority to make exemptions under certain *biosecurity* conditions, for example for transport of *aquatic animal* ~~waste~~ *waste* to another location for disposal; |
| 4.7.5. | 72 | Following collection, *aquatic animal* ~~waste~~ *waste* should be stored for the minimum time practical; however, where storage is necessary there should be sufficient capacity for the expected ~~waste~~ *aquatic animal waste* and the *Competent Authority* may require additional measures.<br>[…]<br>The containers of stored *aquatic animal* ~~waste~~ *waste* should be leak-proof and secured to prevent contact with *aquatic animals*, other animals or birds and unauthorised personnel.<br>*Aquatic animal* ~~waste~~ *waste* infected or contaminated by an agent causing a *disease* referred to in the *Aquatic Code* or suspected of being so, may not be transported without permission from the *Competent Authority*. […]<br>Containers used for transport of *aquatic animal* ~~waste~~ *waste* should be leak-proof and labelled regarding content. […] |

| Article | Page number | Proposed change |
|---|---|---|
| 4.7.6. | 72 | 1. Requirement for approval<br>All disposal plants dealing with *aquatic animal* ~~waste~~ *waste* should be approved by the *Competent Authority*. […]<br>2. Conditions for approval<br>For a disposal plant to be approved to deal with *aquatic animal* ~~waste~~ *waste*, it should:<br>[…]<br>d) fulfil requirements for handling the *aquatic animal waste* and products specified by the *Competent Authority*. […]<br>3. Operating requirements<br>[…]<br>c) handling and treatment of *aquatic animal* ~~waste~~ *waste* should take place as soon as possible after being received;<br>[…] |
| 4.7.7. | 73 | 1. Rendering<br>[…]<br>The process typically involves pre-heating to 50–60°C, followed by cooking of the raw *aquatic animal waste* at 95–100°C for 15 to 20 minutes. […]<br>2. Incineration<br>[…] Mobile air curtain incinerators enable the process to be carried out on site thus removing the need to transport the *aquatic animal waste*.<br>Incinerators may only be capable of handling limited volumes of *aquatic animal* ~~waste~~ *waste*. |
| 4.7.7. | 74 | 6. Ensiling<br>[…]<br>Ensiling of *aquatic animal* ~~waste~~ *waste* in an organic acid such as formic acid is an effective method of inactivating most *pathogenic agents* within 48 hours. […] |
| 4.7.7. | 74 | 7. Burial<br>[…]<br>Whenever possible, the *aquatic animal* ~~waste~~ *waste* should be subjected to a treatment that ensures inactivation of the *pathogenic agents* prior to burial.<br>In selecting an acceptable burial site, consideration should be given to the following:<br>[…]<br>b) Access – easy access for equipment and delivery of *aquatic animal* ~~waste~~ *waste*. Fencing and restricted admittance may be necessary.<br>c) Pit construction – […] Pit dimensions depend on the volume of the *aquatic animal* ~~waste~~ *waste* to be buried and should be easy to fill.<br>d) Pit closure – contents should be covered with unslaked lime (CaO) at a rate of 85 kg per 1,000 kg of *aquatic animal* ~~waste~~ *waste* to hasten decomposition and prevent scavenging.<br>8. Pyre-burning<br>Pyre-burning may not be suitable for large amounts of *aquatic animal* ~~waste~~ *waste*.<br>[…]<br>b) Access – for equipment to construct the pyre and maintain the fire, for the delivery of fuel and *aquatic animal waste*.<br>[…] If the pyre-burning is carried out correctly, *aquatic animal* ~~wastes~~ *waste* will be destroyed within 48 hours. |

| Article | Page number | Proposed change |
|---|---|---|
| 4.7.8. | 75 | 1. Ensiling<br>Ensiling of *aquatic animal* ~~waste~~ *waste* in an organic acid such as formic acid is an effective method of inactivating most *pathogenic agents* within 48 hours. |
| 5.4.2. | 93 | […] The criteria for inclusion of *aquatic animal products* in point 1 of Article X.X.11. (mollusc disease-specific chapters), Article X.X.12. (amphibian, crustacean and fish disease-specific chapters) and Article 10.4.16. include consideration of the form and presentation of the product, the expected volume of ~~waste~~ *aquatic animal waste* ~~tissues~~generated by the consumer and the likely presence of viable *pathogenic agent* in the ~~waste~~ *aquatic animal waste*.<br>[…]<br>It is assumed that: (i) the *aquatic animal products* are used for human consumption only; (ii) ~~waste~~ *aquatic animal waste* may not always be handled in an appropriate manner that mitigates the introduction of the *pathogenic agent*; the level of risk is related to the ~~waste~~ *aquatic animal waste* disposal practices in each Member's country or territory; […] |
| 5.4.2. | 93 | **Criteria**<br>[…]<br>EITHER<br>2) it includes an amount of raw ~~waste~~ *aquatic animal waste* ~~tissues~~ generated by the consumer that is unlikely to result in the introduction and establishment of the *pathogenic agent*;<br>OR<br>3) the *pathogenic agent* is not normally found in the ~~waste~~ *aquatic animal waste* ~~tissues~~ generated by the consumer. |
| 6.5.3. | 129 | 3. Entry assessment<br>[…]<br>– data on trends and occurrence of resistant microorganisms obtained through *surveillance* of *aquatic animals* and *aquatic animal products* and ~~waste~~ *aquatic animal waste* ~~products~~.<br>4. Exposure assessment<br>[…]<br>– disposal practices for ~~waste~~ *aquatic animal waste* and the likelihood for human exposure to resistant microorganisms or resistance determinants through those ~~waste~~ *aquatic animal waste* ~~products~~;<br>[…] |

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**New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) – track changes**

CHAPTER 4.X.

# BIOSECURITY FOR AQUACULTURE ESTABLISHMENTS

**EU position**

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

Article 4.X.1.

**Purpose**

To provide recommendations on the development and implementation of *biosecurity* measures primarily to mitigate the *risk* of the introduction of specific *pathogenic agents* into *aquaculture establishments*, and if *pathogenic agents* are introduced, to mitigate the *risk* of further spread within, or release from, the *aquaculture establishment*.

Article 4.X.2.

**Scope**

*Biosecurity* principles are relevant to the application of the standards in the *Aquatic Code* at the level of a country, *zone*, *compartment* or *aquaculture establishment* ~~as appropriate~~. This chapter describes recommendations on *biosecurity* to be applied to *aquaculture establishments*, including semi-open, semi-closed and closed systems. The chapter describes general principles of *biosecurity* planning, categories of *aquaculture* production systems, ~~major transmission pathways,~~ area management, mitigation measures for transmission pathways, ~~the use of~~ the application of *risk analysis* and approaches for *biosecurity plan* development. ~~to develop a *biosecurity plan*, and the key components of a plan.~~

For further guidance on *disease* prevention and control refer to other chapters of Section 4 ~~of the *Aquatic Code*~~.

Article 4.X.3.

**Introduction**

~~The fundamental measures that underpin *aquatic animal disease* prevention at the level of country, *zone* or *compartment* is the application of *biosecurity*.~~ *Biosecurity* at the level of an *aquaculture establishment* is integral to effective *biosecurity* at the level of a country, *zone* or *compartment* and thus the optimal health status and welfare of *aquatic animal* populations. This chapter describes *biosecurity* principles designed to mitigate the *risks* associated with the introduction of *pathogenic agents* into, the spread within, or the release from *aquaculture establishments*. ~~The application of *biosecurity* at the level of an *aquaculture establishment* may be integral to effective *biosecurity* at the level of a country, *zone* or *compartment* to maintain the optimal health status of *aquatic animal* populations.~~

Given the unique challenges posed by varied *aquaculture* production systems and the vast diversity of farmed *aquatic animal* species, the development of *biosecurity plans* for *aquaculture establishments* requires the assessment of *disease risks* posed by specific *pathogenic agents* and their potential transmission pathways. A *biosecurity plan* describes management and physical ~~and management~~ measures to mitigate the identified *risks* according to the circumstances of the *aquaculture establishment*. *Aquaculture establishment* personnel ~~s~~Staff, ~~and~~ service providers and *aquatic animal health professionals* or *veterinarians* should be engaged in developing and implementing the *biosecurity plan* to ensure it is practical and effective.

The outcome achieved through the implementation of *biosecurity* at *aquaculture establishments* is improved health and welfare ~~status~~ of *aquatic animals* throughout the production cycle. The benefits may include improved market access, ~~and~~ increased productivity ~~directly~~ (through improved survival, growth rates and *feed* conversion), and ~~indirectly through~~ ~~the~~ a reduction in the use ~~in treatments~~ of veterinary medicinal products (including *antimicrobial agents*), thus leading to a reduction in ~~and associated~~ production costs and the rate of emergence of antimicrobial resistance ~~(AMR)~~.

Article 4.X.4.

**General principles**

*Biosecurity* is a set of physical and management measures which, when used together, cumulatively reduce the *risk* of *infection* in *aquatic animal* populations ~~at~~ within an *aquaculture establishment*. Planning and implementation of *biosecurity* within an *aquaculture establishment* requires ~~planning to~~ ~~identifying~~ identification of *risks* and ~~consider~~ ~~cost-effective~~ cost-effective measures to achieve the identified *biosecurity* objectives of the plan. The measures required will vary among ~~between~~ *aquaculture establishments*, depending on factors such as ~~risk~~ likelihood of exposure to *pathogenic agents*, the species of farmed *aquatic animal* ~~farmed species~~, the category of *aquaculture* production system, husbandry practices, environmental conditions and geographical location. ~~Although~~ ~~different~~ Different approaches may be used to achieve an identified *biosecurity* objective~~,~~; however, the general principles for developing and implementing a *biosecurity plan* are consistent and are described ~~as~~ below:

~~1) Planning is necessary to document the objectives of the biosecurity plan, the identified risks to be managed, the measures that will be put in place to manage the disease risks, required operating procedures and monitoring, as described in Articles 4.X.6. and 4.X.7.~~

~~2~~1) Potential pathways for *pathogenic agents* to be transmitted into, spread within and released from the *aquaculture establishment* must be identified, as described in Article~~s 4.X.5. and~~ 4.X.6.,~~and~~ giving consideration to the category of *aquaculture* production system and design of the *aquaculture establishment*.

~~3~~2) *Risk analysis* should be undertaken to identify and evaluate ~~*biosecurity*~~ *disease* threats and ensure that the plan addresses *risks* appropriately and efficiently. The *risk analysis* may range from a simple to a complex analysis depending on the objectives of the *biosecurity plan* ~~and~~, the circumstances of the *aquaculture establishment* and the *disease risks*, as described in Article 4.X.7.

~~4~~3) *Biosecurity* measures to address identified *disease risks* should be evaluated ~~based~~ on the basis of their potential effectiveness, initial and ongoing costs (e.g. building works, maintenance), and management requirements, as described in Article 4.X.7.

~~5~~4) Management practices should be integrated into the *aquaculture establishment's* operating procedures and ~~associated~~ relevant training ~~are is~~ provided to personnel, as described in ~~Article 4.X.7. and~~ Article 4.X.8.

~~5) Clear signage should be displayed to promote awareness and compliance with *biosecurity plan* measures by personnel, visitors and the public.~~

~~5~~65) Appropriate records and documentation are essential to demonstrate effective implementation of the *biosecurity plan*. Examples are ~~provided~~ described in Article 4.X.8.

~~6~~76) A ~~routine review~~ schedule for routine reviews and audits of the *biosecurity plan* should be described. ~~and identified t~~Triggers for ~~ad hoc~~ *ad hoc* review must be determined (e.g. *outbreaks* of *disease*, and changes to infrastructure, production techniques, ~~*disease outbreaks,*~~ or *risk* profiles). Third party audits may be required where recognition of the *biosecurity* measures is required by customers, or regulators, or for market access, as ~~described~~ ~~provided~~ described in Article 4.X.8.

Article 4.X.5.

**Categories of aquaculture production systems**

~~*Aquatic animals* can be produced in f~~Four different categories of *aquaculture* production systems~~, which~~ are defined based on the capacity to treat water entering and exiting the system, and the level of control ~~of~~ over *aquatic animals* and *vectors*. These ~~measures~~ factors need to be considered in *biosecurity* planning.

Open systems

In an open ~~Open~~ *aquaculture* production system~~s,~~ it is not possible to have ~~no~~ control of the water, environmental conditions, ~~and~~ animals ~~and~~ or *vectors*. These production systems may include stock enhancement of wild populations with *aquatic animals* originating from *aquaculture establishments* or from the wild. As these systems cannot be considered '*aquaculture establishments'*, they are not considered further in this chapter. However, movements of *aquatic animals* between *aquaculture establishments* ~~to~~ and open systems should ~~still~~ be ~~subject to~~ assessed to determine the need for *disease* mitigation measures.

Semi-open systems

In a semi-open *aquaculture* production system, it is not possible to have control over the water entering or exiting the system, or ~~of~~ over the environmental conditions. Some *aquatic animals* and *vectors* may also enter and exit the system. Examples of semi-open *aquaculture* production systems are net pens or cages for finfish and suspended baskets or rope systems for molluscs ~~aquaculture~~ in natural water bodies ~~and mollusc *aquaculture*, either suspended in the water column or on the ocean floor~~.

Semi-closed systems

In a semi-closed *aquaculture* production system, there is some control ~~of~~ over the water entering and exiting the system and ~~of~~ over the environmental conditions. *Aquatic animals* and *vectors* ~~may~~ can be prevented from entering and exiting the system; however, there is limited control to prevent the entry or exit of *pathogenic agents*. Examples of semi-closed *aquaculture* production systems are ponds, raceways, ~~enclosed~~ floating enclosures ~~pens~~, and flow-through tanks.

Closed systems

In a closed *aquaculture* production system, ~~the~~ there is sufficient control ~~of~~ over water entering and exiting the system ~~can~~ to exclude *aquatic animals*, *vectors* and *pathogenic agents*. Environmental conditions can also be controlled. Examples of closed *aquaculture* systems include recirculating *aquaculture* production systems, production systems with a safe water supply free from *pathogenic agents* or *aquatic animals* (e.g. ground water), or those with high levels of treatment (and redundancy) of water entering ~~or~~ and exiting the system.

Article 4.X.5. bis

**Area management**

It may not be possible to control the transmission of *pathogenic agents* among semi-open or semi-closed *aquaculture establishments* that are in close proximity within shared water bodies. In these circumstances, a consistent set of *biosecurity* measures should be applied by all of the *aquaculture establishments* considered to be epidemiologically linked. Area management agreements can formalise the coordination of common *biosecurity* measures among all of the epidemiologically linked *aquaculture establishments*.

Article 4.X.6.

**Transmission pathways~~, and associated risks~~ and mitigation measures**

*Pathogenic agents* can move into, spread within, and be released from *aquaculture establishments* via various transmission pathways. The identification of all potential transmission pathways is essential for the development of an effective *biosecurity plan*. ~~Mitigation of p~~Pathways that are likely to result in transmission of specific ~~may expose susceptible *aquatic animals* to high loads of~~ *pathogenic agents* should be prioritised for mitigation.

The *risks* associated with the introduction into, spread within, and release of *pathogenic agents* from the *aquaculture establishment* need to be considered for each of the following transmission pathways.

1. Aquatic animals

   Movement of *aquatic animals* into, within and from *aquaculture establishments*, either intentionally or unintentionally, ~~may usually~~ may pose ~~pose has~~ a high likelihood ~~*risk*~~ of transmitting *pathogenic agents* ~~transmission~~. This is particularly the case when clinically and sub-clinically infected *aquatic animals*, or *aquatic animals* with unknown health status are moved into a susceptible population.

   *Aquatic animals* intentionally ~~brought~~ introduced into, or moved within, an *aquaculture establishment*~~, or moved within it~~, may include broodstock, larvae, juvenile stock for on-growing, and genetic material such as *eggs* and milt. Both horizontal and vertical transmission mechanisms of *pathogenic agents* should be considered for *aquatic animals*. The *risk* of transmitting *pathogenic agents* via *aquatic animals* should be managed~~; possible mitigation measures include the~~ giving consideration to the following mitigation measures ~~can be managed by~~:

   a) Only ~~introducing~~ introduce into the *aquaculture establishment* *aquatic animals* with a known health status ~~into the *aquaculture establishment* with known health status~~, which is of equal or higher status than the existing animals in the establishment.

b) ~~Quarantining~~ ~~Placing introduced~~ If *aquatic animals* of unknown *disease* status are introduced, they should be placed into *quarantine* ~~from other farm populations in separate production units or dedicated *quarantine* facilities~~.

c) Where appropriate, ~~treating~~ treat ~~treatment of~~ *quarantined aquatic animals* to mitigate *disease risks* (for example, treatment for external parasites).

d) ~~Ensuring~~ Ensure biosecure transport of *aquatic animals* that avoids exposure to and release of *pathogenic agents.*

e) Only ~~moving~~ move *aquatic animals* between different populations within the establishment following consideration of the *disease risks* and with a view to maintaining the highest possible health status of the *aquatic animal* population.

f) ~~Isolating~~ ~~Isolate~~ Where possible, isolate *aquatic animal* populations that display clinical signs of *disease* from other populations until the cause is known and the situation is resolved.

g) ~~Removing~~ Remove ~~sick~~ moribund or dead *aquatic animals* from production units as soon as possible and ~~disposing~~ dispose of them in a biosecure manner in accordance with Chapter 4.7.

h) ~~Reporting of~~ Report unexplained or unusual mortalities, or suspicion of a notifiable *disease* or an *emerging disease* in *aquatic animals* to the *Competent Authority* in accordance with local requirements. Investigation and *diagnosis* of the cause of mortality should be undertaken by *aquatic animal health professionals* or *veterinarians*.

i) If possible, completely remove *aquatic animals* from all or parts of ~~totally~~ ~~depopulating~~ ~~depopulate~~ the *aquaculture establishment* at intervals, for instance between *aquatic animal* generations or production cycles, followed by cleaning, ~~and~~ *disinfection* and drying of production installations. Sites should be fallowed for a period sufficient to interrupt *infection* cycles and reduce or eliminate pathogen challenge to restocked *aquatic animals*. *Fallowing* should be coordinated for *aquaculture establishments* that are epidemiologically linked through shared water bodies.

j) ~~Where possible, preventing unintended movement of *aquatic animals* into, within or from the establishment.~~ ~~Considering~~ Consider physical measures to minimise the likelihood of escape of farmed *aquatic animals* or the entry of wild *aquatic animals* into the *aquaculture establishment*. The likelihood of entry or escape of *aquatic animals* will be higher for semi-open than for closed or semi-closed systems.

~~The *risk* of unintentional movements of *aquatic animals* will be influenced by the category of *aquaculture* production system, with the likelihood being higher for semi-open than closed systems. If *risks* are found to be high, physical mitigation measures may be necessary~~.

2. Aquatic animal products and aquatic animal waste

*Aquatic animal products* may also be brought into, moved within ~~and~~ or moved out of ~~an~~ *aquaculture establishments*~~or moved within it~~; for example, *aquatic animal products* derived from *aquatic animals* harvested at other sites. *Aquatic animal* ~~waste~~ *waste* may ~~include the~~ be generated ~~entire body or parts of~~ when *aquatic animals* ~~that~~ have died or been killed for *disease* control purposes, ~~as~~ or when they ~~through~~ killing and processing of ~~*aquatic animals*~~ have been killed and processed ~~and their parts, that are not intended~~ for human consumption or other purposes.

Movement of *aquatic animal products* and *aquatic animal* ~~waste~~ *waste* into, within ~~and out of~~ or from *aquaculture establishments* may pose a *risk* of *pathogenic agent* transmission. This is particularly the case when a susceptible population is exposed to *aquatic animal products* and *aquatic animal* ~~waste~~ *waste* derived from clinically or sub-clinically infected *aquatic animals*. ~~High *risk* waste includes *aquatic animal* waste *waste* that constitutes, or is suspected of constituting, a high significant health *risk* to *aquatic animals*.~~ Movement of *aquatic animal waste* into *aquaculture establishments* should be avoided ~~where possible~~. *Aquatic animal waste* should be stored, transported, disposed of and treated as ~~following the guidance~~ described in Chapter 4.7. ~~Handling, disposal and treatment of *aquatic animal waste*~~.

For intentional movements of *aquatic animal products* and *aquatic animal* ~~waste~~ *waste*, the likelihood of presence of *pathogenic agents* in the *aquatic animals* from which ~~products~~ *aquatic animal products* and *aquatic animal waste* are derived should be evaluated giving consideration to the species, source, and health status.

The *risk* of transmitting *pathogenic agents* via *aquatic animal products* and *aquatic animal* ~~waste~~ *waste* should be assessed and managed~~; possible mitigation measures include the~~ giving consideration to the following mitigation measures ~~can be managed by~~:

a) ~~determining~~ Determine the potential *disease risk* of *aquatic animal products* and *aquatic animal waste* to *aquatic animals* in the establishment and the environment;

b) ~~Manage~~ Manage *aquatic animal products* and *aquatic animal waste* in areas within the *aquaculture establishment* that are isolated ~~isolating areas within the *aquaculture establishment* where *aquatic animal products* and *aquatic animal* waste *waste* are managed~~ from *aquatic animal* populations to minimise identified *disease* transmission *risks*;

c) ~~ensuring~~ Ensure procedures ~~systems~~ are implemented for appropriate collection, treatment (inactivating *pathogenic agents*), transport, storage or disposal of *aquatic animal products* and *aquatic animal waste* ~~waste~~ to minimise identified *disease* transmission *risks* ~~the *risks* of transmitting *pathogenic agents*~~.

3. Water

Water ~~is an important asset that supports productivity and *aquatic animal* health but~~ may present a *risk* of the introduction of *pathogenic agents* into, spread within, and release from *aquaculture establishments.* The source of the water, and how it may provide~~s~~ an epidemiological link between the *aquaculture establishment* and other farmed or wild populations or processing plants, should be identified and considered. Exposure to transport water and ballast water should be considered.

The *risk* of the *aquaculture establishment* being exposed to water containing *pathogenic agents* may be influenced by the category of *aquaculture* production system, the likelihood being higher for semi-open than for semi-closed and closed systems. Any water that is flowing from *aquatic animals* with lower or unknown health status presents a potential *risk* of transmitting *pathogenic agents* to *aquatic animals* of a higher health status.

The *risk* of transmitting *pathogenic agents* via water should be assessed, and managed~~; possible mitigation measures include the~~ giving consideration to the following mitigation measures ~~can be managed by~~:

a) Where possible, ~~choosing~~ choose a water source that ~~are~~ is entirely free of susceptible *aquatic animal* populations and *pathogenic agents* of concern. Such water sources may include saline or fresh groundwater, de-chlorinated municipal water, and artificial seawater. These water sources may be particularly suitable for ~~high health status~~ *aquatic animals* with high health status, such as broodstock.

b) ~~Providing~~ Provide an appropriate level of screening, filtration or *disinfection* (in accordance with Chapter 4.3.) of water from sources that are likely to contain *susceptible species* and which may present a *risk* of *pathogenic agent* transmission (e.g. oceans, streams or lakes). The type and level of treatment required will depend on the identified *risks*.

c) Provide an appropriate level of filtration and *disinfection* ~~or holding~~ (in accordance with Chapter 4.3.) of effluent water (and associated filtered waste) from *aquaculture establishments* (or associated slaughterhouses or processing facilities) where it may present a *risk* of *pathogenic agent* transmission to wild *aquatic animals* or other *aquaculture establishments* with *susceptible species*. The type and level of treatment required will depend on the identified *risks*.

~~c~~d) ~~Ensuring~~ Ensure the position of water intakes and outlets for semi-closed and closed *aquaculture establishments*, and the location of semi-open *aquaculture establishments*, minimises contamination from other farmed or wild populations or processing plants, taking into account factors such as distance and water currents.

e) The likelihood of ingress of contaminated water either through flooding from external sources or from defective infrastructure (e.g. leaking pipes, blocked drains, bund wall failure) should be assessed and appropriate management or infrastructure measures applied.

f) Assess the *risk* and establish procedures to treat and dispose of *waste* water resulting from the transport of *aquatic animals*.

4. Feed

*Feed* can be an important pathway for transmission of *pathogenic agents* to *aquatic animals*. *Feed* manufactured from infected *aquatic animals* may contain *pathogenic agents*, or become contaminated during harvest, transport, storage or processing. ~~*Feed* may be initially infected with contain *pathogenic agents* or become contaminated during harvest, transport, storage and processing of *commodities* used as *feed ingredients*.~~ Poor hygiene may contribute to contamination during manufacture, transport, storage and use of *feed*.

In closed or semi-closed production systems there can be a high level ~~on~~ of control of *aquatic animal feed~~s~~*. However, in semi-open production systems, *aquatic animals* may obtain food from their environment (e.g. filter-feeding molluscs or predation of wild fish ~~which may be preyed on predated~~ by farmed fish in net pens or cages). The *risk* of *disease* transmission from *feed* to the environment also needs to be managed.

The *risk* of transmitting *pathogenic agents* via *aquatic animal feed* ~~can~~ should be assessed, and managed by mitigation measures as ~~described provided~~ described in Chapter 4.8., for example using *feed* and *feed ingredients* that:

a) have undergone sufficient processing to inactivate *pathogenic agents* of concern;

b) are from sources that are declared free from the *pathogenic agents* of concern or have been confirmed (e.g. by testing) that *pathogenic agents* are not present in the *feed* or *feed ingredients* ~~*commodity*~~;

c) have been processed, manufactured, stored, ~~and~~ transported and delivered during feeding to *aquatic animals* in a manner to prevent contamination by *pathogenic agents*.

5. Fomites

Equipment, *vehicles*, packaging material, clothing, footwear, sediments, infrastructure and other fomites can mechanically transfer *pathogenic agents* into, within and from an *aquaculture establishment*.

The ~~level of *risk*~~ likelihood of transferring *pathogenic agents* will depend on the stability of the *pathogenic agent* in the environment, the presence and nature of organic matter on the fomite surface, as well as the type of surface and its ~~ability~~ capacity to hold water. The ~~*risk*~~ likelihood of transferring *pathogenic agents* may be higher for fomites which are difficult to clean and disinfect. Sharing equipment~~Equipment that is shared~~ between *aquaculture establishments,* ~~between *aquaculture establishments* and processing facilities~~, or between different production units ~~with~~ within an *aquaculture establishment*, or between *aquaculture establishments* and processing facilities, ~~with unequal health status,~~ may result in the spread of *pathogenic agents* ~~present a higher *risk* than compared to new or dedicated equipment.~~ The ~~risk~~ *risk* ~~likelihood~~ of transmitting *pathogenic agents* via fomites should be assessed and managed~~; possible mitigation measures include the~~ giving consideration to the following mitigation measures ~~can be managed by~~:

a) ~~Assessing~~ Assess the *disease risk* associated with any fomites ~~brought~~ moved into, within or from the *aquaculture establishment* ~~for their *disease risk*~~.

b) ~~Ensuring~~ Ensure procedures and infrastructure are in place to clean and disinfect fomites, including at designated delivery and loading areas, prior to entry into the *aquaculture establishment*. Recommendations for the cleaning and ~~disinfection~~ *disinfection* of fomites are described in Chapter 4.3.

~~c) Assigning dedicated equipment for use in production units of different health status. Where equipment must be used in multiple production units it should be cleaned and disinfected prior to movement between units.~~

c) ~~Wherever possible, dedicating~~ Dedicate~~,~~ ~~where possible~~, items that are difficult to disinfect, or those with a high likelihood of contamination, to a specific *aquaculture establishment* or to areas within an establishment ~~rather than~~ instead of moving them ~~between *aquaculture establishments*~~ after *disinfection*.

d) ~~Applying~~ Apply the mitigation measures described at points a) to c) above to the movement of fomites between production units within an *aquaculture establishment* with the measures determined based on an evaluation of the *risk* of *disease* transmission ~~*disease risks*~~.

6. Vectors

*Vectors* can ~~transport~~ ~~transfer~~ transmit *pathogenic agents* to susceptible *aquatic animals* in *aquaculture establishments*. ~~These~~ They may include ~~wild~~ *aquatic animals* entering via the water supply, predators, wild birds, ~~and~~ scavengers, and pest animals such as rodents~~, and people~~. *Vectors* can also ~~transfer~~ transmit *pathogenic agents* ~~into,~~ within and from an *aquaculture establishment*~~, either by mechanical transfer or as a developmental stage of the *pathogenic agent* within the *vector*~~. ~~The *risk* of unintentional exposure to *vectors* will be influenced by the category of *aquaculture* production system.~~

The ~~*risk*~~ likelihood of ~~transferring~~ transmitting *pathogenic agents* via *vectors* varies with the type of *vector* ~~species~~, the nature of the *pathogenic agent*, the category of *aquaculture* production system, and the level of *biosecurity*. ~~Measures identified to mitigate *risks* associated with *aquatic animals,* as described in point 1, can also be applied to mitigate *risks* associated with *vectors*. Mitigation measures for other *vectors* include:~~

The *risk* of transmitting *pathogenic agents* via *vectors* should be assessed~~.~~ and managed giving consideration to the following mitigation measures:

a) ~~netting (to prevent access by birds);~~ Physical mitigation measures ~~should be used~~ to prevent the access of *vectors* to *aquaculture establishments* ~~including~~ may include:

   i) filtering or screening of water entering and exiting semi-closed and closed *aquaculture* production systems to prevent entry of wild *aquatic animals*;

   ii) surrounding land-based *aquaculture* production systems by a fence or a wall to prevent entry of animals and people, with a gate for controlled access for authorized personnel and visitors;

   iii) surrounding floating *aquaculture* production systems by barriers on the establishment perimeter to prevent contact with or entry of wild *aquatic animals* and other animals;

   iv) covering ~~outdoor~~ outdoor or unenclosed *aquaculture* production systems with nets to prevent access by birds.

~~b) barriers on the establishment perimeter to prevent entry by of other animals (e.g. electric fencing);~~

~~b) Controlling Access of personnel to *aquaculture establishments* should be controlled by creating a defined border between the outer risk area and the inner biosecure area comprising facilities for:~~

   ~~i) changing of clothes and shoes, or use of disposal coverings (hoods, coats, shoe coverings);~~

   ~~ii) *disinfection* of hands, and the use of foot baths for shoe disinfection.~~

~~c~~b) Ppest control. ~~and secure storage of feed and mortalities~~

7. Personnel and visitors

a) Access of personnel and visitors to *aquaculture establishments* should be controlled by creating a defined border between the outer *risk* area and the inner biosecure area comprising facilities for:

   i) completion of a register, which should include visitors' names, contact information, and details of exposure to *aquatic animals* or *pathogenic agents* over a preceding period, including visits to other *aquaculture establishments* or other facilities;

   ii) changing of clothes and shoes, or use of disposable coverings (e.g. hoods, coats, gloves, shoe coverings);

   iii) *disinfection* of hands, and the use of foot baths ~~for shoe disinfection~~.

b) All visitors should be briefed and supervised to ensure compliance with the *biosecurity plan*.

c) Clear signage should be displayed to promote awareness and compliance with *biosecurity plan* measures by personnel, visitors and the public.

**Risk analysis**

*Risk analysis* is an accepted approach for evaluating *biosecurity* threats and is used to support the development of mitigation measures. A formal *risk analysis* has four components: *hazard* identification, *risk assessment*, *risk management* and *risk communication* ~~(see Chapter 2.1.)~~. This article elaborates the principles described in Chapter 2.1. and applies them ~~for~~ to guide the development of *biosecurity plans* for *aquaculture establishments*.

A *biosecurity plan* may not necessarily require a comprehensive *risk analysis* to evaluate *disease risks* linked to transmission pathways. The chosen approach may depend on the objectives of the *biosecurity plan*, the level of *biosecurity* that is appropriate for the specific production requirements of the *aquaculture establishment*, the complexity of the threats to be addressed, and the availability of information and resources. Depending on these circumstances, a partial analysis may be appropriate, and can build on previous experiences to identify the *hazards* associated with relevant transmission pathways.

The three formal steps of the *risk analysis* process to underpin a ~~the~~ *biosecurity plan* are:

**Step 1 – Hazard ~~i~~identification**

*Hazard* identification determines which *pathogenic agents* should be the subject of the *risk assessment*. A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents*. This step includes identifying and collecting relevant information on the *pathogenic agents* that have ~~a~~ potential to cause *diseases* in *aquatic animal* populations within an *aquaculture establishment*. This process must consider the *aquatic animal health status* of the establishment and, for semi-open and semi-closed *aquaculture* production systems, the *aquatic animal health status* of the epidemiologically linked environments. ~~The following step is to identify both known and *emerging diseases*, not present in the *aquaculture establishment*, which may negatively impact the farmed population.~~ Known and *emerging diseases* which could negatively impact the farmed population should be identified, regardless of whether they are present in the *aquaculture establishment*.

To complete the next steps of the *risk assessment*, ~~required~~ information on the identified *hazards* is required ~~needed~~ and includes: i) the frequency of occurrence, ii) the biophysical characteristics, iii) the likelihood of detection if present and iv) the possible transmission pathways (described in Article 4.X.6.). Many of the *hazards* will share the same pathways. ~~A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents*.~~

**Step 2 – Risk ~~A~~assessment**

A *risk assessment* can be initiated once it has been identified that a ~~biological~~ *hazard* exists, and the required information listed under step 1 has been gathered. The aim of the *risk assessment* is to establish a *risk* estimate, which is the product of the likelihood and consequences of entry of a *pathogenic agent* ~~entry~~ into, spread within or release from the *aquaculture establishment*.

A *risk assessment* can be quantitative or qualitative. Both methods require the same conceptual pathway which identifies the necessary steps for *hazard* introduction, establishment and spread to be constructed. In a qualitative assessment, introduction and establishment are estimated using descriptors of likelihood. A quantitative assessment requires data on which to estimate likelihood. In most circumstances, the likelihood of *disease* transmission and associated consequences ~~pathways~~ will be assessed qualitatively but within a formal *risk assessment* framework. Examples of descriptors for qualitative estimates of likelihood and consequence are given in Tables 1 and 2. Table 3 illustrates how estimates of likelihood and consequence can be combined in a matrix to give an estimate of *risk*. Table 4 provides an interpretation of *risk* estimates.

**Table 1. Qualitative descriptors of likelihood**

| Estimate | Descriptor |
|---|---|
| Remote | ~~Never heard of~~ Very unlikely, but not impossible. |
| Unlikely | May occur ~~here~~, but only in rare circumstances. |
| Possible | Clear evidence to suggest this is possible in this situation. |
| Likely | It is likely, but not certain, to occur ~~here~~. |
| Certain | It is certain to occur. |

**Table 2. Qualitative descriptors of consequences**

| **Estimate** | **Descriptor of consequences at level of the aquaculture establishment** |
|---|---|
| Insignificant | Impact not detectable or minimal. No trade impacts. |
| Minor | ~~Impact~~ Limited decreased production ~~on *aquaculture establishment* productivity limited to some~~ affecting only a small number of ~~production~~ units or short-term, and/or very limited and transitory disruption to trade. ~~only.~~ |
| Moderate | ~~Widespread impact on *aquaculture establishment* productivity due to increased mortality or decreased performance.~~ Decreased production (e.g. sustained increased mortality or decreased growth rate) and/or some short-term to medium-term disruption to trade, resulting in financial loss. |
| Major | Considerable, decreased ~~impact on *aquaculture establishment*~~ production, and/or some medium-term to long-term disruption to trade, resulting in significant financial loss ~~resulting in serious supply constraints and financial impact~~. |
| Catastrophic | Complete ~~depopulation~~ production loss, ~~in~~ of the ~~*aquaculture establishment*~~ and possibly barriers to resumption of production, and/or complete loss of trade, resulting in extreme financial loss. |

**Table 3. Matrix for ~~assessing~~ estimating risk**

| **Likelihood estimate** | Consequence rating | | | | |
|---|---|---|---|---|---|
| | insignificant | minor | moderate | major | catastrophic |
| remote | negligible | low | low | low | medium |
| unlikely | low | low | medium | medium | high |
| possible | low | medium | medium | high | high |
| likely | low | medium | high | high | extreme |
| certain | ~~medium~~ low | high | high | extreme | extreme |

~~Results of~~ *~~r~~Risk assessments* inform~~s~~ which ~~biological~~ *hazards* need to be addressed, which critical control points on the transmission pathway should be targeted for management, and the measures ~~which~~ that are most likely to be effective in reducing *risk*.

**Table 4. Interpretation of risk estimates**

| ***Risk*** ~~**level**~~ **estimate*** | **Explanation and management response** |
|---|---|
| Negligible | Acceptable level of *risk*. No action required. |
| Low | Acceptable level of *risk*. On-going monitoring may be required. |
| Medium | Unacceptable level of *risk*. ~~Active management~~ Review and strengthen the *risk* mitigation measures ~~is required to reduce the level of *risk*.~~ |
| High | Unacceptable level of *risk*. ~~Intervention~~ Identify and implement additional *risk* mitigation measures ~~is required to mitigate the *risk*.~~ |
| Extreme | Unacceptable level of *risk*. Take immediate action to mitigate the *risk*. ~~Urgent intervention is required to mitigate the level of *risk*.~~ |

*~~The~~ ~~*R*risk~~ ~~level~~ ~~estimate~~ ~~is~~ ~~determined by~~ ~~from~~ ~~a~~ ~~combination of~~ ~~the~~ ~~likelihood and consequence~~ ~~estimates~~ ~~score obtained using the *risk* matrix (Table 3)~~. Likelihood and consequence estimates are combined using the *risk* matrix (Table 3) to produce the *risk* estimate.

**Step 3 – Risk ~~M~~management**

*Risk management* is used to determine the appropriate management response for the assessed level of *risk* as described in Table 4. The *risk assessment* process identifies the steps within transmission pathways necessary for a *risk* to be realised and thus allows the most effective mitigation measures to be determined. Many of the *hazards* will share the same pathways and ~~thus~~ therefore mitigation measures may be effective against more than one *hazard*. Information on *hazards* and their pathways of introduction (step 1) should be combined with an assessment of *risk* associated with each ~~the assessment of the~~ pathway~~s~~ (step 2) to identify the most appropriate and cost-effective *risk* mitigation measures.

Article X.X.6. describes some possible mitigation measures relevant ~~for~~ to different transmission pathways. The most appropriate mitigation measures for a specific *aquaculture establishment* will depend on ~~the *risks* *hazards* identified,~~ the effectiveness and reliability of the mitigation measure, the category of *aquaculture* production system and cost.

After the implementation of the *biosecurity plan*, *hazards* should be regularly reassessed, and measures adjusted according to any changed *risk* estimates.

Article 4.X.8.

**Biosecurity plan development**

The purpose of a *biosecurity plan* is primarily to reduce the *risk* of introducing *pathogenic agents* into an *aquaculture establishment*, and if *pathogenic agents* are introduced, to reduce the *risk* of further spread within or release from the *aquaculture establishment*. The plan will document identified transmission pathways and the outputs of any *risk analysis* performed (*hazards, risk* estimate and mitigation measures), and information relevant to ongoing implementation, monitoring and review of the plan.

1. Development of a biosecurity plan

   The process ~~to~~ of developing a *biosecurity plan* will vary depending on its objectives ~~of the *biosecurity plan*~~, the level of *biosecurity* appropriate to the specific production system requirements, the complexity of the *disease risks* to be addressed, and availability of information and resources. Consideration and documentation of the following issues are recommended:

   a) objectives, scope and regulatory requirements for the *biosecurity plan*;

   b) information about the *aquaculture establishment* including an up-to-date plan of the layout of buildings and production units (including *epidemiological units*, if any, and structures and ~~the~~ processes to maintain separation~~methods~~), loading/unloading, unpacking, processing, *feed* storage, ~~waste~~ *aquatic animal waste* storage, reception areas, access points and maps showing major movements of *aquatic animals*, *aquatic animal products* and *aquatic animal* ~~waste~~ *waste*, water, *feed* and fomites ~~(including staff, equipment and *vehicles*)~~;

   c) the potential pathways for entry of *pathogenic agents* into, spread within or release from the *aquaculture establishment* (refer to Article X.X.6. above);

   d) a *risk analysis*, including identification of the major *disease hazards* to the *aquaculture establishment* (refer to Article X.X.7. above);

   e) the mitigation measures that have been determined to address ~~identified~~ *risks*;

   f) emergency procedures in the event of a *biosecurity* failure. These ~~They~~ may include reporting requirements, and emergency measures to eradicate *pathogenic agents* such as *aquatic animal* depopulation and disposal, and site *disinfection*, in accordance with Chapters 4.3. and 7.4.;

   ~~g) standard operating procedures required to support implementation of the mitigation measures, emergency procedures and the training requirements of personnel;~~

   ~~h~~g) internal and external communication procedures, ~~and~~ roles and responsibilities of ~~personnel~~ *aquaculture establishment* personnel ~~staff~~ and essential contact information, e.g. for ~~personnel,staff~~ personnel, *aquatic animal health professionals* or *veterinarians* ~~farm *veterinarian*~~ and the *Competent Authority*;

   ~~i~~h) monitoring and audit schedule;

   ~~j~~i) performance evaluation;

   j) standard operating procedures required to support ~~all~~ implementation of the mitigation measures described by the *biosecurity plan*, emergency procedures and the training requirements of establishment personnel.

2. Key components of a biosecurity plan

a) Standard operating procedures (SOPs)

SOPs describe routine management processes that must be performed to support the effectiveness of the *biosecurity plan*. Each SOP should clearly describe its objectives, ~~staff~~ personnel responsibilities, the procedure (including record keeping), precautions and a review date.

~~Staff Personnel should be trained in the application of the SOPs including completion of forms, checklists and other records associated with each procedure, as well as routine communication requirements.~~

b) Training of personnel

Personnel should be trained in the application of the SOPs including completion of forms, checklists and other records associated with each procedure, as well as routine communication requirements.

The *biosecurity plan* should include a training programme to ensure that all personnel are capable of playing their role in the implementation of *biosecurity* at the *aquaculture establishment*.

~~b~~c) Documentation and record keeping

The *biosecurity plan* describes the documentation necessary to provide evidence of compliance with the ~~mitigation measures~~ plan. The level of detail required in the documentation depends on the outcomes of the transmission pathway assessment.

Examples of documentation required ~~may~~ include: *aquaculture establishment* layout, movements of *aquatic animals*, ~~escapees,~~ origin and destination and health status of the *aquatic animals* introduced to the *aquaculture establishment*, *quarantine* measures, records of visitors to the establishment, escapees, stocking densities, feeding and growth rates, records of ~~staff~~ personnel training, treatments/vaccination, water quality, cleaning and *disinfection* events, morbidity and mortality (including removal and disposal of mortalities), *surveillance* and laboratory records.

~~c~~d) Emergency procedures

Procedures should be developed and, when necessary, implemented to minimise the impact of emergencies, *disease* events, or unexplained mortality in *aquatic animals*. These procedures should include clearly defined thresholds that help to identify an emergency incident and activate response protocols, including reporting requirements.

~~d~~e) Health monitoring

Health monitoring as part of the *biosecurity plan* involves monitoring of the health status of *aquatic animals* in *aquaculture establishments*. Monitoring should be performed at a production unit and establishment level. Activities may include *disease surveillance*, routine monitoring of stock for important health and production parameters (e.g. by personnel ~~staff~~, an *aquatic animal health professional* or a *veterinarian*), recording of clinical signs of *disease*, morbidity and mortality, laboratory test results and analysis of these data (e.g. calculation of rates of morbidity and mortality ~~and diseases~~).

~~e~~f) Routine review and auditing

The *biosecurity plan* should describe a systematic auditing schedule to verify implementation and compliance with the requirements of the *biosecurity plan*. Routine revision of the *biosecurity plan* is necessary to ensure that it continues to effectively address *biosecurity risks*.

The *biosecurity plan* should also be reviewed at least annually or in response to changes to the *aquaculture establishment* operations, changes in facility design, changes ~~to~~ in husbandry approaches, identification of a new *disease risk*, or the occurrence of a *biosecurity* incident. *Biosecurity* incidents, and actions taken to remedy them, should be documented to enable ~~SOP~~ re-assessments of SOPs.

~~f) Training of personnel~~

~~The *biosecurity plan* should include a training programme to ensure that all personnel are capable of playing their role in the implementation of *biosecurity* at the *aquaculture establishment*.~~

**New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) – clean version**

CHAPTER 4.X.

# BIOSECURITY FOR AQUACULTURE ESTABLISHMENTS

Article 4.X.1.

**Purpose**

To provide recommendations on the development and implementation of *biosecurity* measures primarily to mitigate the *risk* of the introduction of specific *pathogenic agents* into *aquaculture establishments*, and if *pathogenic agents* are introduced, to mitigate the *risk* of further spread within, or release from, the *aquaculture establishment*.

Article 4.X.2.

**Scope**

*Biosecurity* principles are relevant to the application of the standards in the *Aquatic Code* at the level of a country, *zone*, *compartment* or *aquaculture establishment*. This chapter describes recommendations on *biosecurity* to be applied to *aquaculture establishments*, including semi-open, semi-closed and closed systems. The chapter describes general principles of *biosecurity* planning, categories of *aquaculture* production systems, area management, mitigation measures for transmission pathways, the application of *risk analysis* and approaches for *biosecurity plan* development.

For further guidance on *disease* prevention and control refer to other chapters of Section 4.

Article 4.X.3.

**Introduction**

*Biosecurity* at the level of an *aquaculture establishment* is integral to effective *biosecurity* at the level of a country, *zone* or *compartment* and thus the optimal health status and welfare of *aquatic animal* populations. This chapter describes *biosecurity* principles designed to mitigate the *risks* associated with the introduction of *pathogenic agents* into, the spread within, or the release from *aquaculture establishments.*

Given the unique challenges posed by varied *aquaculture* production systems and the vast diversity of farmed *aquatic animal* species, the development of *biosecurity plans* for *aquaculture establishments* requires the assessment of *disease risks* posed by specific *pathogenic agents* and their potential transmission pathways. A *biosecurity plan* describes management and physical measures to mitigate the identified *risks* according to the circumstances of the *aquaculture establishment*. *Aquaculture establishment* personnel, service providers and *aquatic animal health professionals* or *veterinarians* should be engaged in developing and implementing the *biosecurity plan* to ensure it is practical and effective.

The outcome achieved through the implementation of *biosecurity* at *aquaculture establishments* is improved health and welfare of *aquatic animals* throughout the production cycle. The benefits may include improved market access, increased productivity (through improved survival, growth rates and *feed* conversion), and a reduction in the use of veterinary medicinal products (including *antimicrobial agents*), thus leading to a reduction in production costs and the rate of emergence of antimicrobial resistance.

Article 4.X.4.

**General principles**

*Biosecurity* is a set of physical and management measures which, when used together, cumulatively reduce the *risk* of *infection* in *aquatic animal* populations within an *aquaculture establishment*. Planning and implementation of *biosecurity* within an *aquaculture establishment* requires identification of *risks* and cost-effective measures to achieve the identified *biosecurity* objectives of the plan. The measures required will vary among *aquaculture establishments*, depending on factors such as likelihood of exposure to *pathogenic agents*, the species of farmed

*aquatic animal*, the category of *aquaculture* production system, husbandry practices, environmental conditions and
geographical location. Different approaches may be used to achieve an identified *biosecurity* objective; however, the general principles for developing and implementing a *biosecurity plan* are consistent and are described below:

1) Potential pathways for *pathogenic agents* to be transmitted into, spread within and released from the *aquaculture establishment* must be identified, as described in Article 4.X.6., giving consideration to the category of *aquaculture* production system and design of the *aquaculture establishment*.

2) *Risk analysis* should be undertaken to identify and evaluate *disease* threats and ensure that the plan addresses *risks* appropriately and efficiently. The *risk analysis* may range from a simple to a complex analysis depending on the objectives of the *biosecurity plan*, the circumstances of the *aquaculture establishment* and the *disease risks*, as described in Article 4.X.7.

3) *Biosecurity* measures to address identified *disease risks* should be evaluated on the basis of their potential effectiveness, initial and ongoing costs (e.g. building works, maintenance), and management requirements, as described in Article 4.X.7.

4) Management practices should be integrated into the *aquaculture establishment's* operating procedures and relevant training provided to personnel, as described in Article 4.X.8.

5) Appropriate records and documentation are essential to demonstrate effective implementation of the *biosecurity plan*. Examples are described in Article 4.X.8.

6) A schedule for routine reviews and audits of the *biosecurity plan* should be described. Triggers for *ad hoc* review must be determined (e.g. *outbreaks* of *disease*, and changes to infrastructure, production techniques, or *risk* profiles). Third party audits may be required where recognition of the *biosecurity* measures is required by customers, or regulators, or for market access, as described in Article 4.X.8.

Article 4.X.5.

**Categories of aquaculture production systems**

Four different categories of *aquaculture* production systems are defined based on the capacity to treat water entering and exiting the system, and the level of control over *aquatic animals* and *vectors*. These factors need to be considered in *biosecurity* planning.

Open systems

In an open *aquaculture* production system it is not possible to have control of the water, environmental conditions, animals or *vectors*. These production systems may include stock enhancement of wild populations with *aquatic animals* originating from *aquaculture establishments* or from the wild. As these systems cannot be considered '*aquaculture establishments'*, they are not considered further in this chapter. However, movements of *aquatic animals* between *aquaculture establishments* and open systems should be assessed to determine the need for *disease* mitigation measures.

Semi-open systems

In a semi-open *aquaculture* production system, it is not possible to have control over the water entering or exiting the system, or over the environmental conditions. Some *aquatic animals* and *vectors* may also enter and exit the system. Examples of semi-open *aquaculture* production systems are net pens or cages for finfish and suspended baskets or rope systems for molluscs in natural water bodies.

Semi-closed systems

In a semi-closed *aquaculture* production system, there is some control over the water entering and exiting the system and over the environmental conditions. *Aquatic animals* and *vectors* can be prevented from entering and exiting the system; however, there is limited control to prevent the entry or exit of *pathogenic agents*. Examples of semi-closed *aquaculture* production systems are ponds, raceways, floating enclosures, and flowthrough tanks.

Closed systems

In a closed *aquaculture* production system, there is sufficient control over water entering and exiting the system to exclude *aquatic animals*, *vectors* and *pathogenic agents*. Environmental conditions can also be controlled. Examples of closed *aquaculture* systems include recirculating *aquaculture* production systems, production

systems with a safe water supply free from *pathogenic agents* or *aquatic animals* (e.g. ground water), or those with high levels of treatment (and redundancy) of water entering and exiting the system.

Article 4.X.5.bis

**Area management**

It may not be possible to control the transmission of *pathogenic agents* among semi-open or semi-closed *aquaculture establishments* that are in close proximity within shared water bodies. In these circumstances, a consistent set of *biosecurity* measures should be applied by all of the *aquaculture establishments* considered to be epidemiologically linked. Area management agreements can formalise the coordination of common *biosecurity* measures among all of the epidemiologically linked *aquaculture establishments*.

Article 4.X.6.

**Transmission pathways and mitigation measures**

*Pathogenic agents* can move into, spread within, and be released from *aquaculture establishments* via various transmission pathways. The identification of all potential transmission pathways is essential for the development of an effective *biosecurity plan*. Pathways that are likely to result in transmission of specific *pathogenic agents* should be prioritised for mitigation.

The *risks* associated with the introduction into, spread within, and release of *pathogenic agents* from the *aquaculture establishment* need to be considered for each of the following transmission pathways.

1. Aquatic animals

Movement of *aquatic animals* into, within and from *aquaculture establishments*, either intentionally or unintentionally, may pose a high likelihood of transmitting *pathogenic agents*. This is particularly the case when clinically and sub-clinically infected *aquatic animals*, or *aquatic animals* with unknown health status are moved into a susceptible population.

*Aquatic animal*s intentionally introduced into, or moved within, an *aquaculture establishment* may include broodstock, larvae, juvenile stock for on-growing, and genetic material such as *eggs* and milt. Both horizontal and vertical transmission mechanisms of *pathogenic agents* should be considered for *aquatic animals*. The *risk* of transmitting *pathogenic agents* via *aquatic animals* should be managed giving consideration to the following mitigation measures:

a) Only introduce into the *aquaculture establishment aquatic animals* with a known health status, which is of equal or higher status than the existing animals in the establishment.

b) If *aquatic animals* of unknown *disease* status are introduced, they should be placed into *quarantine*.

c) Where appropriate, *quarantined aquatic animals* to mitigate *disease risks* (for example, treatment for external parasites).

d) Ensure biosecure transport of *aquatic animals* that avoids exposure to and release of *pathogenic agents.*

e) Only move *aquatic animals* between different populations within the establishment following consideration of the *disease risks* and with a view to maintaining the highest possible health status of the *aquatic animal* population.

f) Where possible, isolate *aquatic animal* populations that display clinical signs of *disease* from other populations until the cause is known and the situation is resolved.

g) Remove moribund or dead *aquatic animals* from production units as soon as possible and dispose of them in a biosecure manner in accordance with Chapter 4.7.

h) Report unexplained or unusual mortalities, or suspicion of a notifiable *disease* or an *emerging disease* in *aquatic animals* to the *Competent Authority* in accordance with local requirements. Investigation and *diagnosis* of the cause of mortality should be undertaken by *aquatic animal health professionals* or *veterinarians*.

i) If possible, completely remove *aquatic animals* from all or parts of the *aquaculture establishment* at intervals, for instance between *aquatic animal* generations or production cycles, followed by cleaning, *disinfection* and drying of production installations. Sites should be fallowed for a period sufficient to interrupt *infection* cycles and reduce or eliminate pathogen challenge to restocked *aquatic animals*.

*Fallowing* should be coordinated for *aquaculture establishments* that are epidemiologically linked through shared water bodies.

j) Consider physical measures to minimise the likelihood of escape of farmed *aquatic animals* or the entry of wild *aquatic animals* into the *aquaculture establishment*. The likelihood of entry or escape of *aquatic animals* will be higher for semi-open than for closed or semi-closed systems.

2. Aquatic animal products and aquatic animal waste

*Aquatic animal products* may also be brought into, moved within or moved out of *aquaculture establishments*; for example, *aquatic animal products* derived from *aquatic animals* harvested at other sites. *Aquatic animal waste* may be generated when *aquatic animals* have died or been killed for *disease* control purposes, or when they have been killed and processed for human consumption or other purposes.

Movement of *aquatic animal products* and *aquatic animal waste* into, within or from *aquaculture establishments* may pose a *risk* of *pathogenic agent* transmission. This is particularly the case when a susceptible population is exposed to *aquatic animal products* and *aquatic animal waste* derived from clinically or sub-clinically infected *aquatic animals*. Movement of *aquatic animal waste* into *aquaculture establishments* should be avoided. *Aquatic animal waste* should be stored, transported, disposed of and treated as described in Chapter 4.7.

For intentional movements of *aquatic animal products* and *aquatic animal waste*, the likelihood of presence of *pathogenic agents* in the *aquatic animals* from which *aquatic animal products* and *aquatic animal waste* are derived should be evaluated giving consideration to the species, source, and health status.

The *risk* of transmitting *pathogenic agents* via *aquatic animal products* and *aquatic animal waste* should be assessed and managed giving consideration to the following mitigation measures:

a) Determine the potential *disease risk* of *aquatic animal products* and *aquatic animal waste* to *aquatic animals* in the establishment and the environment;

b) Manage *aquatic animal products* and *aquatic animal waste* in areas within the *aquaculture establishment* that are isolated from *aquatic animal* populations to minimise identified *disease* transmission *risks*;

c) Ensure procedures are implemented for appropriate collection, treatment (inactivating *pathogenic agents*), transport, storage or disposal of *aquatic animal products* and *aquatic animal waste* to minimise identified *disease* transmission *risks*.

3. Water

Water may present a *risk* of the introduction of *pathogenic agents* into, spread within, and release from *aquaculture establishments.* The source of the water, and how it may provide an epidemiological link between the *aquaculture establishment* and other farmed or wild populations or processing plants, should be identified and considered. Exposure to transport water and ballast water should be considered.

The *risk* of the *aquaculture establishment* being exposed to water containing *pathogenic agents* may be influenced by the category of *aquaculture* production system, the likelihood being higher for semi-open than for semi-closed and closed systems. Any water that is flowing from *aquatic animals* with lower or unknown health status presents a potential *risk* of transmitting *pathogenic agents* to *aquatic animals* of a higher health status.

The *risk* of transmitting *pathogenic agents* via water should be assessed, and managed giving consideration to the following mitigation measures:

a) Where possible, choose a water source that is entirely free of susceptible *aquatic animal* populations and *pathogenic agents* of concern. Such water sources may include saline or fresh groundwater, de-chlorinated municipal water, and artificial seawater. These water sources may be particularly suitable for *aquatic animals* with high health status, such as broodstock.

b) Provide an appropriate level of screening, filtration or *disinfection* (in accordance with Chapter 4.3.) of water from sources that are likely to contain *susceptible species* and which may present a *risk* of *pathogenic agent* transmission (e.g. oceans, streams or lakes). The type and level of treatment required will depend on the identified *risks*.

c) Provide an appropriate level of filtration and *disinfection* (in accordance with Chapter 4.3.) of effluent water (and associated filtered waste) from *aquaculture establishments* (or associated slaughterhouses or processing facilities) where it may present a *risk* of *pathogenic agent* transmission to wild *aquatic animals* or other *aquaculture establishments* with *susceptible species*. The type and level of treatment required will depend on the identified *risks*.

d) Ensure the position of water intakes and outlets for semi-closed and closed *aquaculture establishments*, and the location of semi-open *aquaculture establishments*, minimises contamination from other farmed or wild populations or processing plants, taking into account factors such as distance and water currents.

e) The likelihood of ingress of contaminated water either through flooding from external sources or from defective infrastructure (e.g. leaking pipes, blocked drains, bund wall failure) should be assessed and appropriate management or infrastructure measures applied.

f) Assess the *risk* and establish procedures to treat and dispose of *waste* water resulting from the transport of *aquatic animals*.

4. Feed

*Feed* can be an important pathway for transmission of *pathogenic agents* to *aquatic animals*. *Feed* manufactured from infected *aquatic animals* may contain *pathogenic agents*, or become contaminated during harvest, transport, storage or processing. Poor hygiene may contribute to contamination during manufacture, transport, storage and use of *feed*.

In closed or semi-closed production systems there can be a high level of control of *aquatic animal feed*. However, in semi-open production systems, *aquatic animals* may obtain food from their environment (e.g. filter-feeding molluscs or predation of wild fish by farmed fish in net pens or cages). The *risk* of *disease* transmission from *feed* to the environment also needs to be managed.

The *risk* of transmitting *pathogenic agents* via *aquatic animal feed* should be assessed, and managed by mitigation measures as described in Chapter 4.8., for example using *feed* and *feed ingredients* that:

a) have undergone sufficient processing to inactivate *pathogenic agents* of concern;

b) are from sources that are declared free from the *pathogenic agents* of concern or have been confirmed (e.g. by testing) that *pathogenic agents* are not present in the *feed* or *feed ingredients*;

c) have been processed, manufactured, stored, transported and delivered during feeding to *aquatic animals* in a manner to prevent contamination by *pathogenic agents*.

5. Fomites

Equipment, *vehicles*, packaging material, clothing, footwear, sediments, infrastructure and other fomites can mechanically transfer *pathogenic agents* into, within and from an *aquaculture establishment*.

The likelihood of transferring *pathogenic agents* will depend on the stability of the *pathogenic agent* in the environment, the presence and nature of organic matter on the fomite surface, as well as the type of surface and its capacity to hold water. The likelihood of transferring *pathogenic agents* may be higher for fomites which are difficult to clean and disinfect. Sharing equipment between *aquaculture establishments,* or between different production units within an *aquaculture establishment,* or between *aquaculture establishments* and processing facilities, may result in the spread of *pathogenic agents*. The *risk* of transmitting *pathogenic agents* via fomites should be assessed and managed giving consideration to the following mitigation measures:

a) Assess the *disease risk* associated with any fomites moved into, within or from the *aquaculture establishment*.

b) Ensure procedures and infrastructure are in place to clean and disinfect fomites, including at designated delivery and loading areas, prior to entry into the *aquaculture establishment*. Recommendations for the cleaning and *disinfection* of fomites are described in Chapter 4.3.

c) Dedicate items that are difficult to disinfect, or those with a high likelihood of contamination, to a specific *aquaculture establishment* or to areas within an establishment instead of moving them after *disinfection*.

d) Apply the mitigation measures described at points a) to c) above to the movement of fomites between production units within an *aquaculture establishment* with the measures determined based on an evaluation of the *risk* of *disease* transmission.

6. Vectors

*Vectors* can transmit *pathogenic agents* to susceptible *aquatic animals* in *aquaculture establishments*. They may include *aquatic animals* entering via the water supply, predators, wild birds, scavengers, and pest animals such as rodents. *Vectors* can also transmit *pathogenic agents* within and from an *aquaculture establishment*.

The likelihood of transmitting *pathogenic agents* via *vectors* varies with the type of *vector*, the nature of the *pathogenic agent*, the category of *aquaculture* production system, and the level of *biosecurity*.

The *risk* of transmitting *pathogenic agents* via *vectors* should be assessed and managed giving consideration to the following mitigation measures:

a) Physical mitigation measures to prevent the access of *vectors* to *aquaculture establishments* may include:

i) filtering or screening of water entering and exiting semi-closed and closed *aquaculture* production systems to prevent entry of wild *aquatic animals*;

ii) surrounding land-based *aquaculture* production systems by a fence or a wall to prevent entry of animals and people, with a gate for controlled access for authorized personnel and visitors;

iii) surrounding floating *aquaculture* production systems by barriers on the establishment perimeter to prevent contact with or entry of wild *aquatic animals* and other animals;

iv) covering outdoor or unenclosed *aquaculture* production systems with nets to prevent access by birds.

b) Pest control.

7. Personnel and visitors

a) Access of personnel and visitors to *aquaculture establishments* should be controlled by creating a defined border between the outer *risk* area and the inner biosecure area comprising facilities for:

i) completion of a register, which should include visitors' names, contact information, and details of exposure to *aquatic animals* or *pathogenic agents* over a preceding period, including visits to other *aquaculture establishments* or other facilities;

ii) changing of clothes and shoes, or use of disposable coverings (e.g. hoods, coats, gloves, shoe coverings);

iii) *disinfection* of hands, and the use of foot baths.

b) All visitors should be briefed and supervised to ensure compliance with the *biosecurity plan*.

c) *C*lear signage should be displayed to promote awareness and compliance with *biosecurity plan* measures by personnel, visitors and the public.

Article 4.X.7.

**Risk analysis**

*Risk analysis* is an accepted approach for evaluating *biosecurity* threats and is used to support the development of mitigation measures. A formal *risk analysis* has four components: *hazard* identification, *risk assessment*, *risk management* and *risk communication*. This article elaborates the principles described in Chapter 2.1. and applies them to guide the development of *biosecurity plans* for *aquaculture establishments*.

A *biosecurity plan* may not necessarily require a comprehensive *risk analysis* to evaluate *disease risks* linked to transmission pathways. The chosen approach may depend on the objectives of the *biosecurity plan*, the level of *biosecurity* that is appropriate for the specific production requirements of the *aquaculture establishment*, the complexity of the threats to be addressed, and the availability of information and resources. Depending on these circumstances, a partial analysis may be appropriate, and can build on previous experiences to identify the *hazards* associated with relevant transmission pathways.

The three formal steps of the *risk analysis* process to underpin a *biosecurity plan* are:

**Step 1 – Hazard identification**

*Hazard* identification determines which *pathogenic agents* should be the subject of the *risk assessment*. A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents.* This step includes identifying and collecting relevant information on the *pathogenic agents* that have potential to cause *diseases* in *aquatic animal* populations within an *aquaculture establishment*. This process must consider the *aquatic animal health status* of the establishment and, for semi-open and semi-closed *aquaculture* production systems, the *aquatic animal health status* of the epidemiologically linked environments. Known and *emerging diseases* which could negatively impact the farmed population should be identified, regardless of whether they are present in the *aquaculture establishment*.

To complete the next steps of the *risk assessment*, information on the identified *hazards* is required and includes: i) the frequency of occurrence, ii) the biophysical characteristics, iii) the likelihood of detection if present and iv) the possible transmission pathways (described in Article 4.X.6.). Many of the *hazards* will share the same pathways.

**Step 2 – Risk assessment**

A *risk assessment* can be initiated once it has been identified that a *hazard* exists, and the required information listed under step 1 has been gathered. The aim of the *risk assessment* is to establish a *risk* estimate, which is the product of the likelihood and consequences of entry of a *pathogenic agent* into, spread within or release from the *aquaculture establishment*.

A *risk assessment* can be quantitative or qualitative. Both methods require the same conceptual pathway which identifies the necessary steps for *hazard* introduction, establishment and spread to be constructed. In a qualitative assessment, introduction and establishment are estimated using descriptors of likelihood. A quantitative assessment requires data on which to estimate likelihood. In most circumstances, the likelihood of *disease* transmission and associated consequences will be assessed qualitatively but within a formal *risk assessment* framework. Examples of descriptors for qualitative estimates of likelihood and consequence are given in Tables 1 and 2. Table 3 illustrates how estimates of likelihood and consequence can be combined in a matrix to give an estimate of *risk*. Table 4 provides an interpretation of *risk* estimates.

**Table 1. Qualitative descriptors of likelihood**

| Estimate | Descriptor |
|---|---|
| Remote | Very unlikely, but not impossible. |
| Unlikely | May occur, but only in rare circumstances. |
| Possible | Clear evidence to suggest this is possible in this situation. |
| Likely | It is likely, but not certain, to occur. |
| Certain | It is certain to occur. |

**Table 2. Qualitative descriptors of consequences**

| Estimate | Descriptor of consequences at level of the aquaculture establishment |
|---|---|
| Insignificant | Impact not detectable or minimal. No trade impacts. |
| Minor | Limited decreased production affecting only a small number of units or short-term, and/or very limited and transitory disruption to trade. |
| Moderate | Decreased production (e.g. sustained increased mortality or decreased growth rate) and/or some short-term to medium-term disruption to trade, resulting in financial loss. |

| | |
|---|---|
| Major | Considerable, decreased production, and/or some medium-term to long-term disruption to trade, resulting in significant financial loss. |
| Catastrophic | Complete production loss, possibly barriers to resumption of production, and/or complete loss of trade, resulting in extreme financial loss. |

**Table 3. Matrix for estimating risk**

| **Likelihood estimate** | Consequence rating | | | | |
|---|---|---|---|---|---|
| | insignificant | minor | moderate | major | catastrophic |
| remote | negligible | low | low | low | medium |
| unlikely | low | low | medium | medium | high |
| possible | low | medium | medium | high | high |
| likely | low | medium | high | high | extreme |
| certain | low | high | high | extreme | extreme |

*Risk assessments* inform which *hazards* need to be addressed, which critical control points on the transmission pathway should be targeted for management, and the measures that are most likely to be effective in reducing *risk*.

**Table 4. Interpretation of risk estimates**

| ***Risk* estimate*** | **Explanation and management response** |
|---|---|
| Negligible | Acceptable level of *risk*. No action required. |
| Low | Acceptable level of *risk*. On-going monitoring may be required. |
| Medium | Unacceptable level of *risk*. Review and strengthen the *risk* mitigation measures. |
| High | Unacceptable level of *risk*. Identify and implement additional *risk* mitigation measures. |
| Extreme | Unacceptable level of *risk*. Take immediate action to mitigate the *risk*. |

* Likelihood and consequence estimates are combined using the *risk* matrix (Table 3) to produce the *risk* estimate.

**Step 3 – Risk management**

*Risk management* is used to determine the appropriate management response for the assessed level of *risk* as described in Table 4. The *risk assessment* process identifies the steps within transmission pathways necessary for a *risk* to be realised and thus allows the most effective mitigation measures to be determined. Many of the *hazards* will share the same pathways and therefore mitigation measures may be effective against more than one *hazard*. Information on *hazards* and their pathways of introduction (step 1) should be combined with an assessment of *risk* associated with each pathway (step 2) to identify the most appropriate and cost-effective *risk* mitigation measures.

Article X.X.6. describes some possible mitigation measures relevant to different transmission pathways. The most appropriate mitigation measures for a specific *aquaculture establishment* will depend on the effectiveness and reliability of the mitigation measure, the category of *aquaculture* production system and cost.

After the implementation of the *biosecurity plan*, *hazards* should be regularly reassessed, and measures adjusted according to any changed *risk* estimates.

Article 4.X.8.

**Biosecurity plan development**

The purpose of a *biosecurity plan* is primarily to reduce the *risk* of introducing *pathogenic agents* into an *aquaculture establishment*, and if *pathogenic agents* are introduced, to reduce the *risk* of further spread within or release from the *aquaculture establishment*. The plan will document identified transmission pathways and the outputs of any *risk analysis* performed (*hazards, risk* estimate and mitigation measures), and information relevant to ongoing implementation, monitoring and review of the plan.

1. Development of a biosecurity plan

The process of developing a *biosecurity plan* will vary depending on its objectives, the level of *biosecurity* appropriate to the specific production system requirements, the complexity of the *disease risks* to be

addressed, and availability of information and resources. Consideration and documentation of the following issues are recommended:

a) objectives, scope and regulatory requirements for the *biosecurity plan*;

b) information about the *aquaculture establishment* including an up-to-date plan of the layout of buildings and production units (including *epidemiological units*, if any, and structures and processes to maintain separation), loading/unloading, unpacking, processing, *feed* storage, *aquatic animal waste* storage, reception areas, access points and maps showing major movements of *aquatic animals*, *aquatic animal products* and *aquatic animal waste*, water, *feed* and fomites;

c) the potential pathways for entry of *pathogenic agents* into, spread within or release from the *aquaculture establishment* (refer to Article X.X.6. above);

d) a *risk analysis*, including identification of the major *disease hazards* to the *aquaculture establishment* (refer to Article X.X.7. above);

e) the mitigation measures that have been determined to address *risks*;

f) emergency procedures in the event of a *biosecurity* failure. These may include reporting requirements, and emergency measures to eradicate *pathogenic agents* such as *aquatic animal* depopulation and disposal, and site *disinfection*, in accordance with Chapters 4.3. and 7.4.;

g) internal and external communication procedures, roles and responsibilities of *aquaculture establishment* personnel and essential contact information, e.g. for personnel, *aquatic animal health professionals* or *veterinarians* and the *Competent Authority*;

h) monitoring and audit schedule;

i) performance evaluation;

j) standard operating procedures required to support implementation of the mitigation measures described by the *biosecurity plan*, emergency procedures and the training requirements of establishment personnel.

2. Key components of a biosecurity plan

a) Standard operating procedures (SOPs)

SOPs describe routine management processes that must be performed to support the effectiveness of the *biosecurity plan*. Each SOP should clearly describe its objectives, personnel responsibilities, the procedure (including record keeping), precautions and a review date.

b) Training of personnel

Personnel should be trained in the application of the SOPs including completion of forms, checklists and other records associated with each procedure, as well as routine communication requirements.

The *biosecurity plan* should include a training programme to ensure that all personnel are capable of playing their role in the implementation of *biosecurity* at the *aquaculture establishment*.

c) Documentation and record keeping

The *biosecurity plan* describes the documentation necessary to provide evidence of compliance with the plan. The level of detail required in the documentation depends on the outcomes of the transmission pathway assessment.

Examples of documentation required include: *aquaculture establishment* layout, movements of *aquatic animals*, origin and destination and health status of the *aquatic animals* introduced to the *aquaculture establishment*, *quarantine* measures, records of visitors to the establishment, escapees, stocking densities, feeding and growth rates, records of personnel training, treatments/vaccination, water quality, cleaning and *disinfection* events, morbidity and mortality (including removal and disposal of mortalities), *surveillance* and laboratory records.

d) Emergency procedures

Procedures should be developed and, when necessary, implemented to minimise the impact of emergencies, *disease* events, or unexplained mortality in *aquatic animals*. These procedures should include clearly defined thresholds that help to identify an emergency incident and activate response protocols, including reporting requirements.

e) Health monitoring

Health monitoring as part of the *biosecurity plan* involves monitoring of the health status of *aquatic animals* in *aquaculture establishments*. Monitoring should be performed at a production unit and establishment level. Activities may include *disease surveillance*, routine monitoring of stock for important health and production parameters (e.g. by personnel, an *aquatic animal health professional* or a *veterinarian*), recording of clinical signs of *disease*, morbidity and mortality, laboratory test results and analysis of these data (e.g. calculation of rates of morbidity and mortality).

f) Routine review and auditing

The *biosecurity plan* should describe a systematic auditing schedule to verify implementation and compliance with the requirements of the *biosecurity plan*. Routine revision of the *biosecurity plan* is necessary to ensure that it continues to effectively address *biosecurity risks*.

The *biosecurity plan* should also be reviewed at least annually or in response to changes to the *aquaculture establishment* operations, changes in facility design, changes in husbandry approaches, identification of a new *disease risk*, or the occurrence of a *biosecurity* incident. *Biosecurity* incidents, and actions taken to remedy them, should be documented to enable re-assessments of SOPs.

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Back to Agenda

CHAPTER 1.3.

# DISEASES LISTED BY THE OIE

**EU position**

**The EU supports the adoption of this revised article.**

[…]

Article 1.3.3.

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

– Acute hepatopancreatic necrosis disease

– Infection with *Aphanomyces astaci* (crayfish plague)

– Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)

– Infection with infectious hypodermal and haematopoietic necrosis virus

– Infection with infectious myonecrosis virus

– Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

– Infection with decapod iridescent virus 1

– Infection with Taura syndrome virus

– Infection with white spot syndrome virus

– Infection with yellow head virus genotype 1.

# ASSESSMENT OF INFECTION WITH DECAPOD IRIDESCENT VIRUS 1 (DIV1) FOR LISTING IN CHAPTER 1.3 OF THE *AQUATIC ANIMAL HEALTH CODE*

## Overall assessment

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) assessed infection with decapod iridescent virus 1 (DIV1) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code* and agreed that infection with (DIV1) meets the OIE criteria for listing, notably 1.: International spread of the disease is likely; 2.: At least one country may demonstrate country or zone freedom from the disease; 3.: A precise case definition is available and a reliable means of detection and diagnosis exists, and 4b.: The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level (see Table 1 below).

**Table 1.** Summary of assessment of infection with (DIV1)

| | Listing criteria | | | | | | Conclusion |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4a | 4b | 4c | |
| Infection with DIV1 | + | + | + | NA | + | - | The disease meets the criteria for listing |

NA = not applicable.

## Background

A novel member of family *Iridoviridae*, named as decapod iridescent virus 1 (DIV1) (ICTV, 2019), with a double-stranded DNA genome of about 166K bp (Li *et al*., 2017; Qiu *et al*., 2017b), has been identified as the cause of mass mortalities in shrimp, prawn and crayfish production (Xu *et al*., 2016; Qiu *et al*., 2017a; Qiu *et al*., 2019a). Infection with DIV1 has so far been detected in red claw crayfish (*Cherax quadricarinatus*) (Xu *et al*., 2016), white-leg shrimp (*Penaeus vannamei*) (Qiu *et al*., 2017a, b), giant freshwater prawn (*Macrobrachium rosenbergii*) (Qiu *et al*., 2019a), red swamp crayfish (*Procambarus clarkii*) (Qiu *et al*., 2019 a, b), oriental river prawn (*Macrobrachium nipponense*) (Qiu *et al*., 2019a), ridgetail white prawn (*Exopalaemon carinicauda*) and giant tiger prawn (*Penaeus monodon)* (OIE, 2020; Srisala *et al*., 2020). Two species of crab, Chinese mitten crab (*Eriocheir sinensis*) and striped shore crab (*Pachygrapsus crassipes*) have been shown to become infected with DIV1 in experimental challenge through unnatural pathways (Pan *et al*., 2017). The Commission has recognised the potential significance of infection with DIV1 to many countries given the worldwide importance of crustacean farming and trade. At the moment, infection with DIV1 is considered an "emerging disease" and, as such, should be reported in accordance with Article 1.1.4. of the *Aquatic Code*.

## Criteria for listing an aquatic animal disease (Article 1.2.2.)

**Criterion No. 1.** ***International spread of the pathogenic agent (via aquatic animals, aquatic animal products, vectors or fomites) is likely.***

*Assessment*

The virus has been detected by PCR or nested PCR method in white-leg shrimp *(P. vannamei),* giant freshwater prawn (*M. rosenbergii*), red swamp crayfish (*P. clarkii*), oriental river prawn (*M. nipponense*) and ridgetail white prawn (*E. carinicauda*) in farms in China (People's Rep. of) (Xu *et al*., 2016; Qiu *et al*., 2017a; Qiu *et al*., 2018b; Qiu *et al*., 2019b). Additionally, DIV1 has been detected in farmed *P. monodon* in Chinese Taipei (OIE, 2020) and in wild *P. monodon* in the Indian Ocean (Srisala *et al*., 2020). Historically, *P. vannamei*, *P. monodon* and other susceptible crustacean species have been traded internationally as broodstock and postlarvae for production in new geographic regions. Thus, pathways for transmission are present and international spread is likely. Histopathology, visualization under TEM and *in-situ* hybridisation provide evidence that the virus can be found in haematopoietic tissue, lymphoid organs, gills, hepatopancreas, epithelium, periopods and muscle (Sanguanrut *et al*., 2020; Qiu *et al*., 2020a). Quantitative PCR detection in experimentally infected shrimp showed that haemolymph and haemopoietic tissues had the highest DIV1 load and muscle tissues had the lowest load (Qiu *et al*., 2018a; Qiu *et al*., 2019a).

*Conclusion*

The criterion is met.

**AND**

**Criterion No. 2. At least one country may demonstrate country or zone freedom from the disease in susceptible aquatic animals.**

Assessment

Currently, infection with DIV1 has been detected in China (People's Rep. of), Chinese Taipei and in the Indian Ocean, but the geographic distribution of the virus may be wider if more mortality events had been investigated. However, because of the broad distribution of *P. vannamei*, *P. monodon*, *M. rosenbergii*, and other susceptible species to infection with DIV1, as well as extensive trade in these species, and likely expression of clinical disease and mortality, it is expected that the disease would have been reported elsewhere if the virus had spread widely.

In addition, the disease has been listed as a notifiable disease by the Network of Aquaculture Centres in Asia-Pacific (NACA) in its 'Quarterly Aquatic Animal Disease report' (Asia and Pacific Region) since January 2019. It is likely, therefore, that at least one country may be able to demonstrate country or zone freedom from the disease in susceptible aquatic animals.

*Conclusion*

The criterion is met.

**AND**

**Criterion No. 3.** ***A precise case definition is available, and a reliable means of detection and diagnosis exists.***

*Assessment*

Infected *P. vannamei* exhibit empty stomach and guts in all diseased shrimp, slight loss of colour on the surface and around the hepatopancreas, and soft shell. In some individuals slight reddening of the body is observed. Moribund shrimp lose their swimming ability and sink to the bottom of the pond (Qiu *et al*., 2017a). Diseased *M. rosenbergii* exhibit a white triangle inside the carapace at the base of rostrum which is the location of hematopoietic tissue (Qiu *et al*., 2019a).

To date, a PCR method (Xu *et al*., 2016), a nested PCR method (Qiu *et al*., 2017a), a TaqMan probe based real-time PCR (TaqMan qPCR) method target ATPase gene (Qiu *et al*., 2018a), an *in situ* hybridization method (Qiu *et al*., 2017a) and an *in situ* DIG-labelling-loop-mediated DNA amplification (ISDL) method (Chen *et al*., 2019), a recombinase polymerase amplification method (Chen *et al*., 2019) and a TaqMan qPCR method target MCP gene (Qiu *et al*., 2020b) have been published and are available for DIV1 detection. The PCR primers and TaqMan probe target MCP gene have been shown to be specific for DIV1 (no cross-reaction with other shrimp pathogens), with a low detection limit (41.2 copies per reaction) and high diagnostic sensitivity and diagnostic specificity (97.2% and 98.7%, respectively). Validation of the nested PCR method and 2 TaqMan probe based real-time PCR method has occurred.

It can be concluded that reliable means of detection and diagnosis are available, and a precise case definition can be developed based on clinical signs and available diagnostic tests.

*Conclusion:*

Criterion is met.

**AND**

**Criterion No. 4. a.** ***Natural transmission to humans has been proven, and human infection is associated with severe consequences.***

*Assessment:*

No available data to assess.

*Conclusion*

Criterion not applicable.

**OR**

**Criterion No. 4.b.** ***The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level.***

*Assessment*

High mortality (>80%) has been observed in affected *P. vannamei* and *M. rosenbergii* populations in farms in China (People's Rep. of) (Qiu *et al*., 2017a; Qiu *et al*., 2019a). Experimental infection trials mimicking the natural infection pathway (*per os*) in *P. vannamei* have shown 100% cumulative mortality within two weeks (Qiu *et al.*, 2017a). Injection challenges in *P. vannamei*, *C. quadricarinatus*, and *P. clarkii* also resulted in 100% cumulative mortalities (Xu *et al.*, 2016; Qiu *et al.*, 2017a). Since 2014, some disease events with massive losses of *P. vannamei* and *M. rosenbergii* in coastal provinces of China (People's Rep. of) have been associated with infection with DIV1 (Qiu *et al.*, 2017a; Qiu *et al*., 2019a; Qiu *et al*., 2020a). Targeted surveillance in China from 2017 to 2019 detected DIV1 in 13 of 16 provinces (Qiu *et al*., 2018b; Qiu *et al*., 2019b). In 2020, DIV1 was reported associated with disease and mortality in crustacean farms in Chinese Taipei (OIE, 2020; Qiu *et al*., 2020c). Losses are significant at a country level.

*Conclusion*

Criterion is met.

**OR**

**Criterion No. 4.c.** ***The disease has been shown to, or scientific evidence indicates that it would affect the health of wild aquatic animals resulting in significant consequences e.g. morbidity or mortality at a population level, reduced productivity or ecological impacts.***

*Assessment*

Infection with DIV1 has been shown to have a significant effect on the health of cultured shrimp or crayfish resulting in significant consequences including morbidity and mortality. From a survey of wild *P. monodon* from the Indian Ocean in April 2018, the test results of nested PCR for DIV1 were 5 positive of 26 shrimp in a different lot (Srisala *et al*., 2020). It is possible that the disease would affect wild aquatic animals; however, there are no available data to demonstrate impact (e.g. morbidity or mortality) of the disease on wild aquatic animals at a population level.

*Conclusion*

Criterion is not met.

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# MODEL ARTICLE 10.X.13 FOR CHAPTERS 10.5, 10.6 AND 10.10 (AND ARTICLE 10.4.17 FOR CHAPTER 10.4) - TRACK CHANGES

**EU position**

**The EU thanks the OIE and supports the adoption of this revised model article.**

[…]

Article 10.X.13.

**Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with [pathogenic agent X]**

1) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should assess ~~in accordance with Chapter 4.4.~~the ~~*risk* associated with~~ at least the following:

   a) the ~~infection with pathogenic agent X~~ likelihood that ~~status of the~~ water ~~to be~~ used during the *disinfection* of the eggs is contaminated with [pathogenic agent X];

   b) the prevalence of infection with [pathogenic agent X] in broodstock (including ~~by~~ results from testing of 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 request that ~~apply the following~~ *risk* mitigation measures are applied, including:

   a) *disinfection* of the eggs prior to importing, in accordance with recommendations in Chapter 4.4.~~ or those specified by the *Competent Authority* of the *importing country*~~; and

   b) that between *disinfection* and importation, ~~the import~~ eggs should not come into contact with anything which may affect their health status.

   The *Competent Authority* should consider internal measures, such as additional ~~renewed~~ *disinfection* of the eggs upon arrival in the *importing country*.

3) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* certifying that the procedures described in point 2 a) and b) of this article have been fulfilled.

[…]

# MODEL ARTICLE 10.X.13 FOR CHAPTERS 10.5, 10.6 AND 10.10 (AND ARTICLE 10.4.17 FOR CHAPTER 10.4) – CLEAN VERSION

[…]

Article 10.X.13.

**Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with [pathogenic agent X]**

1) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should assess at least the following:

   a) the likelihood that water used during the *disinfection* of the eggs is contaminated with [pathogenic agent X];

   b) the prevalence of infection with [pathogenic agent X] in broodstock (including results from testing of ovarian fluid and milt).

2) If the *Competent Authority* of the *importing country* concludes that the importation is acceptable, it should request that *risk* mitigation measures are applied, including:

   a) *disinfection* of the eggs prior to importing, in accordance with recommendations in Chapter 4.4.; and

   b) that between *disinfection* and importation, eggs should not come into contact with anything which may affect their health status.

   The *Competent Authority* should consider internal measures, such as additional *disinfection* of the eggs upon arrival in the *importing country*.

3) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* certifying that the procedures described in point 2 a) and b) of this article have been fulfilled.

[…]

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CHAPTER 10.9.

# INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

**EU position**

**The EU supports the adoption of this revised article.**

[…]

Article 10.9.2.

**Scope**

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.:

~~all varieties and subspecies of common carp *(Cyprinus carpio)*, bighead carp *(Aristichthys nobilis)*, bream *(Abramis brama)*, Caspian white fish *(Rutilus kutum)*, fathead minnow *(Pimephales promelas)*, golden shiner *(Notemigonus crysoleucas)*, goldfish *(Carassius auratus)*, grass carp (*Ctenopharyngodon idella)*, roach *(Rutilus rutilus)* and sheatfish (also known as European or wels catfish) *(Silurus glanis)*.~~

| Family | Scientific name | Common name |
|---|---|---|
| Cyprinidae | *Abramis brama* | Bream |
| | *Aristichthys nobilis* | Bighead carp |
| | *Carassius auratus* | Goldfish |
| | *Ctenopharyngodon idella* | Grass carp |
| | *Cyprinus carpio* | Common carp (all varieties and subspecies) |
| | *Danio rerio* | Zebrafish |
| | *Notemigonus crysoleucas* | Golden shiner |
| | *Pimephales promelas* | Fathead minnow |
| | *Rutilus kutum* | Caspian white fish |
| | *Rutilus rutilus* | Roach |
| Siluridae | *Silurus glanis* | ~~Sheatfish (also known as European or wels catfish)~~ Wels catfish |

[…]

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CHAPTER 10.10.

# INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

**EU position**

**The EU supports the adoption of this revised article.**

[…]

Article 10.10.2.

**Scope**

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.~~: rainbow trout *(Oncorhynchus mykiss)*, brown trout *(Salmo trutta)*, grayling *(Thymallus thymallus)*, white fish (*Coregonus* spp.), pike *(Esox lucius)*, turbot *(Scophthalmus maximus)*, herring and sprat (*Clupea* spp.), Pacific salmon (*Oncorhynchus* spp.), Atlantic cod *(Gadus morhua)*, Pacific cod *(Gadus macrocephalus)*, haddock *(Gadus aeglefinus)* and rockling *(Onos mustelus)*. These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

| **Family** | **Scientific name** | **Common name** | ~~**Genotype**~~ |
|---|---|---|---|
| Ammodytidae | *Ammodytes hexapterus* | Pacific sand lance | ~~IVa~~ |
| ~~Aralichthyidae~~ | ~~*Paralichthys olivaceus*~~ | ~~Bastard halibut~~ | ~~IVa~~ |
| Carangidae | *Trachurus mediterraneus* | Mediterranean horse mackerel | ~~Ie~~ |
| Centrarchidae | *Ambloplites rupestris* | Rock bass | ~~IVb~~ |
| | *Lepomis gibbosus* | Pumpkinseed | ~~IVb~~ |
| | *Lepomis macrochirus* | Bluegill | ~~IV, IVb~~ |
| | *Micropterus dolomieu* | Smallmouth bass | ~~IVb~~ |
| | *Micropterus salmoides* | Largemouth bass | ~~IVb~~ |
| | *Pomoxis nigromaculatus* | Black crappie | ~~IVb~~ |
| Clupeidae | *Alosa immaculata* | Pontic shad | ~~Ie~~ |
| | *Sardina pilchardus* | Pilchard | |
| | *Clupea harengus* | Atlantic herring | ~~Ib, III~~ |
| | *Clupea pallasii pallasii* | Pacific herring | ~~IVa~~ |
| | *Dorosoma cepedianum* | American gizzard shad | ~~IVb~~ |
| | *Sardinops sagax* | South American pilchard | ~~IVa~~ |
| | *Sprattus sprattus* | European sprat | ~~Ib~~ |
| Cyclopteridae | *Cyclopterus lumpus* | Lumpfish | ~~IVd~~ |
| Cyprinidae | *Danio rerio* | Zebra fish | ~~IVa~~ |
| | *Notropis hudsonius* | Spottail shiner | ~~IVb~~ |
| | *Notropis atherinoides* | Emerald shiner | ~~IVb~~ |
| | *Pimephales notatus* | Bluntnose minnow | ~~IVb~~ |
| | *Pimephales promelas* | Fathead ~~Minnow~~ minnow | ~~IVb~~ |
| Embiotocidae | *Cymatogaster aggregata* | Shiner perch | ~~IVa~~ |
| Engraulidae | *Engraulis encrasicolus* | European anchovy | ~~Ie~~ |
| Esocidae | *Esox lucius* | Northern pike | ~~IVb~~ |
| | *Esox masquinongy* | Muskellunge | ~~IVb~~ |

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| Fundulidae | *Fundulus heteroclitus* | Mummichog | IVc |
| Gadidae | *Gadus macrocephalus* | Pacific cod | IVa |
| | *Gadus morhua* | Atlantic cod | Ib, III |
| | *Merlangius merlangus* | Whiting | Ie |
| | *Micromesistius poutassou* | Blue Whiting whiting | Ib, III |
| | *Trisopterus esmarkii* | Norway pout | Ib, III |
| Gasterosteidae | *Gasterosteus aculeatus* | Three-spine stickleback | IVc |
| Gobiidae | *Neogobius melanostomus* | Round goby | IVb |
| | *Pomatoschistus minutus* | Sand goby | Ib |
| Ictaluridae | *Ictalurus Ameiurus nebulosus* | Brown bullhead | IVb |
| Labridae | *Centrolabrus exoletus* | Rock cook wrasse | III |
| | *Ctenolabrus rupestris* | Goldsinny wrasse | III |
| | *Labrus bergylta* | Ballan wrasse | III |
| | *Labrus mixtus* | Cuckoo wrasse | III |
| | *Symphodus melops* | Corkwing wrasse | III |
| Lotidae | *Gaidropsarus vulgaris* | Three-bearded rockling | Ie |
| Moronidae | *Morone americana* | White Perch perch | IVb |
| | *Morone chrysops* | White Bass bass | IVb |
| | *Morone saxatilis* | Striped bass | IVb, IVc |
| Mullidae | *Mullus barbatus* | Red mullet | Ie |
| Osmeridae | *Thaleichthys pacificus* | Eulachon | IVa |
| Percidae | *Sander vitreus* | Walleye | IVb |
| | *Perca flavescens* | Yellow perch | IVb |
| AParalichthyidae | *Paralichthys olivaceus* | Bastard halibut | IVa |
| Petromyzontidae | *Lampetra fluviatilis* | River lamprey | II |
| Pleuronectidae | *Limanda limanda* | Common dab | Ib |
| | *Platichthys flesus* | European flounder | Ib |
| | *Pleuronectes platessus* | European plaice | III |
| Rajidae | *Raja clavata* | Thornback ray | Ie |
| Salmonidae | *Coregonus artedii* | Lake cisco | IVb |
| | *Coregonus clupeaformis* | Lake whitefish | IVb |
| | *Coregonus lavaretus* | Common whitefish | Ia |
| | *Oncorhynchus kisutch* | Coho salmon | IVa |
| | *Oncorhynchus mykiss* | Rainbow trout | Ia-e, III, IVb |
| | *Oncorhynchus mykiss X Oncorhynchus kisutch hybrids* | Rainbow trout X coho salmon hybrids | Ia |
| | *Oncorhynchus tshawytscha* | Chinook salmon | IVa, IVb |
| | *Salmo marmoratus* | Marble trout | Ia |
| | *Salmo salar* | Atlantic salmon | Ia, Ib, II, III, IVa |
| | *Salmo trutta* | Brown trout | Ia, Ib |
| | *Salvelinus namaycush* | Lake trout | Ia, IVa, IVb |
| | *Thymallus thymallus* | Grayling | I |
| Scophthalmidae | *Scophthalmus maximus* | Turbot | Ib, III |
| Sciaenidae | *Aplodinotus grunniens* | Freshwater drum | IVb |
| Scombridae | *Scomber japonicus* | Pacific Cchub mackerel | IVa |
| Soleidae | *Solea senegalensis* | Senegalese sole | III |
| Uranoscopidae | *Uranoscopus scaber* | Atlantic stargazer | Ie |

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CHAPTER 11.3

# INFECTION WITH *BONAMIA OSTREAE*

**EU position**

**The EU supports the adoption of these revised articles.**

Article 11.3.1.

For the purposes of the *Aquatic Code*, infection with *Bonamia ostreae* means *infection* with the *pathogenic agent ~~B.~~ Bonamia ostreae* of the Family Haplosporidiidae.

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

Article 11.3.2.

**Scope**

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: European flat oyster *(Ostrea edulis)*, ~~Australian mud oyster *(Ostrea angasi)*~~, Argentinean flat oyster ~~*(Ostrea puelchana)*~~, Chilean flat oyster *(Ostrea chilensis)*, ~~Asiatic oyster *(Ostrea denselammellosa)*~~ and Suminoe oyster *(Crassostrea ariakensis)*. ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

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*Original: English*
January–July 2020

# REPORT OF THE OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF MOLLUCS SPECIES TO INFECTION WITH OIE LISTED DISEASES

## January–June 2020

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This report covers the work of the OIE *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases (the *ad hoc* Group) between January and June 2020. During this period, the *ad hoc* Group met twice (a three-day physical meeting followed by a series of virtual meetings).

The list of participants and the Terms of Reference are presented in Annex I and Annex II, respectively.

**Methodology**

The *ad hoc* Group applied the criteria to potential host species to determine susceptibility and non-susceptibility to infection with *Bonamia ostreae*. This was done by the three-stage approach, outlined in Article 1.5.3 of the *Aquatic Code*, to assess susceptibility of a species to infection with *B. ostreae*, as described below:

**1) Criteria to determine whether the route of transmission is consistent with natural pathways for the infection (as described in Article 1.5.4):**

*Stage 1: Criteria to determine whether the modality of exposure is consistent with natural pathways (as described in Article 1.5.4)*

Consideration was given to whether experimental procedures mimic natural pathways for disease transmission. Consideration was also given to environmental factors given that these may affect host response, virulence and transmission of infection with *B. ostreae*.

The table below describes additional considerations made by the *ad hoc* Group when applying Stage 1 to support susceptibility to infection with *B. ostreae*

| Stage 1: Source of infection | Comment |
|---|---|
| Natural exposure includes situations where infection has occurred without experimental intervention (e.g. infection in wild or farmed populations)<br>OR<br>Non-invasive experimental procedures[1]: cohabitation with infected hosts; infection by immersion or feeding | *In vitro* experimental assays (contact between haemocytes and parasites) are not considered appropriate to answer the question of susceptibility or non-susceptibility. |

[1] Invasive experimental procedures including injection can only be used to demonstrate non-susceptibility.

**2) Criteria to determine whether the pathogenic agent has been adequately identified (as described in Article 1.5.5):**

*Stage 2: Criteria to determine whether the pathogenic agent has been adequately identified (as described in Article 1.5.5)*

The *ad hoc* Group noted that unambiguous pathogenic agent identification might not have been carried out in older publications because molecular techniques were not available at the time. In these circumstances a weight of evidence approach, whereby the combined information from subsequent studies and additional information provided by the authors, was considered and used to conclude sufficiency of pathogen identification.

The table below describes the pathogen identification methods used by the *ad hoc* Group including some considerations.

| Stage 2: Pathogen Identification | Comment |
|---|---|
| Molecular sequence information (species-specific regions of 18S sequence)<br>OR<br>PCR-RFLP (as described in Cochennec *et al*., 2000)<br>OR<br>Species-specific Real-time or conventional PCR (for example Ramilo *et al*., 2013) | Molecular data should be associated with microscopical examination wherever possible to confirm the presence of the pathogen.<br>ISH is currently not sufficiently specific to resolve species level identifications.<br>For early studies without molecular information, corroborating evidence from later studies was considered.<br>ITS rDNA sequence has a higher resolution than 18s rDNA and can add information about the intra-species diversity between populations. |

**3) Criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection (as described in Article 1.5.6):**

*Stage 3: Criteria to determine whether the evidence indicates that presence of the pathogenic agent constitutes an infection as described in Article 1.5.6*

Criteria A to D in Article 1.5.6 were used to determine if there was sufficient evidence for infection with *B. ostreae* in the suspected host species. Evidence to support criterion A alone was sufficient to determine infection. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D were required to determine infection.

A. The pathogenic agent is multiplying in the host, or developing stages of the pathogenic agent are present in or on the host;

B. Viable pathogenic agent is isolated from the proposed susceptible species, or infectivity is demonstrated by way of transmission to naïve individuals;

C. Clinical or pathological changes are associated with the infection;

D. The specific location of the pathogen corresponds with the expected target tissues.

The table below describes the criteria for assessment of Stage 3 to support susceptibility to infection with *B. ostreae*

| Stage 3: Evidence for infection | | | |
|---|---|---|---|
| A: Replication | B: Viability / Infectivity | C: Pathology / Clinical signs* | D: Location |
| 1) Presence of multiple intracellular cells or presence of multinucleated cells (including plasmodial stage) demonstrated **by:**<br><br>Histopathology<br><br>OR<br><br>Cytology (usually gill or heart imprint or haemolymph smears)<br><br>OR<br><br>*In-situ* hybridization (ISH)<br><br>OR<br><br>TEM<br><br>OR<br><br>2) Demonstration of increasing copy number over time with qPCR (targeting DNA) or reverse transcription qPCR (targeting RNA) in tissues | 1) Transmission via co-habitation with uninfected individuals of a known-susceptible (e.g. *Ostrea edulis*) species<br><br>OR<br><br>3) Demonstration of viability of cells isolated from tissues<br><br>**by:**<br><br>Flow cytometry<br><br>OR<br><br>Vital stains<br><br>OR<br><br>Successful infection of uninfected animals by inoculation | Mortality<br><br>OR<br><br><u>Macroscopic lesions</u> such as<br>- Discolouration of tissue<br><br>- Gill ulceration<br><br>OR<br><br>Rapid loss of condition<br><br>OR<br><br><u>Microscopic lesions</u> such as generalized haemocyte infiltration in connective tissues of several organs including gills and mantle | Within haemocytes circulating in the connective tissue in different organs, in particular gills** or heart (rarely extracellular) |

* non-specific signs and inconsistent presentation

** inside gills, as opposed to potential external contaminant

An assessment of non-susceptibility was made when there was a 'Yes' for criterion D and a 'No' for other assessed criteria A, B, or C based on multiple sources with no conflicting results.

The table below describes the outcomes of the assessment undertaken by the ad hoc Group.

| | |
|---|---|
| **1.** | Species that were assessed as susceptible (as described in Article 1.5.7) were proposed for inclusion in Article 11.3.2 of Chapter 11.3, Infection with *B. ostreae,* of the *Aquatic Code* and Section 2.2.1 of Chapter 2.4.3 of the *Aquatic Manual.* |
| **2.** | Species that were assessed as species for which there is partial evidence for susceptibility (as described in Article 1.5.8) were proposed for inclusion in Section 2.2.2, Species with incomplete evidence for susceptibility, of Chapter 2.4.3, Infection with *B. ostreae,* of the *Aquatic Manual*. |
| **3.** | Species that were assessed not to meet the criteria or for which there was unresolved conflicting information were not proposed for inclusion in either the *Aquatic Code* or *Aquatic Manual*. The exception were species where there had been reported pathogen-specific positive PCR results, but an active infection had not been demonstrated. These species were included in a separate paragraph in Section 2.2.2, Species with incomplete evidence for susceptibility, of Chapter 2.4.3 of the *Aquatic Manual.* |
| **4.** | Species that were assessed to have evidence of non-susceptibility were to be included in the revised Section 2.2.3 when applying the new template to Chapter 2.4.3 of the *Aquatic Manual*. |
| **5.** | Vector - at the time of the assessments, the *ad hoc* Group were waiting for a decision to be made by the Aquatic Animals Commission to determine/clarify the definition of 'vector'. Until this decision is made, the *ad hoc* Group did not consider 'vector' as an outcome. |
| **NS** | Not scored due to insufficient or irrelevant information. |

**Assessments of host susceptibility to infection with *B. ostreae***

***Summary***

The *ad hoc* Group found that of the six species currently listed in Article 11.3.2 as susceptible to infection with *B. ostreae*, three species, Australian mud oyster (*Ostrea angasi*), Argentinean flat oyster (*Ostrea puelchana*) and Asiatic oyster (*Ostrea denselammellosa*), did not meet the criteria for listing as a susceptible species and were proposed to be deleted from Article 11.3.2.

No new species were found to meet the criteria for listing as susceptible species to infection with *B. ostreae.*

The assessments, outcomes, and relevant references for host susceptibility to infection with *B. ostreae* conducted by the *ad hoc* Group are shown in the table below.

| Family | Scientific name | Common name | Stages 1: Route of infection | Stage 2: Pathogen identification | Stage 3: Evidence for infection | | | | Outcome | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | A | B | C | D | | |
| ***Score 1*** | | | | | | | | | | |
| Ostreidae | *Ostrea edulis* | European flat oyster | ND | Yes | Yes | ND | Yes | Yes | 1 | Cochennec *et al*., 2000 |
| | | | N | Yes | Yes | ND | Yes | Yes | 1 | Marty *et al*., 2006 |
| Ostreidae | *Ostrea chilensis* | Chilean flat oyster | N | Yes | Yes | ND | Yes | Yes | 1 | Lane *et al*., 2016 |
| | | | N | Yes[2] | ND | ND | Yes | Yes | 1 | Grizel *et al.,* 1983 |
| Ostreidae | *Crassostrea ariakensis* | Suminoe oyster | N | Yes[3] | Yes | ND | Yes | Yes | 1 | Cochennec *et al.*, 1998 |
| | | | E | Yes | ND | ND | No | Yes | 3 | Audemard *et al*., 2005 (conference abstract), and personal communication (R. Carnegie) |
| ***Score 2*** | | | | | | | | | | |
| Ostreidae | *Ostrea puelchana* | Argentinean flat oyster | N | Yes[4] | ND | ND | Inconclu-sive[5] | Yes | 2 | Pascual *et al*., 1991 |

[2] Study sites referred in Grizel *et al*., 1983 were in areas known to be infected with *B. ostreae* (later characterized by molecular test in addition to histology or cytology).

[3] The parasite described by Cochennec *et al*., 1998 was later confirmed to be *B. ostreae* by DNA sequencing by the OIE reference laboratory as stated in Engelsma *et al*., 2014.

[4] Study sites referred in Pascual *et al.,* 1991 were in areas known to be infected with *B. ostreae* (later characterized by molecular test in addition to histology or cytology).

[5] Criterion C was considered as inconclusive because the cause of mortality was not clear (*B. ostreae* versus *M. refringens* and/or environmental.

| Family | Scientific name | Common name | Stages 1: Route of infection | Stage 2: Pathogen identification | Stage 3: Evidence for infection | | | | Outcome | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | A | B | C | D | | |
| ***Score 3*** | | | | | | | | | | |
| Ophiotrichidae | *Ophiothrix fragilis* | Brittle star | N and E | Yes | ND | ND | ND | ND | 3 | Lynch *et al*., 2007 |
| Actiniidae | *Actina equina* | Beadlet anemone | N | Yes | ND | ND | ND | ND | 3 | Lynch *et al.,* 2007 |
| Ascidiidae | *Ascidiella aspersa* | European sea squirt | N | Yes | ND | ND | ND | ND | 3 | Lynch *et al*., 2007 |
| | | Grouped zooplankton | N | Yes | ND | ND | ND | ND | 3 | Lynch *et al*., 2007 |
| Ostreidae | *Crassostrea gigas* | Pacific cupped oyster | N and E and EI | Yes[6] | No | No | No | No | 4 | Culloty *et al*., 1999 |
| | | | N and E and EI | Yes | Yes | Inconclusive[7] | No | Yes | 1 | Lynch *et al*., 2010 |
| | | | EI | Yes | No | ND | No | No | 4 | Gervais, 2016 |
| ***Score 4*** | | | | | | | | | | |
| Veneridae | *Ruditapes decussatus* | European clam | E and EI | Yes | No | No | No | No | 4 | Culloty *et al*., 1999 |
| Veneridae | *Ruditapes philippinarum* | Manila clam | E and EI | Yes | No | No | No | No | 4 | Culloty *et al*., 1999 |
| Mytilidae | *Mytilus edulis* | Blue mussel | E and EI | Yes | No | No | No | No | 4 | Culloty *et al*., 1999 |
| Mytilidae | *Mytilus galloprovincialis* | Mediterranean mussel | E and EI | Yes | No | No | No | No | 4 | Culloty *et al*., 1999 |

[6] Study sites referred in Culotty *et al*., 1999 were in areas known to be infected with *B. ostreae* (later characterized by molecular test in addition to histology or cytology).

[7] Criterion B was considered as inconclusive because parasites *B. ostreae* detected in exposed *C. gigas* were detected in shell fluids and not in tissues.

| Family | Scientific name | Common name | Stages 1: Route of infection | Stage 2: Pathogen identification | Stage 3: Evidence for infection | | | | Outcome | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | A | B | C | D | | |
| ***Not scored (NS) because pathogen ID was inconclusive*** | | | | | | | | | | |
| Ostreidae | *Ostrea angasi* | Australian mud oyster | N | No | ND | ND | Inconclu-sive[8] | Yes | NS | Bougrier *et al*., 1986 |
| Ostreidae | *Ostrea denselamellosa* | Lamellated oyster | ND | No | ND | ND | ND | ND | NS | Le Borgne and le Pennec, 1983 |
| Ostreidae | *Ostrea lurida* *(O. conchaphila)* | Olympia oyster | N | No | Yes | ND | Yes | Yes | NS | Farley, 1988 |
| Ostreidae | *Crassostrea angulata* | Portuguese oyster | ND | No | ND | ND | ND | ND | NS | Katkansky *et al*.,1969, Engelsma *et al*., 2014 |

The scientific names of the species are in line with World Register of Marine Species (WoRMS) https://www.marinespecies.org/index.php (for *Crassostrea gigas* see explanatory note below).

The common names of mollusc species are in line with FAOTERM (http://www.fao.org/faoterm/collection/faoterm/en/) and https://www.sealifebase.ca. Where the common mollusc name was not found in FAOTERM, the naming was done in line with sealifebase.

[8] Criterion C was considered as inconclusive because reported mortality could possibly be due to an unidentified *Haplosporidium* parasite.

**Comments on the *ad hoc* Group's rationale and decision-making**

- The *ad hoc* Group decided to focus on studies published from the year 2000 onwards, when molecular testing was available. Papers published in earlier years were referred to where necessary to increase confidence of assessment or when no recent paper was available for the assessment of a specific host species.

- The *ad hoc* Group decided that either two papers with a score of '1', or a single study with a second study providing corroborative information, were enough to conclude susceptibility of a species. Additional studies were still checked and considered for conflicting evidence.

- The Brittle star only has a PCR positive and was thus scored as a "3" (Lynch *et al*., 2007). Although natural infection and feeding trials were carried out, information related to viability and pathology were inconclusive and information on location was not documented. *Actina equina, Ascidiella aspersa* and grouped zooplankton only have a PCR positive and were thus scored as a "3" Lynch *et al*., 2007.

- *Crassostrea ariakensis*: Cochennec *et al*., 1998, ID was based on histology and eccentric nuclei, but later confirmed by DNA sequencing (Engelsma *et al*., 2014). Limited corroborating evidence was provided by the Audemard 2005 abstract (and personal communication with co-author) regarding a cohabitation exposure trial (1/30 PCR positives following 6 mo exposure).

- *Ostrea puelchana* is currently listed as susceptible in the *Aquatic Code* but the *ad hoc* Group considered that it should be more accurately regarded as a species for which there is partial evidence for susceptibility (i.e., scored as a '2'). The study reporting this occurrence (Pascual *et al*., 1991) did not fulfil the criteria for evidence of infection (Stage 3) where only column D (Location) was scored as 'Y'.

- *Ostrea angasi* is currently listed as susceptible in the *Aquatic Code* but the *ad hoc* Group did not score this host species because pathogen identification was not provided unambiguously and it was not stated that experimental oysters were surveyed for existing infection prior to cohabitation in natural beds. Furthermore, experimental oysters were derived from an Australian locality that is now known to be endemic for *B. exitiosa*.

- *Ostrea denselamellosa* is currently listed as susceptible in the *Aquatic Code* but the *ad hoc* Group did not score this host species since the literature (Le Borgne & Le Pennec, 1983) provided no information with respect to infection with *B. ostreae*.

- *Crassostrea gigas* is currently listed as a 'carrier' in the *Aquatic Manual*, but the *ad hoc* Group found information regarding this host species to be conflicting and gave it a score of '3'. Two formal studies (Culloty *et al.,* 1999; Renault *et al*., 1995), in full or in part, met criteria for identifying a non-susceptible species. This was corroborated by the absence of detections by reference labs despite ongoing EU surveillance (extracted from EURL website, partial survey results show > 7200 animals tested from > 359 lots from areas known to be infected with *Bonamia* sp.). However, there have also been records that detect *Bonamia* sp. RNA (Gervais, 2016). Positive histology for three animals in one study (Lynch *et al*., 2010) clearly questions non-susceptibility. What is unclear is whether these histological findings reflect an early stage of phagocytosis by the host or indicate potential vector status. Consequently, further assessment of *C. gigas* is recommended pending additional information on the viability of detected organisms and/or a finalized definition for vector species.

- The *ad hoc* Group considered Article 1.5.9 in the *Aquatic Code* (Listing of susceptible species at a taxonomic ranking of Genus or higher) but felt that it was not applicable for the hosts of *B. ostreae* identified at this time.

- The *ad hoc* Group had difficulties with the current 'vector' definition and requested the Aquatic Animals Commission to discuss a new proposal and decide.

- The *ad hoc* Group noted that the inconsistency in the lists of susceptible species for infection with *B. ostreae* between Chapter 11.3 of the *Aquatic Code* and Chapter 2.4.3 of the *Aquatic Manual* should be addressed by the application of the recommendations of this *ad hoc* Group. For example, *O. denselamellosa* is currently listed as a susceptible species in the *Aquatic Code* but does not appear in the *Aquatic Manual*.

- According to WoRMS, the accepted name for *Crassostrea gigas* should be *Magallana gigas*. However, Bayne *et al.*, 2017, consider that the report by Salvi & Mariottini, 2017, is not sufficiently robust to support the proposed taxonomic change.

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# OIE *AD HOC* GROUP ON SUSCEPTIBILITY OF MOLLUCS SPECIES TO INFECTION WITH OIE LISTED DISEASES

## January–June 2020

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# OIE AD HOC GROUP ON SUSCEPTIBILITY OF MOLLUSC SPECIES TO INFECTION WITH OIE LISTED DISEASES

## January–June 2020

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## Terms of reference

**Background**

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

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

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

**Purpose**

The *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases will undertake assessments for the seven OIE listed mollusc diseases.

**Terms of Reference**

1) Consider evidence required to satisfy the criteria in Chapter 1.5.

2) Review relevant literature documenting susceptibility of species for OIE listed mollusc diseases.

3) Propose susceptible species for OIE listed diseases for molluscs based on Article 1.5.7.

4) Propose susceptible species for OIE listed diseases for molluscs based on Article 1.5.8.

**Expected outputs of the *ad hoc* Group**

1) Develop a list of susceptible species for inclusion in the relevant Article X.X.2 of mollusc disease-specific chapters in the *Aquatic Code.*

2) Develop a list of species with incomplete evidence for susceptibility for inclusion in Section 2.2.2 of the *Aquatic Manual.*

3) Draft a report for consideration by the Aquatic Animals Commission at their September 2020 meeting.

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CHAPTER 2.3.3.

# INFECTION WITH *GYRODACTYLUS SALARIS*

**EU position**

**The EU thanks the OIE and in general supports the adoption of this revised chapter. A comment is inserted in the text below.**

## 1. Scope

~~For the purpose of this chapter,~~ Infection with *Gyrodactylus salaris* means infection with the pathogenic agent *Gyrodactylus salaris*, a viviparous freshwater ectoparasite ~~(*G. salaris*)~~ of the ~~Genus *Gyrodactylus* and~~ Family *Gyrodactylidae*, ~~Order *Gyrodactylidea*,~~ and Class *Monogenea*.

**EU comment**

**The EU suggests amending the wording of the scope as follows:**

**"Infection with *Gyrodactylus salaris* means infection with the pathogenic agent *Gyrodactylus salaris*, a viviparous ~~freshwater~~ ectoparasite of the Family *Gyrodactylidae* and Class *Monogenea*, which is found in fresh and brackish waters".**

**Indeed, this parasite can also survive in brackish / esturine water. This in fact appears to be supported by the text in Section 2.3.5. which says that "*Gyrodactylus salaris* is a cold-water adapted parasite and mainly lives in freshwater".**

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

Several strains ~~or clades~~ of *G. salaris* have been identified on the basis of genotyping with the mitochondrial cytochrome oxidase 1 (CO1) marker (Hansen *et al*., 2003; 2007b; Meinilä *et al*., 2002; 2004; Mieszkowska *et al*., 2018). Although there does not seem to be an unambiguous correspondence between parasite strains as identified by CO1 and pathogenicity (Hansen *et al*., 2007a), all strains recovered from Atlantic salmon (*Salmo salar*) that have been studied in laboratory experiments, so far, are highly pathogenic to strains of Atlantic salmon. Strains non-pathogenic to Atlantic salmon have been recovered from non-anadromous Arctic charr (Salvelinus alpinus) in Norway (Olstad *et al*., 2007a; Robertsen *et al*., 2007) and rainbow trout (*Oncorhynchus mykiss*) in Denmark (Jørgensen *et al*., 2007; Lindenstrøm *et al*., 2003).

There has been a long taxonomic/scientific debate on whether *Gyrodacytlus thymalli*, a species described from grayling (*Thymallus thymallus*), is a junior synonym of *G. salaris* (see e.g. Hansen *et al*., 2003; 2007a, 2007b; Meinilä *et al*, 2004, Fromm *et al*, 2014), and most evidence favours such a synonymisation. The National Center for Biotechnology Information (NCBI) has accepted ~~the~~ a synonymisation of *G. salaris* and *G. thymalli* with the result that all accessions of DNA sequences previously assigned to *G. thymalli* are now assigned to *G. salaris*. Irrespective of this debate, strains isolated from grayling have never been found to be pathogenic to Atlantic salmon in experimental trials (see e.g. Sterud *et al*., 2002), and have not been observed ~~do not seem to occur~~ on Atlantic salmon when in sympatry with grayling (Anttila *et al*., 2008). ~~In~~ For the purpose of this chapter, ~~it is assumed that~~ *G. salaris* and *G. thymalli* are treated as two separate species.

#### 2.1.2. Survival and stability off the host or in processed or stored samples

Survival of detached *G. salaris* is temperature dependent: approximately 24 hours at 19°C, 54 hours at 13°C, 96 hours at 7°C and 132 hours at 3°C (Olstad *et al*., 2006). *Gyrodactylus salaris* is known to survive between temperatures of 0°C to 25°C. Tolerance to temperatures above 25°C is unknown. *Gyrodactylus salaris* is sensitive to freezing and desiccation. It dies after a few days at pH≤5. It is more

sensitive to low pH (5.1<pH<6.4) in association with aluminium and zinc than the host Atlantic salmon (Poleo *et al*., 2004; Soleng *et al*., 1999). ~~and recently, it was also found that *G. salaris* is sensitive to low doses of chlorine (Hagen *et al*., 2014).~~ For inactivation methods, see Section 2.4.5.

#### 2.1.3. Survival and stability on host tissues

Survival of *G. salaris* attached to a dead host is temperature dependent: maximum survival times for *G. salaris* on dead Atlantic salmon are 72, 142 and 365 hours at 18°C, 12°C and 3°C, respectively (Olstad *et al*., 2006).

## 2.2. Host factors

#### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) ~~include~~ are: Arctic char (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), ~~North American~~ brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*) and rainbow trout (*Oncorhynchus mykiss*).

#### 2.2.2. Species with incomplete evidence for susceptibility

~~None known.~~

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Code* are: none known.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have identified *G. salaris* on the following organisms, but a long-term active infection has not been demonstrated: [Under study].

~~**2.2.3. Non-susceptible species**~~

~~Species that have been found non-susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Code* are: none known [under study].~~

#### 2.2.~~4~~ 3. Likelihood of infection by species, host life stage, population or sub-populations

The prevalence and abundance of *G. salaris* on Atlantic strains of ~~Atlantic salmon (*Salmon* *S. salar*)~~ are higher than ~~in~~ on other susceptible species and Baltic strains of *S. salar*. All life stages are susceptible, but prevalence and abundance ~~in~~ on Atlantic salmon are highest in fry and parr stages, where mortality is also most likely to be observed.

For the purposes of Table 4.1 Atlantic salmon alevins and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, parr and smolts can be considered as juveniles and all fish post smoltification as adults.

#### 2.2.~~5~~ 4. Distribution of the pathogen on the host

*Gyrodactylus salaris* usually occurs on the fins of infected Atlantic salmon, but the parasite distribution on the host may vary depending on intensity of infection (Jensen & Johnsen, 1992; Mo, 1992; Paladini *et al*., 2014). Parasites are also commonly found on the body but less commonly on the gills. On other hosts, the distribution may be different, but in general the parasite is relatively less abundant on the fins and relatively more common on the body compared with Atlantic salmon.

#### 2.2.~~6~~ 5. Aquatic animal reservoirs of infection

There are a number of combinations of host species and *G. salaris* strains which do not result in clinical signs of disease and may, therefore, act as reservoirs of infection. ~~Some~~ Several stocks of Atlantic salmon in the Baltic region are infected with *G. salaris* but do not generally show clinical signs or suffer mortality (Anttila *et al.,* 2008). *Gyrodactylus salaris* has been found in wild Arctic char without any observable signs or mortality (Robertsen *et al.,* 2007). Rainbow trout can be infected with some strains of *G. salaris* at a very low prevalence and abundance without observable signs (Paladini *et al.,* 2014).

#### 2.2.~~7~~ 6. Vectors

*Gyrodactylus salaris* parasites may attach themselves to any fish species not considered a susceptible species, for short periods of time. On some species limited reproduction takes place, but insufficient for the parasite to maintain a persistent infection (Paladini *et al.,* 2014). Thus, whilst any fish species could act as a vector, those on which reproduction occurs, are more likely to act as vectors. However, there is no evidence from the published literature that fish vectors ~~they are important in the epidemiology of~~ have transmitted *G. salaris*.

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

Mortality in farmed Atlantic salmon fry and parr can be 100% if not treated. Mortality in wild Atlantic salmon fry and parr in Norwegian rivers can be as high as 98%, with an average of about 85% (Johnsen *et al*, 1999). Mortality in other susceptible species is usually low to negligible.

Prevalence in susceptible strains of Atlantic salmon reaches close to 100% in wild parr in rivers (Appleby & Mo, 1997); similarly, prevalence in farmed Atlantic salmon (in freshwater) rises to close to 100% within a short time after introduction of the parasite. Prevalence in resistant strains of Atlantic salmon in rivers and farms is ~~unknown~~ likely to be low~~, but has not been well documented~~ (Bakke *et al.,* 2007) and highly variable depending on season, location and age of the fish (Anttila *et al.,* 2008). Prevalence in other susceptible species is usually much lower than in Atlantic salmon and can be below 10% (e.g. in farmed rainbow trout; Buchmann & Bresciani, 1997).

### 2.3.2. Clinical signs, including behavioural changes

~~Usually there are no clinical signs in~~ Wild Atlantic salmon with low infection~~s~~ intensities (one or up to a few tens) of *G. salaris* parasites usually do not exhibit any clinical signs. Increased parasite mean intensity over time often leads to increased flashing (fish scratch their skin on the substrate), increased mucous production (giving the fish a greyish appearance) and erosion of the fins. ~~In the early disease phase in susceptible stocks of wild Atlantic salmon, increased flashing (fish scratch their skin on the substrate) is typical. Later, fish may become greyish because of increased mucous production and the fins may be eroded. Diseased fish are lethargic and are usually found in slower-moving water.~~

~~Flashing is common among moderate to heavily infected farmed Atlantic salmon as they scratch their skin on the bottom or wall of a tank or pond. Heavily infected fish may have reduced activity and stay in low current areas.~~

Susceptible species other than Atlantic salmon usually only carry low numbers of *G. salaris* parasites and do not show clinical signs. ~~Rainbow trout usually only carry low numbers of *G. salaris* parasites and do not show clinical signs.~~

### 2.3.3 Gross pathology

Heavily infected Atlantic salmon may become greyish as a result of increased mucification, and at a later stage the dorsal and pectoral fins may become whitish as a result of increased thickness (mainly ~~hypertrophy~~ hyperplasia) of the epidermis. As the infestation continues, fish may have eroded fins, especially dorsal, tail and pectoral fins, because of parasite feeding. Secondary fungal infections (*Saprolegnia* spp.) are commonly observed in fish with infection with *G. salaris*.

### 2.3.4. Modes of transmission and life cycle

*Gyrodactylus salaris* is an obligate parasite with a direct life cycle. Parasites give birth to live offspring, and there are no other life stages. *Gyrodactylus salaris* can transfer to a new host via contact with live hosts, dead hosts, detached parasites ~~drifting~~ in the water column, or parasites attached to the substrate.

*Gyrodactylus salaris* has spread between rivers and farms mainly by the translocation of live fish. Fish migrating through brackish water can also spread the parasite between neighbouring rivers (see also Section 2.3.5). The risk of transmission is greater between rivers located within the same brackish water system.

### 2.3.5. Environmental ~~and management~~ factors

*Gyrodactylus salaris* is a cold-water-adapted parasite and mainly lives in freshwater, reproducing normally at salinities up to 5–6 ppt (Malmberg, 1973; 1988). The average number of offspring per parasite peaks between 6.5°C and 13.0°C (Jansen & Bakke ~~*et al.*~~, 1991). At lower temperatures, *Gyrodactylus salaris* can survive longer in higher salinities ~~at lower temperatures~~ (Soleng & Bakke, 1997). For example at 1.4°C, *G. salaris* may survive for 240 hours, 78 hours and 42 hours at 10 ppt, 15 ppt and 20 ppt salinity, respectively, while at 12°C it may survive for 72 hours, 24 hours and 12 hours at the same three salinities, respectively (Soleng & Bakke, 1997).

Although *G. salaris* mainly lives in freshwater, it reproduces normally at salinities up to 5–6 ppt. Survival at higher salinities is temperature dependent. For example at 1.4°C, *G. salaris* may survive for 240 hours, 78 hours and 42 hours at 10 ppt, 15 ppt and 20 ppt salinity, respectively, while at 12°C it may survive for 72 hours, 24 hours and 12 hours at the same three salinities, respectively (Soleng & Bakke, 1997).

*Gyrodactylus salaris* is sensitive to changes in the chemical composition of the water. It is sensitive to the most commonly used chemicals for bath treatment of farmed salmon parr and eggs (e.g. high salinity salt water, formaldehyde and compounds containing chlorine and iodine). Furthermore, *G. salaris* is sensitive to acidic solutions (pH 5.0–6.0) of aluminium sulphate ($[Al_2(SO_4)_3]$) and zinc (ZN) (Poleo *et al*., 2004; Soleng *et al*., 1999). As aluminium sulphate is less toxic to fish than to *G. salaris* in moderately acidified waters, and this chemical has been used to eradicate the parasite from one river system in Norway (Pettersen *et al*., 2007). *Gyrodactylus salaris* is sensitive to low doses of chlorine (Hagen *et al*. 2014).

#### 2.3.6. Geographical distribution

The original distribution of *Gyrodactylus salaris* is considered to be within the eastern parts of the Baltic area including the drainages of the Russian lakes Onega and Ladoga (Ergens, 1983; Malmberg & Malmberg, 1993). From these areas, the parasite has spread and it has been reported from several countries in Europe (Paladini *et al.,* 2021 in press) in both wild and farmed populations. *Gyrodactylus salaris* is restricted in its distribution to Europe. It has been recovered from farmed Atlantic salmon or farmed rainbow trout in several (mainly northern) European countries. In the wild, The parasite has been found on

wild salmonids, mainly Atlantic salmon parr, in rivers in Finland, Norway, Russia and Sweden. , Finland and Norway. In some areas, the parasite continues to spread, and in 2015 it was detected on salmon parr in a new area in the north of Russia. In 2006, Infection with *G. salaris* was reported from fish farms in Italy (Paladini *et al*., 2009) and, in 2007, from fish farms in Poland (Rokicka *et al*., 2007) and Macedonia (Zietara *et al*., 2007). In 2009, *G. salaris* was identified from fish farms in Romania (Hansen *et al*., 2014). The parasite has never been detected in the United Kingdom or in the Republic of Ireland.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

### 2.4. Biosecurity and disease control strategies

#### 2.4.1. Vaccination

Vaccines are not available.

#### 2.4.2. Chemotherapy including blocking agents

Not applicable.

#### 2.4.3. Immunostimulation

Immunostimulation is not available.

#### 2.4.4. Breeding resistant strains

In laboratory experiments, selected breeding of Atlantic salmon has resulted in increased survival among the offspring (Salte *et al*., 2010). However, stocking rivers with resistant strains has not been attempted because the stock will remain infected and thus the parasite may spread to other rivers with susceptible hosts. In addition, stocking with resistant strains of Atlantic salmon (e.g. Baltic Neva strain) in affected rivers is not considered compatible with existing strain management of Atlantic salmon (i.e. preservation of the genetic integrity of wild stocks) (Karlsson *et al.*, 2019).

#### 2.4.5. Inactivation methods

Not applicable. *Gyrodactylus salaris* is killed by exposure to water at 40°C for 5 minutes (Koski *et al.*, 2016) and by a commonly used oxidising to disinfectant (e.g. 1% Virkon S for 15 minutes) (Koski *et al.*, 2016), which may be used to eliminate transfer of the parasite with and can be used to disinfect equipment.

#### 2.4.6. Disinfection of eggs and larvae

Eggs that are transferred from infected farms should be disinfected (iodine-containing compounds have been used).

#### 2.4.7. General husbandry

*Gyrodactylus salaris* is sensitive to changes in the chemical composition of the water. It is killed by sensitive to the most commonly used chemicals for bath treatment of farmed salmon parr and eggs

(e.g. high salinity salt water, formaldehyde and compounds containing chlorine or iodine) (Thrush *et al.*, 2019). Treatment of farmed salmonid populations with formaldehyde or other bath treatments will reduce the prevalence and abundance of *G. salaris* and may therefore render detection more difficult.

*Gyrodactylus salaris* is sensitive to acidic solutions (pH 5.0–6.0) of aluminium sulphate ($[Al_2(SO_4)_3]$) and zinc (Zn) (Poleo *et al.*, 2004; Soleng *et al*., 1999). Aluminium sulphate is less toxic to fish than to *G. salaris* in moderately acidified waters, and has been used to eradicate the parasite from one river system in Norway (Pettersen *et al*., 2007). Recently, it was also found that *G. salaris* is sensitive to low doses of chlorine (Hagen *et al.*, 2014).

~~The spread of *G. salaris* between freshwater fish farms and between rivers may be avoided by disinfection of equipment (e.g. fish nets) before translocation (see section 2.4.5).~~

~~Restocking with resistant strains of Atlantic salmon (e.g. Baltic Neva strain) in affected rivers is not considered compatible with existing strain management of Atlantic salmon (i.e. preservation of the genetic integrity of wild stocks) (Karlsson *et al*., 2019).~~

## 3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

### 3.1. Selection of populations and individual specimens

Sampling wild healthy populations should take place during the late summer or autumn or when the prevalence is known to be at its highest. Atlantic salmon should be targeted. In farms, fish showing clinical signs of infection (as described in Section 2.3.1) should be selected. Sampling should be avoided for a period after treatment for ectoparasites. In the absence of clinical signs, sampling ~~in~~ of wild Atlantic salmon populations should target year class 1+ and 2+ as these are more likely of being infected than 0+ parr. ~~Grayling should not be sampled as they are not highly susceptible to *G. salaris*, and the possible detection of *G. thymalli* will create unnecessary diagnostic investigations.~~

### 3.2. Selection of organs or tissues

Detection of *Gyrodactylus* and identification of *G. salaris* is a two-step process. Firstly, gyrodactylid parasite specimens are detected (e.g. on fish or fins) using optical equipment and picked ~~out~~ off~~,~~. ~~and~~ Individual parasites are identified to species level using other equipment and methods.

Fish should be examined as whole specimens either live under anaesthesia (for example, with MS222), freshly killed, or preserved. In addition, fresh or preserved fins can be examined. Examination of live, anaesthetised fish is very time-consuming and not recommended. When Atlantic strains of Atlantic salmon parr are infected, almost all fish have at least one *G. salaris* on one of the fins. On some fish, *G. salaris* specimens may occur on the body or head, including the nostrils, the gills and the mouth cavity. The distribution of *G. salaris* on fins and other parts of the fish varies among fish species and strains of Atlantic salmon. For all hosts the examination of whole fish is recommended as it will increase the likelihood of detecting low intensity infections.

~~Live anaesthetised fish, freshly cut fins or EtOH-preserved fish or fins should be examined under a binocular dissecting microscope with good illumination. The fish should be placed in a box and completely covered in freshwater. Preserved fish can also be examined in EtOH. Living parasites are more easily detected by their movements, thus disturbing light refraction on the skin of the fish should be avoided. Live *Gyrodactylus* are colourless while EtOH preserved *Gyrodactylus* specimens are usually slightly opaque. Dark field illumination microscopy will increase the contrast and the parasites will be detected more easily. The whole surface of the fish, including gills and mouth cavity, must be examined. It is best to use two forceps for this process. The fins of relatively small fish, usually less than 10 cm, can also be studied using illumination through the bottom of the microscope stage, which makes *Gyrodactylus* specimens easy to observe.~~

### 3.3. Samples or tissues not suitable for pathogen detection

Dead fish, stored on ice, are not acceptable for *Gyrodactylus* examination, even if the fish are kept separately in plastic bags, etc. The parasites die quickly if not covered in water and rapidly disintegrate.

### 3.4. Non-lethal sampling

Fish can be examined as live specimens under anaesthesia (for example, with MS222). Recently, a non-lethal method for isolating specimens of gyrodactylid parasites from fish was developed and tested on brown trout (Thrush *et al*., 2019). The method was shown to have a higher parasite recovery rate compared to whole body examination of killed fish (84.6% and 51.9%, respectively). The method has not yet been used on fish infected with *G. salaris,* but it is likely to be effective.

In addition, environmental DNA (eDNA) methods have been developed for detection of *G. salaris*, and its two main hosts, Atlantic salmon and rainbow trout, in water samples ~~have been developed~~ (Rusch *et al*., 2018). However, detection limits have not been established for these analyses.

## 3.5. Preservation of samples for submission

Fish should be killed immediately and should not be allowed to dry out before preservation. Whole fish should be preserved in 80–100% EtOH in bottles large enough to provide excess space and preservative. The concentration of EtOH after preservation should not be below 70%. As a rule of thumb this concentration is obtained if the proportion of fish tissue to EtOH does not exceed 1:9. If the concentration is lower, the mucous and epidermis may disintegrate and *Gyrodactylus* specimens, even if they are preserved, may drop off. Bottles should have an opening wide enough to avoid the possibility of scraping off *Gyrodactylus* specimens when fish are put into the bottle or when taken out for examination. Bottles should be stored in a horizontal position until the tissue is fixed/preserved to prevent the fish curling. When preservation of the fish is complete, the bottles can be stored in a vertical position.

As *G. salaris* is common on fins of Atlantic salmon, fins cut off from the body and stored in EtOH as described above can also be submitted. This is especially suitable for larger fish and under field conditions where, for example, transport is limited.

Formaldehyde-fixed *Gyrodactylus* specimens are difficult to identify morphologically and are also often unsuitable for DNA analysis.

### 3.5.1. Samples for pathogen isolation

Not applicable.

### 3.5.2. Preservation of samples for molecular detection

Tissue samples, i.e. isolated parasites, whole fish or fins, for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 9:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended.

Template DNA should be prepared from live/fresh or EtOH-preserved specimens using a suitable DNA preparation protocol. DNA extraction kits may be used according to the manufacturers' instructions.

### 3.5.3. ~~Fixed~~ Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Not applicable.

~~**3.5.4. Fixed Samples for electron microscopy**~~

~~Not applicable.~~

### 3.5.~~5~~ 4. Samples for other tests

*Preservation of samples for environmental DNA (eDNA) analyses*

Several methods for filtering water for eDNA analyses exist and the method has also been developed for ~~use on~~ the detection of *G. salaris* and its hosts, Atlantic salmon ~~*Salmo salar*~~ and ~~*Oncorhynchus mykiss*~~ rainbow trout (Rusch *et al*., 2018). In this method, duplicate water samples of 5 litres (2 × 5 litres) ~~should be~~ are collected and filtered on site on to glass fibre filters (47 mm AP25 Millipore, 2 µm pore size, Millipore, Billerica, USA) using a suitable pump, tubing and filter holder. Filters should be placed in separate zip-lock plastic bags containing silica gel and stored dry and dark until further analysis in the laboratory.

## 3.6. Pooling of samples

Sampled fish can be pooled, although each fish should subsequently be examined and analysed separately. Fins of fish from a farm or a river can be pooled and ~~are~~ should also be examined and analysed separately, but in this instance each fin cannot be related to ~~a certain~~ individual fish ~~host~~. Similarly, if fish are pooled for parasite removal using non-lethal bath methods (e.g. Thrush *et al.,* 2019), the parasites recovered cannot be related to individual fish.

Material from parasites should not be pooled for molecular diagnostic methods as data on the impact on diagnostic sensitivity and diagnostic specificity are not currently available.

# 4. Diagnostic methods

The methods currently available for identifying infection for surveillance (in healthy populations), presumptive and confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

| | |
|---|---|
| +++ = | Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; OIE recommended method(s) will be mentioned in the text; |
| ++ = | Suitable method(s) but may need further validation; |
| + = | May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application; |
| Shaded boxes = | Not appropriate for this purpose. |

The selection of a test for a given purpose depends on sensitivity, specificity, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay sensitivity or specificity, such as tissue components inhibiting amplification, nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

***Table 4.1.*** *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Morphological examination** | | + | + | 1 | | + | + | 1 | | | | |
| **Histopathology[3]** | | | | | | | | | | | | |
| **Cytopathology[3]** | | | | | | | | | | | | |
| **Cell culture** | | | | | | | | | | | | |
| **Real-time PCR (using parasite sample)** | | + | + | 1 | | + | + | 1 | | | | |
| **ddPCR/Real-time PCR (using environmental sample)** | + | | | 1 | | | | | | | | |
| **Conventional PCR** | | ± | ± | 1 | | ± | ± | 1 | | ++ | ++ | 2 |
| **Amplicon sequencing[4]** | | | | | | | | | | ++ | ++ | 2 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; ddPCR = droplet digital PCR; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3.
[3]Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. [4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose

### 4.1. Parasite detection

Live anaesthetised fish, freshly cut fins or EtOH-preserved fish or fins should be examined under a binocular dissecting microscope with good illumination. The fish should be placed in a box and completely covered in freshwater. Preserved fish can also be examined in EtOH. Living parasites are more easily detected by their movements, thus disturbing light refraction on the skin of the fish should be avoided. Live *Gyrodactylus* are colourless while EtOH-preserved *Gyrodactylus* specimens are usually slightly opaque. Dark field illumination microscopy will increase the contrast and the parasites will be detected more easily. The whole surface of the fish, including gills and mouth cavity, must be examined. It is best to use two forceps for this process. The fins of relatively small fish, usually less than 10 cm, can also be studied using illumination through the bottom of the microscope stage, which makes *Gyrodactylus* specimens easy to observe.

A non-lethal method (Thrush *et al.*, 2019) results in the collection of ectoparasites from the treated fish on filter paper. The filter can then be screened for the presence of parasites using a stereomicroscope.

Once individual gyrodactylid parasites have been visualised, they can be removed from the fish, fins or filter paper using a pipette. The species of gyrodactylid can be determined using one of the tests described in this section.

### 4.2. Morphological examination

Morphological identification of *Gyrodactylus* species is based on the morphology and morphometry of marginal hooks anchors (hamuli) and bars in the ~~opist~~haptor (the attachment organ). Good preparation of specimens is a prerequisite for species identification. Morphological identification is only recommended for preliminary diagnosis of *G. salaris* and should not be used for confirmation, for which molecular methods are recommended.

Digestion of the soft tissue, leaving the hard parts only, is recommended when high-resolution morphometrics is required for reliable morphometric diagnosis. The soft tissue can be digested in a solution (approx. 1 µl) of 75 mM Tris, 10 mM EDTA (ethylene diamine tetra-acetic acid), 5% SDS (sodium dodecyl sulphate) and 100 mg $ml^{-1}$ proteinase K, pH 8.0. After adding the digestion solution, the reaction should be ~~inspected~~ monitored ~~in the~~ microscopically until completion and then ended by adding a stop solution (1:1 glycerol and 10% neutral buffered formalin). The procedure for digestion is described in detail in Harris *et al*., 1999. Identification of *G. salaris* should be in accordance with references: Cunningham *et al*., 2001; Malmberg, 1957; 1970; McHugh *et al*., 2000; Olstad *et al*., 2007b; Shinn *et al*., 2004.

The size of the ~~opist~~haptoral hard parts in *Gyrodactylus* varies extensively with, for example, temperature, whereas shape is more stable (see e.g. Mo, 1991~~a~~). The capability of linear measurements to capture morphology might therefore not always be sufficient for reliable diagnosis (Olstad *et al*., 2007b).

*Gyrodactylus salaris* can be differentiated from other *Gyrodactylus* species by trained morphologists on the basis of morphology but not from *G. thymalli* (Olstad *et al*., 2007b; and see Section 2.1.1). In addition, *G. salaris* is morphologically similar to *Gyrodactylus teuchis* from brown trout, Atlantic salmon, and rainbow trout, but can be differentiated by trained morphologists on the basis of the shape of the marginal hook sickle. *Gyrodactylus teuchis* has a longer and more constantly curved sickle blade (see Cunningham *et al*., 2001).

### 4.3. Histopathology and cytopathology

Not applicable.

### 4.4. Cell ~~or artificial media~~ culture for isolation

Not applicable.

### 4.5. Nucleic acid amplification

For all molecular tests below DNA can be extracted using standard DNA extraction kits.

#### 4.5.1. Real-time PCR

Both real-time PCR (Collins *et al*., 2010) and digital droplet (dd) PCR (Rusch *et al*., 2018) have been developed for *G. salaris*. Real-time PCR has not been widely applied for diagnostics of *G. salaris,* and ddPCR is developed for use in connection with eDNA-methods. Both these methods target the ribosomal internal transcribed spacers region (ITS) and have the same diagnostic limitations (see below and Section 4.5.2) ~~as described in Sections 4.5.1 and 4.5.2~~. However, real-time PCR is faster than conventional PCR and DNA sequencing (Section 4.4.2) and can be applied as a fast means to exclude species other than *G. salaris/G. thymalli*, and the method is therefore mentioned briefly here. Conventional PCR and sequencing of the mitochondrial cytochrome oxidase ~~I~~ 1 (CO1) gene (Sections 4.4.2 and 4.5.2), ~~which is necessary for species confirmation and haplotype identification,~~ can then be performed on those species with a positive result from real-time PCR ~~to which is necessary~~ for ~~species confirmation and~~ haplotype identification, which will allow *G. salaris* to be differentiated from *G. thymalli* (4.6.2).

The real-time PCR assay of Collins *et al.* (2010) is a TaqMan minor groove binder (MGB) real-time PCR assay that targets a 60 bp unique sequence motif in the ITS1 region of *G. salaris*/*G. thymalli*. It applies the forward primer F (5'-CGA-TCG-TCA-CTC-GGA-ATC-G-3'), reverse primer R (5'-GGT-GGC-GCA-CCT-ATT-CTA-CA-3') and TaqMan MGB probe Gsal2 (5'-FAM-TCT-TAT-TAA-CCA-GTT-CTG-C-3') labelled with the fluorescent reporter dye FAM at the 5'-end and a non-fluorescent quencher MGBNFQ at the 3'-end. Amplifications were performed in a total volume of 20 µl containing TaqMan Universal PCR Master mix (with UNG; Applied Biosystems), 0.9 µM of each forward and reverse primer and 0.25 µM of each probe and dH2O (Sigma) to a final volume of 20 µl. One µl of lysate from a parasite specimen was added to the each test tube. The cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 35 cycles of 95°C for 15 seconds and 60°C for 1 minute and run in an ABI 7000 Sequence Detection System (Applied Biosystems). The efficiency of the singleplex assay was reported as ranging from 93.1% to 101.1% and the limit of detection (dilution of the crude *Gyrodactylus spp.* lysate) was ~~as~~ $10^{-4}$. Further details can be found in Collins *et al.* (2010). ~~**Note**: Low level cross-amplification of *Gyrodactylus derjavinoides* DNA has been observed using the real-time PCR set-up described here (Rusch *et al.*, 2018).~~

#### 4.5.2. Conventional PCR

*Analysis of the ribosomal RNA gene internal transcribed spacer region* (*ITS*)

*For amplification of a 1300 base pair product of the ITS-region, covering ITS1, 5.8S,* and ITS2, primers, such as 5'-TTT-CCG-TAG-GTG-AAC-CT-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3', may be used. The cycling conditions for PCR are as follows, initial denaturation at 95°C for 5 minutes; 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes (Cunningham, 1997). If partially degraded material is analysed or if the PCR above does not give a positive result, the ITS1 and ITS2 spacers can be amplified in two separate reactions using primer sets and PCR conditions described in Matejusová *et al.*, 2001. The amplification of ITS2 alone, using the primers 5'-CAT-CGG-TCT-CTC-GAA-CG-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3' and using the same protocol as above is sufficient.

The primers for amplification of ITS are not specific to *G. salaris* and will amplify all or most species of *Gyrodactylus*. Positive PCR products should thus be sequenced to identify the haplotype, which can be used for species confirmation (see Section 4.5).

*Analysis of the mitochondrial cytochrome oxidase I* (*CO1*) *gene*

For amplification of the CO1-gene, the primers 5'-ATA-TAG-ACG-ATT-TGT-TTT-CA-3' and 5'-ACA-GAT-TAC-TTG-GTA-TTA-CA-3' (Kuusela *et al.,* 2009) may be used to amplify the full-length gene (1600 base pairs) which is recommended. The primers 5'-TAA-TCG-GCG-GGT-TCG-GTA-A-3' and 5'-GAA-CCA-TGT-ATC-GTG-TAG-CA-3') (Meinilä *et al*., 2002) may be used to amplify a 800 base pairs fragment if the first PCR is unsuccessful. The cycling conditions for both PCRs are as follows, initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes. Additional primer sets for amplification of CO1 can be found in references: Hansen *et al*., 2003; Kuusela *et al.,* 2009; Meinilä *et al.,* 2002; 2004.

Primers recommended for amplification of CO1 may not be specific for *G. salaris* and may not amplify all isolates. Positive PCR products should ~~thus~~ be sequenced to identify the haplotype ~~for species confirmation~~ (Section 4.6).

The following controls should be run with each assay: negative extraction control; positive control; no template control.

#### 4.5.3. Other nucleic acid amplification methods

Not applicable.

## 4.6. Amplicon sequencing

#### 4.6.1. ITS sequencing and sequence analysis

Amplified ITS fragments prepared as in Section 4.4.2 should be sequenced using the PCR primers and, in addition, internal sequencing primers (Cunningham, 1997; Matejusová *et al.*, 2001) should be used to obtain overlapping reads of each nucleotide. The resulting ITS sequences should be subjected to a BLAST search in GenBank/EMBL to establish identity with known sequences. Several sequences of other species infecting salmonids, e.g. *G. derjavini*, *G. derjavinoides*, *G. truttae*, and *G. teuchis* are available in GenBank/EMBL. *G. thymalli* cannot be distinguished from *G. salaris* by this method, but sequences of ITS distinguishes *G. salaris* from all other known species. ~~GenBank has synonymised *G. salaris* and *G. thymalli* and~~ Host identity of sequences in GenBank/EMBL should thus always be checked, however, GenBank has synonymised *G. salaris* and *G. thymalli*. Therefore, if the BLAST search of the ITS sequences identifies the parasite as *G. salaris*, CO1 sequencing and sequence analysis ~~should be performed~~ are recommended to identify the haplotype in question (Section 4.6.2).

#### 4.6.2. CO1 sequencing and sequence analysis

Amplified CO1 fragments prepared as described in Section 4.5.2 should be sequenced using the PCR primers and, in addition, internal sequencing primers (Kuusela *et al*., 2009; Meinilä *et al*., 2002) should be used to obtain overlapping reads of each nucleotide. The resulting CO1 sequences should be subjected to a BLAST search in GenBank/EMBL to identify the haplotype.

If the obtained sequence does not have a 100% match in GenBank/EMBL, a phylogenetic analysis can be performed to establish the relationship to other available sequences. Different haplotypes and clades of *G. salaris* and *G. thymalli* can be distinguished with this method. CO1 sequences can be used to assign specimens to a haplotype or clade and thus infer the identity as *G. salaris* or *G. thymalli*. Clades (~~haplogroups~~ i.e. groups of haplotypes with a common ancestor) of *G. salaris* generally correspond well to host preferences ~~and/~~or the geographical distribution of the parasites, with a few exceptions, and some strains, as defined by CO1-sequences (haplotypes), are known to be pathogenic to Atlantic salmon. ~~Host identity can be used to infer potential pathogenicity of a certain strain and thus host identity of sequence hits in GenBank/EMBL should always be checked when BLAST results are returned.~~

GenBank has synonymised *G. salaris* and *G. thymalli*, with the result that all accessions previously listed as *G. thymalli* are now *G. salaris;* the haplotypes in Table 4.6.2 can be retrieved from GenBank. Table 4.6.2 assigns the haplotypes to either *G. salaris* or *G. thymalli,* to support identification of *G. salaris* based on CO1 sequencing (new haplotypes should be compared to the nearest known relative). In rivers where both grayling and Atlantic salmon are found, establishing the *G. thymalli* haplotypes present on grayling will support any subsequent monitoring for *G. salaris* on Atlantic salmon.

***Table 4.6.2*** Gyrodactylus salaris *and* G. thymalli *GenBank accession numbers for CO1 nucleotide sequences*

| ***G. salaris**** | | | | | | | ***G. thymalli**** | | |
|---|---|---|---|---|---|---|---|---|---|
| AF479750 | AY146602 | AY258354 | AY486492 | AY486517 | AY486542 | EU186166 | AF540893 | AY486545 | DQ159928 |
| AF540891 | AY146603 | AY258355 | AY486493 | AY486518 | AY486543 | EU186167 | AF540894 | AY486546 | DQ180333 |
| AF540892 | AY146604 | AY258356 | AY486494 | AY486519 | AY840222 | EU186168 | AF540895 | AY486547 | DQ993195 |
| AF540904 | AY146605 | AY258357 | AY486495 | AY486520 | AY840223 | EU186169 | AF540896 | AY486548 | EF495063 |
| AF540905 | AY146606 | AY258358 | AY486496 | AY486521 | DQ468128 | EU186170 | AF540897 | AY486549 | EF527269 |
| AF540906 | AY146607 | AY258359 | AY486497 | AY486522 | DQ517533 | EU186171 | AF540898 | AY486550 | EF612464 |
| AF542161 | AY146614 | AY258360 | AY486498 | AY486523 | DQ778628 | EU186172 | AF540899 | AY486551 | MG273445 |
| AF542162 | AY258336 | AY258361 | AY486499 | AY486524 | DQ923578 | EU186173 | AF540900 | AY486552 | MG273446 |

| ***G. salaris**** | | | | | | | ***G. thymalli**** | | |
|---|---|---|---|---|---|---|---|---|---|
| AF542163 | AY258337 | AY258362 | AY486500 | AY486525 | DQ988931 | EU186174 | AF540901 | AY486553 | MG273447 |
| AF542164 | AY258338 | AY258363 | AY486501 | AY486526 | DQ993189 | EU186175 | AF540902 | AY840224 | MG273448 |
| AF542165 | AY258339 | AY258364 | AY486502 | AY486527 | DQ993190 | EU186176 | AF540903 | DQ159913 | . |
| AF542166 | AY258340 | AY258365 | AY486503 | AY486528 | DQ993191 | EU186177 | AF542167 | DQ159914 | . |
| AY146589 | AY258341 | AY258366 | AY486504 | AY486529 | DQ993192 | EU223246 | AF542168 | DQ159915 | . |
| AY146590 | AY258342 | AY258367 | AY486505 | AY486530 | DQ993193 | EU304825 | AF542169 | DQ159916 | . |
| AY146591 | AY258343 | AY258368 | AY486506 | AY486531 | DQ993194 | GQ129460 | AF542170 | DQ159917 | . |
| AY146592 | AY258344 | AY258369 | AY486507 | AY486532 | EF117889 | GQ129461 | AF542171 | DQ159918 | . |
| AY146593 | AY258345 | AY258370 | AY486508 | AY486533 | EF524576 | GQ129462 | AY146608 | DQ159919 | . |
| AY146594 | AY258346 | AY258371 | AY486509 | AY486534 | EF524577 | GQ129463 | AY146609 | DQ159920 | . |
| AY146595 | AY258347 | AY258372 | AY486510 | AY486535 | EF524578 | GQ370816 | AY146610 | DQ159921 | . |
| AY146596 | AY258348 | AY258373 | AY486511 | AY486536 | EF570120 | GU187354 | AY146611 | DQ159922 | . |
| AY146597 | AY258349 | AY258374 | AY486512 | AY486537 | EU186161 | KJ941020 | AY146612 | DQ159923 | . |
| AY146598 | AY258350 | AY486488 | AY486513 | AY486538 | EU186162 | KT344124 | AY146613 | DQ159924 | . |
| AY146599 | AY258351 | AY486489 | AY486514 | AY486539 | EU186163 | KT344125 | AY472084 | DQ159925 | . |
| AY146600 | AY258352 | AY486490 | AY486515 | AY486540 | EU186164 | KT344126 | AY472085 | DQ159926 | . |
| AY146601 | AY258353 | AY486491 | AY486516 | AY486541 | EU186165 | KT344127 | AY486544 | DQ159927 | . |
| | | | | | | KT344128 | | | |

*Note: *G. salaris* and *G. thymalli* have been synonymised by NCBI GenBank, i.e. all accessions previously listed as *G. thymalli* are now *G. salaris*.

Where the sequence is not assigned to one of the recognised haplotypes (CO1 sequences) of *G. salaris* or *G. thymalli* advice should be sought from the OIE Reference Laboratory. The OIE Reference Laboratory will keep an updated database of CO1-sequences and will assist in the diagnosis. It is recommended that the OIE Reference Laboratory is informed of any significant detections of *G. salaris* and *G. thymalli* in order to confirm the cases.

### 4.7. *In-situ* hybridisation

Not applicable.

### 4.8. Immunohistochemistry

Not applicable.

### 4.9. Bioassay

Not applicable.

### 4.10. Antibody- or antigen-based detection methods (ELISA, etc.)

Not applicable.

### 4.11. Other methods

Not applicable.

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance to demonstrate freedom of disease in apparently healthy populations. Sequencing of the amplified CO1 amplicon is required for confirmation of infection in any parasite that identified as positive by PCR.

## 6. Corroborative diagnostic criteria

All suspect positive samples of *G. salaris* from country or zone or compartment considered free from infection with *G. salaris* should be referred immediately to the OIE Reference Laboratory ~~for confirmation,~~ to definitively identify the parasite based on the most up-to-date information (see Section 4.6). Submissions should be made whether or not clinical signs ~~are associated with the case~~ have been observed.

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

### 6.1. Detection in apparently healthy animals or animals of unknown health status[9]

Healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations will be sampled in surveys to demonstrate disease freedom.

#### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

i) Identification of *G. salaris* by morphological examination;

ii) A positive result by real-time PCR;

iii) A positive result by ddPCR or real-time-PCR ~~from~~ using an environmental sample.

#### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *G. salaris* is considered to be confirmed if~~, in addition to the criteria in Section 6.1.1.,~~ the following criterion is met:

i) A positive result ~~from~~ by conventional PCR testing of parasite samples and sequencing of one or both of the ITS fragments and the CO1 fragment. The ITS sequences obtained are then analysed according to Section 4.6.1 and the CO1 ~~I~~ sequences according to Table 4.6.2 (see Section 4.6.2) ~~amplified CO1 fragments obtained by conventional PCR~~.

### 6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

#### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality;

9 For example, transboundary commodities.

ii) Identification of *G. salaris* by morphological examination;

iii) A positive result by conventional PCR;

iv) A positive result by real-time PCR.

#### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *G. salaris* is considered to be confirmed if~~, in addition to the criteria in section 6.2.1.~~ the following criterion is met:

~~i) A positive result from sequencing amplified CO1 fragments obtained by conventional PCR~~

i) A positive result by conventional PCR testing of parasite samples and sequencing of one or both of the amplified ITS fragments and the CO1 fragment. The ITS sequences obtained are then analysed according to Section 4.6.1 and the CO1 sequences according to Table 4.6.2 (see Section 4.6.2).

### 6.3. Diagnostic sensitivity and specificity for diagnostic tests: under study

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *G. salaris* is provided in Table 6.3. (note: no data are currently available). This information can be used for the design of surveys for infection with *G. salaris*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3.1.*** *Diagnostic performance of tests recommended for surveillance or diagnosis*

| Test type | Test purpose | Source population | Tissue/ sample type | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Real-time PCR | Surveillance | – | Parasites | – | Not yet available | Not yet available | – | – |
| Amplicon sequencing | Diagnosis | – | Parasites | – | Not yet available | Not yet available | – | – |
| Morphological examination | Diagnosis | – | Parasites | – | Not yet available | Not yet available | – | – |

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study.

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* *

**NB:** There is an OIE Reference Laboratory for infection with *G. salaris*
(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/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on infection with *G. salaris*.

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CHAPTER 2.3.6.

# INFECTION WITH SALMONID ALPHAVIRUS

**EU position**

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

## 1. Scope

Infection with salmonid alphavirus (SAV) means infection with any genotype of the pathogenic agent SAV, Genus *Alphavirus* and Family *Togaviridae.*

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

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

SAV has been divided into six genotypes (SAV 1–SAV 6) based solely on nucleic acid sequences for the proteins E2 and nsP3 (Fringuelli *et al.,* 2008). The level of antigenic variation among genotypes is considered low as monoclonal antibodies (MAbs) raised against a specific SAV genotype are likely to cross react with other SAV isolates (Graham *et al.,* 2014; Jewhurst *et al.,* 2004). ~~The genotype groups by susceptible species and environment are presented in Table 2.1.~~

Infection with SAV causes pancreas disease (PD) or sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.), common dab (*Limanda limanda*), rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007) and Arctic char (*Salvelinus alpinus*) (Lewisch *et al*., 2018). ~~The disease is systemic, characterised microscopically by necrosis and loss of exocrine pancreatic tissue, and heart and skeletal muscle necrosis and atrophy.~~ The genotypes SAV 1 and SAV 2 cause disease in fish both in freshwater and seawater, while the four genotypes SAV 3 – SAV 6 have only been reported from disease outbreaks in seawater.

~~***Table 2.1.*** *SAV genotypes by susceptible host species and environment*~~

| ~~SAV genotype~~ | ~~Fresh water~~ | ~~Sea water~~ |
|---|---|---|
| ~~SAV 1~~ | ~~Rainbow trout~~ | ~~Atlantic salmon~~ |
| ~~SAV 2~~ | ~~Rainbow trout; Atlantic salmon; Arctic charr~~ | ~~Atlantic salmon~~ |
| ~~SAV 3~~ | | ~~Rainbow trout; Atlantic salmon~~ |
| ~~SAV 4~~ | | ~~Atlantic salmon~~ |
| ~~SAV 5~~ | | ~~Atlantic salmon; Common dab~~ |
| ~~SAV 6~~ | | ~~Atlantic salmon~~ |

### 2.1.2. Survival and stability in processed or stored samples

There are no published scientific data specifically on the survival and stability of SAV in processed or stored samples. The OIE Reference Laboratory has found that SAV in serum/plasma samples and virus isolated from cell culture can be stored for many years at –80°C without significant decline in virus titre. This observation is consistent with research on other alphaviruses.

### 2.1.3. Survival and stability outside the host

Laboratory tests suggest that SAV would survive for extended periods in the aquatic environment. In these tests, virus could be detected at the end of the test period of 65 days in a majority of the trials. Virus survival was inversely related to temperature; at 20°C virus was not detectable beyond 35 days, and at 4°C was still present after 65 days. ~~In general, survival time was reduced by the presence of organic matter, markedly longer survival times were observed in sea water compared with fresh water in the water, this effect being most prominent at low water temperatures (Graham *et al.*, 2007b).~~

The half-life of SAV in serum has been found to be inversely related to temperature, being up to 7 times longer at 4°C than at 20°C, emphasising the need for rapid shipment of samples at 4°C to laboratories for virus isolation. For long-term conservation of SAV-positive samples and cultured virus, storage at –80°C is recommended (Graham *et al.,* 2007b).

For inactivation methods, see Section 2.4.5.

## 2.2. Host factors

### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SAV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) ~~include~~ are: ~~Arctic char (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), common dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*).~~

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| Pleuronectidae | *Limanda limanda* | Common dab | SAV 5 |
| Salmonidae | *Oncorhynchus mykiss* | Rainbow trout | SAV 1, 2, 3 |
| | *Salmo salar* | Atlantic salmon | SAV 1, 2, 3, 4, 5, 6 |
| | *Salvelinus alpinus* | Arctic charr | SAV 2 |

### 2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* ~~include~~ are: long rough dab (*Hippoglossoides platessoides*), plaice (*Pleuronectes platessa*) and Ballan wrasse (*Labrus bergylta*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: ~~Argentine hake (*Merluccius hubbsi*), brown trout (*Salmo trutta*), cod (*Gadus morhua*), European flounder (*Platichthys flesus*), haddock (*Melanogrammus aeglefinus*), herring (*Clupea harengus*), Norway pout (*Trisopterus esmarkii)*, saithe (*Pollachius virens*), longhorn sculpin (*Myoxocephalus octodecemspinosus*) and whiting (*Merlangius merlangus*).~~

| Family | Scientific name | Common name |
|---|---|---|
| Clupeidae | *Clupea harengus* | herring |
| Cottidae | *Myoxocephalus octodecemspinosus* | longhorn sculpin |
| Gadidae | *Melanogrammus aeglefinus* | haddock |
| | *Trisopterus esmarkii* | Norway pout |
| | *Pollachius virens* | saithe |
| | *Merlangius merlangus* | whiting |
| | *Gadus morhua* | Atlantic cod |
| Merlucciidae | *Merluccius hubbsi* | Argentine hake |
| Pleuronectidae | *Platichthys flesus* | European flounder |
| Salmonidae | *Salmo trutta* | brown trout |

~~**2.2.3. Non-susceptible species**~~

~~Species that have been found non-susceptible to infection with SVCV~~ SAV ~~according to Chapter 1.5. of the *Aquatic Code* are: None known. No species are listed as non-susceptible.~~

### 2.2.~~4~~ 3. Likelihood of infection by species, host life stage, population or sub-populations

~~Farmed~~ Atlantic salmon and rainbow trout are the species with the highest likelihood of infection with SAV. Experimental studies have demonstrated that all life stages are susceptible to infection (Taksdal & Sindre, 2016). SAV 1–SAV 6 have been detected in Atlantic salmon. SAV 1, SAV 2 and SAV 3 have been detected in rainbow trout.

For the purposes of Table 4.1, Atlantic salmon alevins and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, parr and smolts can be considered as juveniles and all fish post smoltification as adults.

### 2.2.~~5~~ 4. Distribution of the pathogen in the host

The heart and the pancreas are main target organs for infection with SAV. Necrosis and loss of exocrine pancreatic tissue, myocarditis and skeletal myositis are typical histopathological findings. During the viraemic stage, substantial amounts of virus are also found in serum, and during the infection virus can also be found in brain, kidney, spleen, gills, mucous and faeces (Taksdal & Sindre, 2016).

### 2.2.~~6~~ 5. Aquatic animal reservoirs of infection

There is evidence that some survivors of outbreaks will become long-term carriers of the virus (Graham *et al*., 2010 ~~2009~~) and thus farmed Atlantic salmon and rainbow trout can be considered the main reservoir of SAV (Taksdal & Sindre, 2016). Infection with SAV has been detected in some wild flatfish species in Scotland (Bruno *et al.,* 2014; Snow *et al.,* 2010) which could also act as a reservoir of infection.

### 2.2.~~7~~ 6. Vectors

Although most alphaviruses are transmitted by arthropod vectors, vector transmission of SAV has not yet been demonstrated. SAV has been detected by reverse-transcription (RT) PCR in salmon lice (*Lepeophtheirus salmonis*) collected during acute outbreaks of pancreas disease in Atlantic salmon, but transfer to susceptible fish species has not been reported (Petterson *et al.,* 2009).

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

Mortality rates due to infection with SAV may vary with genotype, season, year, use of biosecurity measures and species of fish (Bang Jensen *et al.,* 2012; Graham *et al.,* 2011; Rodger & Mitchell, 2007; Stormoen *et al*., 2013). The cumulative mortality at the farm level ranges from negligible to over 50% in severe cases (Bang Jensen *et al.,* 2012; Graham *et al.,* 2003; Rodger & Mitchell, 2007; Ruane *et al.,* 2008; Stene *et al.,* 2014). Experimental studies have demonstrated that SAV 2 infection in marine fish causes lower mortality than SAV 3 (Taksdal *et al.*, 2015).

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

The prevalence of infection with SAV ~~may vary~~ is variable. During disease outbreaks, the prevalence is usually high; prevalences of 70–100% have been reported in Atlantic salmon farming sites (Graham *et al.,* 2010). Prevalences in wild fish are largely unknown. SAV has been detected by RT-PCR in some marine flatfish species in Scottish waters at prevalences ranging from 0% to 18%, depending on species and location (Snow *et al.,* 2010). A serological survey of wild salmonids in fresh water river systems in Northern Ireland did not detect virus neutralisation antibodies against SAV in any of 188 sera tested, whereas the majority of sera from farmed salmon in sea water in the same area tested positive (Graham *et al.,* 2003).

### 2.3.2. Clinical signs, including behavioural changes

A sudden drop in appetite may be observed 1–2 weeks before the detection of elevated mortality. Clinically diseased fish may be observed swimming slowly at the water surface. In some cases, extremely weak ("sleeping") fish can be found at the bottom of tanks or in net-cages. An increased number of faecal casts may also be observed. However, it is important to note that clinical signs are not pathognomonic.

Initially, nutritional status is usually normal, but in the months after an outbreak or in the later stages of disease, long slender fish ('runts') with poor body condition are typically observed. However, the presentation of long, slender fish can be caused by factors other than SAV.

### 2.3.3 Gross pathology

Yellow mucoid gut contents is a usual post-mortem finding, typically seen in inappetant fish. Occasionally, signs of circulatory disturbances, such as petechial haemorrhages, small mild ascites or reddening of the pancreatic region between the pyloric caeca may be seen. Some diseased fish may show have pale or ruptured hearts or heart ruptures. It is important to note that post-mortem findings are not pathognomonic.

### 2.3.4. Modes of transmission and life cycle

Horizontal transmission of SAV is demonstrated by a range of evidence including: phylogenetic studies, successful transmission among cohabiting fish, proven transmission between farming sites, studies on survival of SAV in sea water and the spread via water currents (Graham *et al.,* 2007b; 2011; Jansen *et al.,* 2010a; Kristoffersen *et al.,* 2009; Stene *et al.,* 2013; Viljugrein *et al.,* 2009).

Long-distance transmission, and thus introduction of SAV into a previously uninfected area is most likely due to movement of infected live fish (Kristoffersen *et al.,* 2009; Rodger & Mitchell, 2007). SAV has been detected in fat leaking from dead fish which accumulates at the sea water surface, contributing to long distance spread of the virus by water currents (Stene *et al.,* 2013 2016). Once SAV has been introduced into an area, farm proximity and water currents influence local transmission (Aldrin *et al.,* 2010; Kristoffersen *et al.,* 2009; Viljugrein *et al.,* 2009).

Vertical transmission of SAV has been suggested (Bratland & Nylund, 2009), but not demonstrated (Kongtorp *et al.,* 2010; McLoughlin & Graham, 2007). The Norwegian Scientific Committee for Food Safety, (2010), carried out a risk assessment and concluded that the risk of vertical transmission of SAV is negligible.

### 2.3.5. Environmental and management factors

Clinical outbreaks and mortality are influenced by water temperature and season (McLoughlin & Graham, 2007; Rodger & Mitchell, 2007; Stene *et al.,* 2014; Stormoen *et al.,* 2013). Stressing the fish by movement, crowding or treatment may initiate disease outbreaks on infected farms.

Risk factors for outbreaks on a farming site include a previous history of infection with SAV, high feeding rate, high sea lice burden, the use of autumn smolts and previous outbreaks of infectious pancreatic necrosis (IPN) (Bang Jensen *et al.,* 2012; Kristoffersen *et al.,* 2009; Rodger & Mitchell, 2007).

### 2.3.6. Geographical distribution

Infection with SAV has been reported from several countries in Europe. See WAHIS (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en) for recent information on distribution at the country level.

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

DNA-based and cell-culture-based virus-inactivated vaccines against SAV are both commercially available. The vaccines may cause a risk of false positives, both in serological and PCR-based tests, according to data presented by vaccine companies. However, reports from the field indicates that false positives to serological tests do not occur after sea transfer. To prevent false positives by RT-PCR, sampling from vaccinated individuals should use heart tissue to avoid opening the abdominal cavity.

### 2.4.2. Chemotherapy including blocking agents

No chemotherapy is available.

### 2.4.3. Immunostimulation

No immunostimulation is available.

### 2.4.4. Breeding resistant strains

Differences in susceptibility among different family groups of Atlantic salmon have been observed in challenge experiments and in the field, indicating the potential for breeding for resistance (Norris *et al*., 2008; Gonen *et al.*, 2015). Breeding programmes in Ireland and Norway have successfully produced fish with increased resistance to disease caused by SAV, which are now commercially available.

### 2.4.5. Inactivation methods

SAV is rapidly inactivated ~~in the presence of high levels of organic matter at 60°C, pH 7.2, and at 4°C, pH 4 and pH 12, suggesting that composting, ensiling and alkaline hydrolysis would all be effective at inactivating virus in fish waste (Graham *et al.*, 2007a). The virus is also readily inactivated by UV-light, but is more resistant to chlorine and ozone treatment.~~ at pH 4 and pH 12, and after heating to 60°C (Graham *et al.,* 2007b). The virus is also readily inactivated by UV-light (Anon). A range of commercially available disinfectants have been tested for efficacy against salmonid alphavirus under different conditions, all being found to be effective under at least some of the conditions tested. The presence of organic matter was shown to decrease the effectiveness of disinfectants ~~be detrimental~~ in some cases (Graham *et al.* 2007a).

### 2.4.6. Disinfection of eggs and larvae

Standard disinfection procedures are considered sufficient to prevent surface contamination of eggs by SAV (Graham *et al.,* 2007a).

### 2.4.7. General husbandry

Stressing the fish by movement, crowding or treatment may initiate disease outbreaks on infected farms. Risk factors for outbreaks on a farming site include a previous history of infection with SAV, high feeding rate, high sea lice burden, the use of autumn smolts and previous outbreaks of infectious pancreatic necrosis (Bang Jensen *et al.,* 2012; Kristoffersen *et al.,* 2009; Rodger & Mitchell, 2007).

To avoid infection with SAV, good husbandry practices should be applied such as use of appropriate sites for farming, segregation of generations, stocking with good quality fish, removal of dead fish, regular cleaning of tanks and pens, control of parasites and other pathogens, as well as careful handling of fish. Once an outbreak has started, mortality may be reduced by minimising handling and ceasing feeding.

## 3. Specimen selection, sample collection, transportation and handling

### 3.1. Selection of populations and individual specimens

~~Clinical inspections should be carried out during a period when the water temperature is below XX°C.~~ All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Extremely weak ('sleeping') fish may be found at the bottom of a tank or ~~in the~~ net-cages. If the number of clinically diseased fish is low, samples from long, thin fish ('runts') may be added ~~(Jansen *et al.*, 2010b)~~. If moribund or thin fish or runts are sampled, the probability of detecting SAV is higher than if randomly selected, apparently healthy fish are sampled (Jansen *et al.,* 2010b). ~~Prevalence estimates will also vary with the diagnostic method used.~~

Fish to be sampled are selected as follows:

i) Susceptible species should be sampled proportionally or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).

ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) ~~If~~ Weak, abnormally behaving or freshly dead (not decomposed) fish ~~are present, such fish~~ should be selected. If such fish are not present, ~~the~~ selected fish ~~selected~~ should include ~~normal appearing,~~ apparently healthy fish collected in such a way that all parts of the farm, as well as all year classes, are proportionally represented in the sample.

### 3.2. Selection of organs or tissues

Heart and mid-kidney are the recommended organs for detection of SAV either by molecular biological methods or by cell culture. During ~~the course of the disease,~~ an outbreak, the heart usually contains more SAV than other tissues and should always be sampled. After disease outbreaks, gill and heart tissue (Graham *et al.,* 2010) and pools of heart and mid-kidney tissue (Jansen *et al.,* 2010b) remained positive by real time RT-PCR for months after initial detection.

~~For sampling from vaccinated fish,~~ For vaccinated fish, the heart should be sampled, ~~from vaccinated fish without~~ and mid-kidney, spleen or other internal organs should not be sampled, because opening the abdominal cavity~~. Sampling of mid-kidney, spleen or other internal organs is not recommended, to avoid~~ may cause contamination ~~of~~ with viral RNA/DNA from the vaccine (See Section 2.4).

During the initial viraemic phase, serum samples are also suitable for detection of SAV either by molecular biological methods or by cell culture, which can provide an early warning of disease outbreaks (Graham *et al.,* 2010). From approximately 3 weeks after SAV infection, blood serum or plasma is suitable for a virus neutralisation test (Graham *et al.,* 2003).

Tissues suitable for histological examinations ~~should~~ include gill, heart, pyloric caeca with attached pancreatic tissue, liver, kidney, spleen and skeletal muscle containing both red (aerobic) and white (anaerobic) muscle. Skin with associated skeletal muscle should be sampled at the lateral line level and deep enough to include both red and white muscle.

### 3.3. Samples or tissues not suitable for pathogen detection

Pancreas, although a target organ for the virus, is not suitable for RT-PCR detection of SAV, as it is impossible to separate this organ from the intestine of the fish during sampling, and in addition loss of pancreas is common in infected fish. Organs other than those recommended in Section 3.2 should not be used for the detection of SAV, as the sensitivity of the diagnostic methods might be reduced.

### 3.4. Non-lethal sampling

There are investigations into using non-lethal sampling methods for surveillance of SAV in fish farms, including detection of virus in water (Bernhard *et al.,* 2021). However, no validated methods are currently available. Serum samples may be collected via non-lethal sampling methods and considered suitable for some SAV test methods as described in Section 3.2.

### 3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

#### 3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (diseases of fish).

~~The success of pathogen isolation and results of bioassay depend heavily on the quality of samples (time since collection, and time and temperature in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should be used only after consultation with the receiving laboratory.~~

~~Before transfer to the laboratory, pieces of the organs to be examined for virus isolation should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml transport medium, i.e. cell culture medium with 10% fetal calf bovine serum (FCS FBS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required. To prepare duplicates (for retesting) it is recommended to aliquot the organ material after homogenisation.~~

~~Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C.~~

~~Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory.~~

~~The virological examination for isolation in cell culture should be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.~~

### 3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1, using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, tissue samples for RT-PCR testing should be preserved in an appropriate medium for preservation of RNA. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

For further storage~~,~~ the samples should ~~can~~ be kept below ~~at~~ –20°C.

### 3.5.3. ~~Fixed~~ Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be fixed in 10% neutral buffered formalin immediately after collection. The recommended ratio of fixative to tissue is 10:1.

### ~~3.5.4. Fixed Samples for electron microscopy~~

~~Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.~~

### 3.5.~~5~~ 4. Samples for other tests

Blood samples should be centrifuged for the collection of serum or plasma as soon as possible after sampling, to avoid lysis of the red blood cells. Serum or plasma samples should be shipped on ice to the laboratory to ensure virus viability.

## 3.6. Pooling of samples

The reliability of a virus isolation and real-time RT-PCR for detecting SAV in pooled samples from apparently healthy and clinically diseased populations of Atlantic salmon has not been evaluated ~~completely~~ thoroughly ~~(Hall *et al.*,2014)~~. ~~The~~ Results suggest that the use of individual samples rather than pools ~~is~~ are more appropriate when testing for freedom from, or for confirmatory diagnosis of, infection with SAV (Hall *et al.,* 2014).

## 4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in Table 4.1 indicate:

Key:

+++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
++ = Suitable method(s) but may need further validation;
+ = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;
Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

***Table 4.1.*** *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology[3]** | | | | | ++ | ++ | ++ | 2 | | | | |
| **Cytopathology[3]** | | | | | | | | | | | | |
| **Cell ~~or artificial media~~ culture** | | | | | + | + | + | 2 | + | + | + | 2 |
| **Real-time RT-PCR** | +++ | +++ | +++ | ~~1~~ 2 | +++ | +++ | +++ | 2 | ~~+++~~ | ~~+++~~ | ~~+++~~ | ~~2~~ |
| **Conventional RT-PCR** | | | | | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing[4]** | | | | | | | | | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab ELISA** | | | | | | | | | | | | |
| **Ag ELISA** | | | | | | | | | | | | |
| **Immunohistochemistry** | | | | | | | | | | ± | ± | 2 |
| **Serum neutralisation assay** | | + | ++ | 1 | ++ | ++ | ++ | 2 | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); RT-PCR = reverse transcription-polymerase chain reaction methods; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3.
[3]Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. [4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose.

### 4.1. Wet mounts

Not ~~relevant~~ applicable.

### 4.2. Histopathology and cytopathology

The pathological changes most commonly found in clinically diseased fish are severe loss of exocrine pancreatic tissue, cardiomyocytic necrosis and inflammation, red (aerobic) skeletal muscle inflammation and white (anaerobic) skeletal muscle degeneration or inflammation. A less frequent but supporting finding is the detection of cells with many cytoplasmic eosinophilic granules along kidney sinusoids.

As the disease progresses, the development of these changes is not simultaneous in all organs: in a very short, early phase, the only lesions present might be necrosis of exocrine pancreatic tissue and a variable inflammatory reaction in the peripancreatic fat. Shortly thereafter, heart muscle cell degeneration and necrosis develop before the inflammation response in the heart becomes more pronounced. The pancreatic necrotic debris will seemingly disappear, and the typical picture of severe loss of exocrine pancreatic tissue will soon appear simultaneously with the increasing inflammation in the heart. ~~Somewhat later,~~ Subsequently, skeletal muscle degeneration, inflammation and fibrosis develop. In a proportion of fish, severe fibrosis of the peri-acinar tissue may occur, and in these cases, the pancreas does not recover (runts) (Christie *et al.,* 2007; Kerbart Boscher *et al.,* 2006; McLoughlin & Graham, 2007; Taksdal *et al.,* 2007).

Cytopathology is not relevant for diagnostic use.

### 4.3. Cell ~~or artificial media~~ culture for isolation

#### ~~4.3.1. Cell lines~~

Isolation of field isolates of SAV in cell culture may be challenging (Christie *et al.,* 1998; Graham *et al.,* 2007b; Petterson *et al.,* 2013).

CHSE-214 cell cultures are commonly used for primary SAV isolation. ~~but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used.~~ Nevertheless, variation in cell line susceptibility among different SAV field isolates has been reported (Graham *et al.,* 2008; Herath *et al.,* 2009). ~~, and it is~~ Therefore ~~recommended that several cell lines are~~ other susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 should be tested for initial cell culture isolation of SAV ~~in a new laboratory or for a new virus strain~~. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

The CHSE-214 cells are grown at 20°C in Eagle's minimal essential medium (EMEM) with non-essential amino acids and 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) buffer, or Leibovitz's L-15 cell culture medium, both supplemented with fetal bovine serum (FBS) (5% or 10%) and L-glutamine (4 mM).

#### ~~4.3.2. Sample preparation and inoculation~~

For virus isolation, cells are grown in tissue culture flasks or multi-well cell culture plates. SAV-positive controls are ~~may be~~ inoculated in parallel with the tissue samples as a test for cell susceptibility to SAV. When positive controls are included, measures must be taken to avoid contamination.

Use the procedure for sample preparation and inoculation described in Chapter 2.3.0 *General information* (on diseases of fish), Section A.2.2.2.

~~i) Inoculation of cell monolayers~~

~~Prepare a 2% suspension of tissue homogenate or a 10% suspension of serum using L-15 medium or EMEM without serum, or other medium with documented suitability. Remove growth medium from actively growing monolayers (1- to 2-day-old cultures or cultures of 70–80% confluency) grown in tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers with a low volume of the 2% tissue homogenate or 10% serum dilution (for 25 $cm^2$ flasks: 1.5 ml). Adjust volume to the respective surface area in use. Allow 2–3 hours of incubation at 15°C, followed by removal of the inoculum, and addition of fresh L-15 or EMEM medium supplemented with 2–5% fetal bovine serum (for 25 $cm^2$ flasks: 5 ml).~~

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

~~ii) Monitoring incubation~~

~~Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of cytopathic effect (CPE). Typical CPE due to SAV appears as plaques of pyknotic, vacuolated cells. However, Norwegian SAV field isolates (both SAV3 and SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV genotypes (Graham *et al.*, 2008; Petterson *et al.*, 2013). If no CPE has developed after 14 days, subculture to fresh cell cultures.~~

~~iii) Subcultivation procedure~~

~~14 days (or earlier when obvious CPE appears) after inoculation, the cultures are freeze–thawed at –80°C to release virus from the infected cells. The procedure can be repeated 1–2 times.~~

~~Following centrifugation at 3000 ***g*** for 5 minutes, the supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 2–3 hours before addition of fresh medium.~~

Inoculated cell cultures are incubated at 15°C for at least 14 days and examined at regular intervals for the occurrence of cytopathic effect (CPE). Typical CPE due to SAV appears as plaques of pyknotic, vacuolated cells. However, Norwegian SAV field isolates (both SAV3 and SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV genotypes (Graham *et al.*, 2008; Petterson *et al.*, 2013). If no CPE has developed after 14 days, subculture to fresh cell cultures~~, as described for the primary inoculation~~. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]) or conventional RT-PCR or real-time RT-PCR as virus replication may occur without development of apparent CPE.

## 4.4. Nucleic acid amplification

### 4.4.1. ~~Reverse transcription,~~ Real-time RT-PCR ~~polymerase chain reaction~~

The primers described below for real-time RT-PCR and RT-PCR with sequencing will detect all known genotypes of SAV.

RT-PCR may be used for detection of SAV from total RNA (or total nucleic acids) extracted from recommended organs or tissues (see Section 3.4). Real-time RT-PCR for the detection of SAV is recommended as it increases the specificity and the sensitivity of the test.

For genotyping, RT-PCR with subsequent sequencing of fragments from the E2 gene is recommended.

The primer~~s~~ and probe sequences for real-time RT-PCR from the nsP1 gene, as well as primers for genotyping, are listed in Table 4.2. ~~The E2-primers may also be used for conventional RT-PCR detection of SAV, if necessary.~~ For RNA extraction, automatic and semi-automatic nucleic acid extractors can be used. In addition, a variety of manual RNA extraction kits can also be used successfully to extract SAV RNA. Various RT-PCR kits and real-time PCR machines can be used. The PCR programme depends on the kit and real-time PCR equipment used in the laboratory. The conditions for performing the real-time RT-PCR in the OIE Reference Laboratory is as follows: 50°C for 10 minutes, 95°C for 3 minutes, and 40 cycles of (95°C for 10 seconds, 60°C for 20 seconds). ~~For the conventional RT-PCRs (sequencing), the following programme is used: 50°C for 30 minutes, 95°C for 15 minutes, and 45 cycles of (94°C for 60 seconds, 55°C for 45 seconds, 72°C for 60 seconds).~~

***Table 4.2.*** *Primers and probe sequences for RT-PCR and real time RT-PCR*

| Primer and probe sequences | Test type | Genomic segment | Product size | Reference |
|---|---|---|---|---|
| QnsP1F: 5'-CCG-GCC-CTG-AAC-CAG-TT-3'<br>QnsP1R: 5'-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3'<br>QnsP1probe: 5'FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3' (Taqman®probe) | Real-time RT- PCR | QnsP1 | 107 nt bp | Hodneland *et al*., 2006 |
| E2F: 5'-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3'<br>E2R: 5'-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3' | RT-PCR | E2 | 516 107 nt bp | Fringuelli *et al*., 2008 |

The following controls should be run with each assay: negative extraction control; positive template control; no template control.

#### 4.4.2. Conventional RT-PCR ~~(PCR)~~

See Section 4.4.1 for comments on conventional PCR kits and PCR machines.

The E2-primers stated in Table 4.2 may be used for conventional RT-PCR detection of SAV, if necessary.

For the conventional RT-PCR ~~(and sequencing)~~, the following programme is used: 50°C for 30 minutes, 95°C for 15 minutes, and 45 cycles of (94°C for 60 seconds, 55°C for 45 seconds, 72°C for 60 seconds).

The following controls should be run with each RT-PCR assay: negative extraction control; positive template control; no template control.

#### 4.4.3. Other nucleic acid amplification methods

Not applicable.

### 4.5. Amplicon sequencing

~~Sequencing to determine the genotype of SAV can be performed using the E2-primer set listed in Table 4.2~~ Nucleotide ~~sequencing~~ sequence analysis of the RT-PCR amplicon (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. SAV-specific sequences will share a higher degree of nucleotide similarity to one of the published reference sequences for SAV.

### 4.6. *In-situ* hybridisation

Not applicable.

### 4.7. Immunohistochemistry

Immunohistochemical testing (Taksdal *et al.,* 2007) is only recommended for samples from fish with acute necrosis of exocrine pancreatic tissue.

#### 4.7.1. Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for immunohistochemistry sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for histopathology and immunohistochemistry as described below.

#### 4.7.2. Staining procedure for immunohistochemistry

All incubations are carried out at room temperature and all washing steps are done with Tris-buffered saline (TBS).

i) Nonspecific antibody binding sites are first blocked in 5% bovine serum albumin (BSA) in TBS for 20 minutes. The solution is then poured off without washing.

ii) Sections are incubated with primary antibody (monoclonal mouse antibody 4H1 against E1 SAV glycoprotein [Todd *et al.,* 2001]), diluted 1/3000 in 2.5% BSA in TBS and then incubated overnight, followed by two wash out baths lasting a minimum of 5 minutes.

iii) Sections are incubated with secondary antibody (biotinylated rabbit anti-mouse Ig) diluted 1/300 for 30 minutes, followed by wash out baths as in step ii above.

iv) Sections are incubated with streptavidin ~~with~~ alkaline phosphatase conjugate (1/500) for 30 minutes followed by wash out baths as in step ii above.

v) For detection of bound antibodies, sections are incubated with Fast Red[10] (1 mg $ml^{-1}$) and Naphthol AS-MX phosphate (0.2 mg $ml^{-1}$) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) and allowed to develop for 20 minutes followed by one wash in tap water before counterstaining with Mayer's haematoxylin and mounting in aqueous mounting medium.

SAV-positive and SAV-negative tissue sections are included as controls in every setup (Taksdal *et al.,* 2007).

## 4.8. Bioassay

Not applicable.

## 4.9. Antibody or antigen-based detection methods

### 4.9.1. Antibody-based verification of SAV growth in cell culture

This technique should not be used as a screening method. All incubations below are carried out at room temperature unless otherwise stated.

i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well plates) or on cover-slips, depending on the type of microscope available (an inverted fluorescence microscope ~~equipped with UV light~~ is necessary for monolayers grown on tissue culture plates). The necessary monolayers for negative and positive controls must be included.

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 9–11 days.

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. If necessary, the fixed cell cultures may be stored dry for 14 days at 4°C until staining.

iv) Incubate the cell monolayers with anti-SAV ~~MAb~~ antibodies in an appropriate dilution in phosphate-buffered saline (PBS) for 1 hour and rinse three times with PBS with 0.05% Tween 20.

v) Incubate with fluorescein isothiocyanate (FITC)-conjugated ~~anti-mouse~~ species-specific immunoglobulin antibody for 1 hour ~~(or if the primary Ab is polyclonal from rabbits, use FITC-conjugated antibody against rabbit immunoglobulin)~~, according to the instructions of the supplier. To increase the sensitivity of the test, FITC-conjugated anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with rinsing as in step d) in between the steps. The nuclei can be stained with propidium iodide (100 µg ml–1 in sterile distilled water). Add PBS (without Tween 20) and examine under fluorescence microscope ~~UV light~~. To avoid fading, the stained plates should be kept in the dark until examination. To reduce photobleaching of FITC due to the exposure to excitation light during microscopy, ~~For long periods of storage (more than 2–3 weeks)~~ a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

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

## 4.10. Other methods

### 4.10.1. ~~Immunoperoxidase-based~~ Serum neutralisation assay

Experimental studies have shown that neutralising antibodies can first be detected 10–16 days post-infection (Graham *et al.,* 2003), and serum neutralisation (SN) assays can be used as a diagnostic tool for the detection of SAV antibodies. SN assays are based on the presence or absence of detectable virus growth in cultured cells following incubation with serum that may contain neutralising antibodies. In addition, the assay allows detection of virus in serum or plasma, if present, as control wells of samples without added SAV are always included in the assay to assess presence of virus in the samples.

CHSE-214 cells are grown as described in Section 4.3.1. A suspension of trypsinised cells, diluted 1/3 in growth medium (10% FBS) is prepared for the SN assay.

i) 1/20 and 1/40 dilutions of each test serum are prepared in maintenance medium (2% FBS), and transferred to two duplicate wells (15 µl per well) on a flat-bottomed tissue culture grade microtitre plate. An equal volume of virus (100 $TCID_{50}$ [median tissue culture infective dose]) is added and the plate is incubated for 2 hours at room temperature.

ii) 70 µl of maintenance medium, and 50 µl of the CHSE-214 cell suspension is added to each well, and the plates are incubated for 3 days at 15°C.

iii) The cell monolayer is then fixed and stained as described in Section 4.9.1 *Antibody-based verification of SAV growth in cell culture*, or using the following procedure: monolayers of CHSE-214 cells are fixed for 30 minutes at room temperature in 10% neutral buffered formalin. Following two washes with 0.01 M PBS, a MAb against SAV is added to the monolayers in an appropriate dilution. Bound MAb is visualised using a labelled streptavidin–biotin system according to the manufacturer's instructions.

iv) SN titres ($ND_{50}$) are then calculated according to the method of Karber (1931), with titres ≥ 1:20 being considered positive. Both known negative serum controls and a control well for each sample (without virus added)$_{\pm}$ and a virus control (without serum added) must always be included in the assay, to ensure valid results. During viremia (as indicated by detection of SAV in the sample control wells) a SN titre cannot be assessed.

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The recommended test to be used in surveillance of susceptible fish populations for declaration of freedom from SAV is real-time RT-PCR as described in Section 4.4.1.

## 6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or in the presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

## 6.1. Apparently healthy animals or animals of unknown health status[11]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with SAV shall be suspected if at least one of the following criteria is met:

i) Positive result by real-time RT-PCR;

~~ii) Positive result by conventional RT-PCR~~

~~iii) SAV-typical CPE in cell culture~~

ii) Detection of neutralising activity against SAV in serum or plasma.

### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SAV is considered to be confirmed if ~~in addition to the criteria in Section 6.1.1.,~~ one or more of the following criteria is met:

i) A positive result on tissue preparations ~~by real-time RT-PCR and a positive result~~ by conventional RT-PCR and sequencing of the amplicon;

ii) ~~A positive result on tissue preparations by real-time RT-PCR and~~ SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon;

iii) A positive result on tissue preparations by immunohistochemistry, and by conventional RT-PCR and sequencing of the amplicon.

~~iii) Detection of neutralising activity against SAV in serum or plasma and SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon~~

~~iv) Detection of neutralising activity against SAV in serum or plasma and a positive result on tissue preparations by conventional RT-PCR and sequencing of the amplicon~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with SAV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with SAV;

ii) Histopathology consistent with SAV infection;

iii) SAV-typical CPE in cell culture;

iv) Positive result by real-time RT-PCR;

v) Positive result by conventional RT-PCR;

~~v) SAV-typical CPE in cell culture~~

vi) Detection of neutralising activity against SAV in serum or plasma.

11 For example, transboundary commodities.

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with SAV is considered to be confirmed if~~, in addition to the criteria Section 6.2.1.,~~ one of the following criteria is met:

i) A positive result on tissue preparations ~~by real-time RT-PCR and a positive result~~ by conventional RT-PCR and sequencing of the amplicon;

ii) ~~A positive result on tissue preparations by real-time RT-PCR and~~ SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon;

iii) A positive result on tissue preparations by immunohistochemistry, and by conventional RT-PCR and sequencing of the amplicon.

~~iii) Detection of neutralising activity against SAV in serum or plasma and SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon~~

~~iv) Detection of neutralising activity against SAV in serum or plasma and a positive result on tissues preparations by conventional RT-PCR and sequencing of the amplicon~~

~~v) A positive result on tissue preparations by conventional RT-PCR and sequencing of the amplicon~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests: under study

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with SAV is provided in Table 6.3. This information can be used for the design of surveys for infection with SAV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3.*** *Diagnostic performance of tests recommended for surveillance or diagnosis on field studies*

| Test type | Test purpose | Test populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Real-time PCR | Surveillance Diagnosis | Infected | Kidney | Atlanntic salmon | 0.39 (598) | >0.99 (598) | n/a (Bayesian probability model) | Hall *et al.*, 2014 |
| Real-time PCR | Surveillance Diagnosis | Infected vs assumed SAV free | Heart and mid-kidney | Atlantic salmon | 0.978 (268) | 0.831 (268) | n/a (Bayesian Latent Class Analysis) | Jansen *et al.*, 2019 |
| Isolation of SAV in cell culture | Diagnosis | Infected | Heart vendticle and head-kidney | Atlanntic salmon | 0.50 (598) | >0.99 (598) | n/a (Bayesian probability model) | Hall *et al.*, 2014 |
| Isolation of SAV in cell culture | Diagnosis | Infected vs assumed SAV free | Heart and mid-kidney | Atlantic salmon | 0.950 (268) | 0.993 (268) | n/a (Bayesian Latent Class Analysis) | Jansen *et al.*, 2019 |
| Detection of neutralising activity against SAV | Surveillance | Infected vs assumed SAV free | Serum or plasma | Atlantic salmon | 0.085 (268) | 0.744 (268) | n/a (Bayesian Latent Class Analysis) | Jansen *et al.*, 2019 |
| Histopathology | Diagnosis | Infected vs assumed SAV free | Heart and mid-kidney | Atlantic salmon | 0.637 (268) | 0.967 (268) | n/a (Bayesian Latent Class Analysis) | Jansen *et al.*, 2019 |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

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**NB:** There is an OIE Reference Laboratory for infection with salmonid alphavirus
(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/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on infection with salmonid alphavirus

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SECTION 2.3.

# DISEASES OF FISH

CHAPTER 2.3.0.

# GENERAL INFORMATION

**EU position**

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

## A. SAMPLING

### 1. Assessing the health status of the epidemiological unit

#### 1.1. Sample material to be used for tests

Sample material and the number of samples to be collected depend~~s~~ on the specific disease or pathogen, the size of the animals and the objective of testing (i.e. diagnosis of overt clinical disease, detection of ~~fish that are~~ subclinical ~~pathogen carriers~~ infection in apparently healthy animals or sampling for targeted surveillance to demonstrate freedom ~~of~~ from infection with a specified ~~disease~~ pathogen). See ~~the OIE *Aquatic Animal Health Code* Chapter 1.4 *Aquatic animal health surveillance* for information on the design and evaluation of surveillance systems for aquatic animals and~~ the individual disease chapters in the *Aquatic Manual* for specific details of sample requirements.

#### 1.2. Specifications according to fish populations

For ~~specific~~ details of fish to sample ~~requirements~~ for a ~~particular~~ specific listed disease, see the relevant disease chapter in the *Aquatic Manual.* The design of a surveillance system for demonstrating disease-free status for a country, zone or compartment should be in accordance with the recommendations of the OIE *Aquatic Code* Chapter 1.4.

Fish to be sampled are selected as follows:

i) Susceptible species should be sampled proportionally or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).

ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include apparently healthy fish collected in such a way that all parts of the farm, as well as all year classes, are proportionally represented in the sample.

#### 1.3. Specifications according to clinical status

For diagnosis of clinical infection for most OIE-listed viruses, appropriate organs to sample include anterior/mid kidney, spleen and either heart or encephalon; for fry whole fish or entire viscera may be used. ~~For koi herpesvirus, gill and gut should be sampled; for epizootic ulcerative syndrome, skin or muscle; and for~~

~~*Gyrodactylus salaris*, whole fish or skin and fins should be examined.~~ Disease-specific recommendations are provided in Section 3.2 *Selection of organs or tissues* of the individual chapters. Samples from five to ten clinically diseased fish consistent with the disease of interest should be sufficient for the pathogen test(s) for each epidemiological unit.

For the appropriate organs to sample to detecting subclinical infections carriers of virus and or for targeted surveillance for case detection or disease freedom, refer to individual disease chapters of the *Aquatic Manual* and chapter 1.4 of the OIE *Aquatic Code* where a large number of samples is required, samples may be combined in pools as specified in each individual disease chapter of the *Aquatic Manual*.

## 1.4. Specifications according to fish size

### 1.4.1. For the listed viral diseases except infection with koi herpesvirus disease and viral encephalopathy and retinopathy

*Fry and yolk sac fry:* Sample the entire fish but remove the yolk sac if present.

*Fish 4 to 6 cm:* Sample the entire viscera including and the kidney. A piece of encephalon can be obtained after severing the head at the level of the rear edge of the operculum and pressing it laterally.

*Fish over 6 cm:* Sample the kidney, spleen, and heart or encephalon and/or other tissues appropriate for the specific pathogen being tested for (see individual disease chapter in the *Aquatic Manual* for details).

*Adult fish Non-lethal sampling:* Sample tissues appropriate for the specific pathogen being tested for (see the specific disease chapter in the *Aquatic Manual* for details). For non-lethal sampling, appropriate sample types are recommended in Section 3.4 of the specific disease chapter. Take the ovarian fluid, milt or tissues appropriate for the specific pathogen being tested for (see individual disease chapter in the *Aquatic Manual* for details).

**1.4.2. For infection with *Aphanomyces invadans* (epizootic ulcerative syndrome [EUS])**

*Any size of fish:* kidney, liver, muscular tissue (See Chapter 2.3.2 Infection with *Aphanomyces invadans* [epizootic ulcerative syndrome] for specific details.

**1.4.3. For infection with *Gyrodactylus salaris***

*Any size of fish:* skin and fins (See Chapter 2.3.3 Infection with *Gyrodactylus salaris* for specific details.

**1.4.4. For Koi herpesvirus (KHV)**

*Fish 4 cm to adult:* Take the gill, kidney, spleen, encephalon and gut tissues depending on test to be used (See Chapter 2.3.7 *Infection with koi herpesvirus disease* for specific details.

**1.4.5. For viral encephalopathy and retinopathy (VER)**

*Fish 2–4 cm:* take the whole head.

*Fish 4 cm to adults:* take the encephalon and possibly the eyes and spinal cord (see Chapter 2.3.12 Viral encephalopathy and retinopathy for specific details).

# 2. General processing of samples

## 2.1. Macroscopic examination

For the listed diseases, macroscopic examination is mostly used for detecting clinical signs of epizootic ulcerative syndrome infection with *Aphanomyces invadans* or *Gyrodactylus salaris,* but this is followed by microscopic examination of histological slides for the former or by identification of parasites removed isolated from of wet mounts of skin/fin scrapings the skin, fins or gills of fish for the latter.

For viral diseases, clinical signs (including increased mortality rate, surface discolouration, distended abdomen, excess mucous production, exophthalmia, pale gills/anaemia, skin/fin/gill lesions, surface haemorrhages, lethargy, abnormal swimming behaviour and inappetence) and increasing mortality rates are non-specific.

## 2.2. Preservation of samples for subsequent virological examination

Samples to be submitted are either (i) fresh and chilled on ice or in vials containing cell culture medium for virus isolation, (ii) fixed in a nucleic acid stabilisation solution (e.g. ~~RNAlater~~ RNA preservative or 80–90% ethanol) for polymerase chain reaction (PCR) detection ~~and~~ or (iii) preserved in ~~4~~ 10% neutral-buffered formalin fixative for histology and *in-situ* hybridisation. See individual sections below for further details. See the individual disease chapters in the *Aquatic Manual* for specific details of preservation requirements for other types of tests.

## 2.3. Virological examination

### 2.3.1. Transportation and antibiotic treatment of samples

Individual or pools of whole fish, organs or secretions ~~Pools of organs or of~~ ovarian fluids/milt are placed in sterile vials and stored at 4°C or on ice until virus ~~extraction~~ isolation is performed in the laboratory. Virus ~~extraction~~ isolation should optimally be carried out within 24 hours after fish sampling, but is still acceptable for up to 48 hours if the storage temperature is maintained at 0–4°C, or for longer periods for clinical disease samples held frozen at –80°C. Freezing at –20°C for storage should be avoided. For testing of apparently healthy fish, freezing of samples (at any temperature) ~~for testing for subclinical carriers~~ should be avoided.

Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks' balanced salt solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable antibiotic concentrations are: gentamycin (1000 µg $ml^{-1}$), or penicillin (800 International Units [IU] $ml^{-1}$) and streptomycin (800 µg $ml^{-1}$). Antifungal compounds~~, such as Mycostatin® or Fungizone®,~~ may also be incorporated into the transport medium at a final concentration of 400 IU $ml^{-1}$. Serum or albumen (5–10%) may be added to stabilise the virus if the transport time will exceed 12 hours.

### 2.3.2. Virus isolation ~~extraction~~

This procedure should be conducted below 15°C (preferably between 0 and 10°C). This can be achieved by using mortars and pestles that have been stored at –20°C or homogenising tissues quickly in a Stomacher or in tubes held in an ice slurry.

1. Decant antibiotic-supplemented medium from the organ sample.
2. Homogenise organ pools (minimum weight of 0.5 g) ~~in transport medium at a final dilution of 1/10~~ using a suitable method (e.g. mortar and pestle, glass or electronic homogeniser, Stomacher or validated equivalent ~~electric homogeniser~~) until a paste is obtained and dilute 1/10 (w/v) with transport medium.
3. Centrifuge the homogenate in a refrigerated (2–5°C) centrifuge ~~at 2–5°C~~ at 2000–4000 ***g*** for 15 minutes, collect the supernatant and treat for either four hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg $ml^{-1}$. If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment makes filtration through membrane filters unnecessary. Alternatively, if gross microbial contamination is suspected, the supernatant can be membrane-filtered (0.45 um) understanding that there may be some loss of virus.
4. Likewise, ovarian fluid/milt samples may be treated with antibiotics to control microbial contamination but should not be diluted more than fivefold in the HBSS and antibiotic medium.
5. Ovarian fluid/milt samples should be centrifuged in the same way as organ homogenates, and their supernatants used directly in subsequent steps.
6. Prepared tissue/ovarian fluids/milt supernatants are used for inoculation of cell cultures for virus isolation and an aliquot may also be used for ~~pre-screening by~~ other tests, for example, PCR.
7. It is recommended to aliquot the homogenised sample material to avoid repeated freeze–thawing of the material. This also ensures reproducibility and comparability of the results.

#### 2.3.3. Treatment to neutralise enzootic viruses

Fish are often subclinically infected with enzootic ~~endemic~~ viruses, such as birnaviruses (e.g. infectious pancreatic necrosis virus [IPNV]), which induce a cytopathic effect in susceptible cell cultures and thus complicate isolation and identification of target pathogens. In such situations, the infectivity of the enzootic viruses should be neutralised, where possible, before testing for the viruses listed in the *Aquatic Code*. However, when it is important to determine whether one of the enzootic viruses is present, samples should be tested with and without the presence of neutralising antibodies (NAbs).

To neutralise aquatic birnaviruses, mix equal volumes (200 µl) of a solution of one or more NAbs against the ~~indigenous~~ enzootic birnavirus serotypes with the supernatant to be tested. Allow the mixture to react for 1 hour at 15°C or overnight at 4°C prior to inoculation on to susceptible cell monolayers. The titre of the NAb solution used should be at least 2000 in a 50% plaque reduction test versus the viral serotypes present in the given geographical area.

When samples are from a country, region, fish population or production unit considered to be free from enzootic viral infections, the NAb treatment of the ~~organ homogenate~~ supernatant may ~~should~~ be omitted.

This approach can also be used to neutralise other viruses enzootic to the area ~~being tested~~ from where the samples were taken.

### 2.4. Parasitic examination

See Chapter 2.3.3 Infection with *Gyrodactylus salaris* for specific details.

### 2.5. Fungal examination

See Chapter 2.3.2 Infection with *Aphanomyces invadans* for specific details.

## B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF FISH PATHOGENS

## 1. Fish viruses

### 1.1. Fish cell lines

The following fish cell lines are used to test for the viral fish pathogens referred to in the *Aquatic Manual:*

*Epithelioma papulosum cyprini* (EPC)

Bluegill fry (BF-2)

Fathead minnow (FHM)

Rainbow trout gonad (RTG-2)

Chinook salmon embryo (CHSE-214)

Salmon head kidney (SHK-1)

Atlantic salmon kidney (ASK)

Chum salmon heart (CHH-1)

Grunt fin (GF)

Koi fin (KF-1)

Common carp ~~*Cyprinus carpio*~~ brain (CCB)

~~Striped snakehead (SSN-1)~~

Grass carp ovary cell lines (GCO)

## 1.2. Culture media

Traditional Eagle's minimal essential medium (MEM) with Earle's salt supplemented with 10% fetal bovine serum (FBS), antimicrobial agents and 2 mM L-glutamine is the most widely used medium for fish cell culture.

Stoker's medium, however, which is a modified form of the above medium comprising a double-strength concentration of certain amino acids and vitamins, is ~~particularly~~ recommended particularly to enhance cell growth, using the same supplements as above + 10% tryptose phosphate.

These media are buffered with either sodium bicarbonate, 0.16 M tris-hydroxymethyl aminomethane (Tris) HCl, or, preferably, 0.02 M N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES). The use of sodium bicarbonate alone is restricted to those cell cultures made in tightly closed cell culture vessels or cultures incubated in an atmosphere supplemented with $CO_2$ to maintain the desired pH (7.3–7.6). As an alternative, MEM with Hanks' salts can be used in both closed cell culture flasks and 24-well or 96-well culture plates without the addition of other buffer salts.

Alternatively, Leibovitz medium (L15) supplemented with FBS (5% or 10%), L-glutamine (4 mM) and gentamicin (50 µg $ml^{-1}$) is recommended for some cell lines, e.g. SHK-1 ~~and SSN-1~~.

For cell growth, the FBS content of the medium is usually 10%, whereas for virus isolation or virus production it may be reduced to 2%. ~~Similarly, the pH of the culture medium for cell growth is 7.3–7.4 and is adjusted to 7.6 for virus production or virus assay.~~

The composition of the most frequently used antimicrobial agent mixture is penicillin (100 IU $ml^{-1}$) and dihydrostreptomycin (100 µg $ml^{-1}$). Add mycostatin (50 IU $ml^{-1}$) if fungal contamination is likely. Other concentrations or other antimicrobial agents may be used as convenient for the operator depending on the antimicrobial sensitivity of the bacterial or fungal strains encountered.

## 1.3. Virus positive controls and antigen preparation

### ~~1.3.1. Virus nomenclature~~

~~Epizootic haematopoietic necrosis virus (EHNV)~~

~~Infectious haematopoietic necrosis virus (IHNV)~~

~~Infectious salmon anaemia virus (ISAV)~~

~~Koi herpesvirus (KHV)~~

~~*Oncorhynchus masou* virus (OMV)~~

~~Red sea bream iridovirus (RSIV)~~

~~Salmonid alphavirus (SAV)~~

~~Spring viraemia of carp virus (SVCV)~~

~~Viral haemorrhagic septicaemia virus (VHSV)~~

~~Viral encepalopathy and retinopathy virus (VERV) also known as viral nervous necrosis virus (VNNV)~~

### 1.3.1. Virus production

For the *in-vitro* production of stock cultures of most of these viruses, monolayer cultures of susceptible cells (see relevant sections in the *Aquatic Manual*) in suitable tissue culture vessels (e.g. plastic flasks) should be inoculated with fairly low multiplicities of infection (m.o.i.), i.e. $10^{-2}$ to $10^{-3}$ plaque-forming units (PFU) per cell or equivalent.

The preferred temperatures for virus propagation are included in the table below.

~~15°C for IHNV, ISAV, OMV, and VERV (genotype BFNNV) and VHSV~~

~~20°C for KHV SVCV and VERV (genotypes BFNNV, SJNNV and TPNNV)~~

~~22°C for EHNV~~

~~25°C for RSIV and VERV (genotypes RGNNV and SJNNV)~~

~~30°C for VERV (genotype RGNNV)~~

| Temperature | Virus |
|---|---|
| 15°C | infectious haematopoietic necrosis virus (IHNV)<br>infectious salmon anaemia virus (ISAV)<br>salmonid alphavirus (SAV)<br>viral haemorrhagic septicaemia virus (VHSV) |
| 20°C | koi herpesvirus (KHV)<br>spring viraemia of carp virus (SVCV) |
| 22°C | epizootic haematopoietic necrosis virus (EHNV) |
| 25°C | red sea bream iridovirus (RSIV) |

#### 1.3.2. Preservation and storage of virus stock cultures

1. Centrifuge infected cell cultures at 2–5°C and 2000–4000 ***g*** for 15 minutes then dilute the virus-containing supernatants in order to obtain virus titres averaging $10^6$ PFU $ml^{-1}$ or equivalent.
2. Dispense the resulting viral suspensions into sterile vials at volumes of 0.3–0.5 ml each.
3. Freeze and store each series of standard virus stocks at –80°C or in liquid nitrogen vapour phase, and check the titre of each virus stock at regular intervals (6–12 months) if it has not been used during that time period.

*Lyophilisation:* long-term storage (decades) of ~~the seeds of~~ standard virus seed strains is achievable by lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% ~~fetal calf serum~~ FBS are mixed (v/v) with an equal volume of cryopreservative medium (such as 20% lactalbumin hydrolysate in distilled water) before processing. Seal or plug under vacuum and store at 4°C, in the dark.

At least every 6 months or if decreased cell susceptibility is suspected, titration of reference isolates is performed to verify cell line susceptibility to infection.

## 2. Techniques

### ~~2.1. Serology~~

#### ~~2.1.1. Production of rabbit antisera and polyclonal antibodies to fish viruses~~

~~There are various ways in which antibodies against fish viruses can be raised in rabbits. Titre and specificity are influenced, however, by the inoculation programme used. The following immunisation protocols may be used to produce antisera for use in the virus isolation and/or identification procedures described later.~~

##### ~~2.1.1.1. Antisera to infectious pancreatic necrosis virus~~

~~Intravenous injection with 50–100 µg of purified virus on day 0, followed by an identical booster on day 21, and bleeding 5–7 days later. Rabbits may be reused if not bled completely.~~

##### ~~2.1.1.2. Antisera to other viruses~~

~~The immunisation protocols alternate an intramuscular or intradermal injection with further intravenous boosters:~~

Day 0: primary injection, 500–1000 µg of purified virus is mixed (v/v) with adjuvant (Freund's incomplete or other[12] adiuvants that are considered more acceptable) giving a total volume of 1.2 ml. This antigen is delivered to the rabbit as multipoint intradermal injections (2 points on each side) after the animal has been shaved.

Day 21: collect about 2 ml of blood and check for reactivity (neutralisation, fluorescence); boost intravenously with the same amount of purified virus as in the primary injection, but without adjuvant. Prior to the intravenous booster injection, the rabbit should be treated with promethazine (12 mg intramuscularly) to prevent a possible anaphylactic response.

Day 28: sample the blood, check the serum reactivity and bleed or boost according to the results.

For rhabdoviruses, this immunisation procedure is well suited to production of antisera to be used in immunofluorescence and in the enzyme-linked immunosorbent assay. However, a more efficient method for production of neutralising antisera is regular intravenous injection without adjuvant (0.2 ml) every 3–4 days (twice a week). As many as 15 injections may be necessary; 1 week after the last injection, a serum sample should be collected and tested.

**2.1.3. Processing and storage of immune sera**

After blood clotting, collect and centrifuge the serum at 20°C and heat it for 30 minutes at 56°C. Filter the resulting heat-inactivated serum through a membrane filter (450 nm pore size) and temporarily store it at 4°C for the time necessary for the screening of its reactivity and specificity and for checking that these properties are not affected by preservation conditions (e.g. freezing or lyophilisation). Sterile rabbit sera can be kept for at least 2 months at 4°C without any change in their properties. Dispense (usually as small volumes) and freeze at –20°C or lyophilise.

Immunoglobulins (Ig) may be extracted from antisera using conventional methods suitable for Ig purification. Selective attachment to protein A constitutes a reliable and effective method. The concentration of Ig solutions is adjusted to the values required for further conjugate preparation or storage.

*Preservation of Ig:* Mix a solution of Ig of concentration 2 mg litre$^{-1}$ with sterile pure glycerol (v/v) and keep at –20°C. Solutions of Ig with a higher concentration may also be prepared in glycerol.

**2.1.4. Mouse monoclonal antibodies**

Monoclonal antibodies (MAbs) to most of the fish viruses have been raised over the past years. Some of them, singly or as two or three associated MAbs, have given rise to biological reagents suitable for the identification of virus groups (IPN, VHS, IHN). Other MAbs, taken individually or as components of Ab panels, allow accurate typing of VHSV and IHNV. These MAbs can be obtained from the Reference Laboratories listed at the end of this *Aquatic Manual*.

In theory, mouse monoclonal IgGs can be processed and stored as for polyclonal IgGs. However, the reactivity of certain MAbs may be impaired by processes such as enzymatic- or radio-labelling or lyophilisation. It is thus necessary to test various MAbs for the conditions under which they will be used.

## 2.1. Direct microscopy

Samples for direct microscopic examination of smears or tissue imprints should be examined as soon as possible after collection. Live specimens should be used whenever possible, or fresh specimens chilled at 4°C, or 10% neutral-buffered formalin-fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection. For *G. salaris*, fresh specimens are examined or fish can be stored in ethanol prior to microscopic examination (see Chapter 2.3.3 Infection with *G. salaris*).

12 Use of Freund's complete adjuvants may be restrictedon animal welfare grounds. Alternative synthetic adjuvants include trehalose dimycolate and monophosphate lipid A.

## 2.2. Histological techniques

### 2.2.1 Preparation of slides for histological examination

~~**2.3.1. Tissue fixation and embedding**~~

Only live or moribund specimens of fish with clinical lesions should be sampled after humane euthanasia for histology. The removed tissues (<5 mm thick) should be fixed immediately in 10% neutral-buffered formalin. Use at least ten volumes of fixative for each volume of tissue sample and allow to fix for at least 24 hours. After removal from the fixative, tissue samples are then dehydrated in ascending ethanol concentrations, cleared in a wax-miscible agent such as xylene and then embedded in paraffin using standard protocols. Cut sections of approximately 3–5 µm thickness from the block. Mount each section on a glass slide, de-wax in a wax-miscible agent, such as xylene or 'Clearene®', and rehydrate. For most disease examinations, the sections can then be stained with haematoxylin and eosin (H&E) using standard procedures (Slaoui & Fiette, 2011). For observing granulomas and fungal hyphae as occur in infection with *A. invadans*, a general fungal stain such as Grocott–Gomori may be used instead of H&E.

~~**2.3.2. Tissue sectioning and staining**~~

~~Cut sections of approximately 5 µm thickness from the block. Mount each section on a glass slide, de-wax in a wax-miscible agent, such as xylene or 'Clearene®', and rehydrate.~~

~~For most disease examinations, the sections can then be stained with haematoxylin and eosin (H&E), by the following procedure:~~

~~**Taking the slides to water**~~

~~1. Place slides in xylene or 'Clearene®' to remove wax for a minimum of 2 minutes.~~

~~2. Repeat step 1 in fresh xylene or 'Clearene'.~~

~~3. Place in 100% alcohol to remove the solvent for a minimum of 2 minutes.~~

~~4. Repeat step 3 in fresh 100% alcohol.~~

~~**Staining**~~

~~5. Wash in running tap water (RTW) for 2–5 minutes. Slides should be clear, not cloudy.~~

~~6. Place in haematoxylin solution for 3 minutes~~

~~7. Turn blue in RTW for 5–10 minutes (or saturated lithium carbonate); cannot over blue.~~

~~8. Dip in acid/alcohol for a maximum of 10 seconds.~~

~~9. Rinse in RTW (or lithium carbonate) until blue.~~

~~10. Microscope check for clear cytoplasm and blue nuclei.~~

~~11. Aqueous eosin for 3 minutes.~~

~~12. Good wash in RTW to differentiate eosin.~~

~~**Dehydration, clearing and mounting**~~

~~13. Rinse well in 70% alcohol but not for too long as it removes eosin.~~

~~14. Place in 100% alcohol for 1–2 minutes.~~

~~15. Repeat step 14 in fresh alcohol.~~

~~16. Place in 50/50 alcohol/Clearene for 1–2 minutes.~~

~~17. Place into Clearene.~~

~~18. Repeat with fresh Clearene bath, slides should be clear.~~

~~19. Mount in DPX (distyrene, plasticizer, and xylene) mountant and leave to dry.~~

~~For observing granulomas and fungal hyphae as occur in epizootic ulcerative syndrome, a general fungal stain such as Grocott-Gomori may be used instead of H&E.~~

### 2.2.2. Preparation of slides for immunohistochemistry

It is important to note that prolonged fixation can mask antigens of interest. Therefore, it is recommended keeping fixation to a minimum whilst still achieving optimal preservation (24–48 hours). This can be reduced further when using small pieces of tissue. Nonetheless, it is recommended to incorporate an antigen retrieval step ~~(included within the protocol below)~~ where possible (Kim *et al.,* 2016). ~~The following outlines a standard immunohistochemistry protocol routinely used in histology laboratories, but~~ Due to variations that may exist between antibodies and commercially available detection kits, it is probable that individuals will need to optimise the technique for their own purposes. This will include factors such as determination of optimal ~~optimum~~ antibody titre. This is the highest dilution that results in the most intense specific staining whilst achieving the least non-specific "background" staining. In addition, individuals may need to consider amending the duration of reagent incubation.

~~1. Carry out steps 1–5 of Section 2.3.2.~~

~~2. Rinse slides in two changes of 0.2% Tween 20 in PBS for 2 minutes.~~

~~3. Perform antigen retrieval by placing slides into plastic coplin jar containing Sodium citrate buffer and place on steamer rack situated inside pressure cooker.~~

~~4. Place cooker on high heat until full pressure is reach indicated by "rocking" of vent.~~

~~5. Reduce temperature and leave on hotplate for approximately 10 minutes whilst maintaining pressure.~~

~~6. Remove from hotplate and allow cooker to cool and vent for approximately 20–30 minutes in a fume hood prior to opening.~~

~~7. Remove coplin jar from pressure cooker and replace Sodium citrate buffer with warm tap water followed by cool tap water and distilled water. This is to cool the slides gradually.~~

~~8. If required, carry out blocking of endogenous biotin/avidin activity (a) incubate slides for 15–20 minutes in 0.005% avidin in PBS (b) rinse in PBS followed by (c) incubation in 0.005% biotin in PBS for 15–20 minutes. Alternatively, employ the use of a commercially available blocking system in accordance to manufacturer guidelines. This is usually undertaken on tissues containing high levels of biotin such as liver, kidney and spleen.~~

~~9. Briefly rinse slides in tap water.~~

~~10. Rinse slides in 0.2% Tween 20 in PBS for 2 minutes.~~

~~11. Tip off reagent and blot dry around tissue section ensuring section is kept moist.~~

~~12. Incubate with primary antibody at 25°C for 30 minutes with gentle orbital rotation if available.~~

~~13. Rinse slides in 0.2% Tween 20 in PBS from a wash bottle.~~

~~14. Tip off reagent and blot dry around tissue section ensuring section is kept moist.~~

~~15. Incubate with biotinylated secondary antibody at 25°C for 10 minutes with gentle orbital rotation if available.~~

~~16. Rinse slides in 0.2% Tween 20 in PBS from a wash bottle.~~

~~17. Quench endogenous peroxidase activity by placing slides into 0.3% Hydrogen peroxide in PBS with 0.1% Sodium azide for 10–15 minutes at room temperature.~~

~~18. Rinse slides in 0.2% Tween 20 in PBS from a wash bottle.~~

~~19. Incubate with preferred commercially available peroxidase-labelled streptavidin detection complex at 25°C for 10 minutes with gentle orbital rotation if available.~~

~~20. Rinse slides in 0.2% Tween 20 in PBS from a wash bottle.~~

~~21. Apply DAB chromogen to slides and develop reaction product by monitoring under microscope for optimum time. Duration will vary depending on DAB product used.~~

~~22. Stop reaction by placing slides into tap water.~~

~~23. Perform chromogenic enhancement (optional) by placing slides into 0.5% Copper sulfate in PBS for 1–5 minutes at 25°C with gentle orbital rotation.~~

~~24. Rinse in distilled water.~~

~~25. Counterstain with Harris's haemotoxylin for 2–3 minutes.~~

~~26. Rinse with water.~~

~~27. Dehydrate, clear and mount.~~

~~*Reagent preparation*~~

| | | |
|---|---|---|
| ~~PBS-Tween 20 (0.2%):~~ | ~~Phosphate-buffered saline~~ | ~~10 litres~~ |
| | ~~Tween 20~~ | ~~2 ml~~ |
| ~~Sodium citrate buffer:~~ | ~~Tri-sodium citrate (dihydrate)~~ | ~~2.94g~~ |
| | ~~Distilled water~~ | ~~1 litre~~ |
| | ~~Tween 20~~ | ~~0.5 ml~~ |

~~Mix to dissolve, adjust pH to 6.0 with 1 N HCl before adding Tween 20. Store this solution at room temperature for 3 months or at 4°C for longer storage.~~

## 2.3. Electron microscopy

Electron microscopy (transmission or scanning) is a valuable research tool for the study of aquatic animal diseases (e.g. Hyatt *et al.,* 1991) and for the detection of previously unknown viruses for which there are no specific diagnostic tests. However, these methods are not normally used for the routine diagnosis of the fish diseases listed by the OIE so are not described in the *Aquatic Manual*.

## 2.4. Virus isolation

### 2.4.1. Introduction

For most viruses, ~~the st-andard surveillance method (to detect subclinical carriers) is virus~~ isolation in cell culture followed by identification of the virus using either antibody-based or~~, more commonly,~~ nucleic acid-based (PCR) methods can be employed in the diagnosis of clinically affected animals or in the surveillance of apparently healthy animals. Isolation of finfish viruses in cultures of a number of established fish cell lines is well-documented (Crane *et al.,* 2005; Devold *et al.,* 2000; Graham *et al.,* 2008; Herath *et al.,* 2009; Lorenzen *et al.,* 1999; Olesen & Vestergård Jørgensen, 1992). However for some viruses, such as ~~While inoculation of fish cell lines with fish tissues processed for virus isolation is considered the reference standard for surveillance programmes with respect to sensitivity, the precise sensitivity of the procedure is unknown. Moreover~~ KHV, cell culture isolation is not as sensitive as the published PCR-based methods and is not considered to be a ~~reliable~~ reproducible diagnostic method for KHV (Haenen *et al.,* 2004). ~~Indeed, real-time or conventional PCR methods and sequencing are, in general, highly sensitive and highly specific and, following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared from fish tissue. The technique has the potential to be used in direct surveillance programmes for obtaining approved free status (e.g. Garver *et al.,* 2011; Jonstrup *et al.,* 2013). Duplicates of unfixed samples testing positive using real-time or conventional PCR methods and sequencing can be processed for virus isolation to confirm presence of infectious virus. At least every 6 months or if decreased cell susceptibility is suspected, titration of frozen viral stocks is performed to verify cell line susceptibility to infection.~~

The success of pathogen isolation and results of bioassay depend heavily on the quality of samples (level of autolysis of fish samples, time since collection, time and temperature in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternative storage methods should be used only after consultation with the receiving laboratory.

Before transfer to the laboratory, pieces of the organs to be examined for virus isolation should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml

transport medium, i.e. cell culture medium with 10% fetal bovine serum (FBS) and antibiotics. The

combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g). To prepare duplicates (for retesting) it is recommended to aliquot the organ material after homogenisation.

Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C, and ice must still be present in the transport box at receipt or at least one freeze block must still be partly or completely frozen.

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with current national and international transport regulations, as appropriate.

The virological examination for isolation in cell culture should be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.

See the individual disease chapters in the *Aquatic Manual* for specific details of virus isolation requirements.

#### 2.4.2. Inoculation of cell monolayers

Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively growing (not confluent) at inoculation.

Prepared tissue samples (see Section A. *Sampling* above) are inoculated onto cell cultures in at least two dilutions, i.e. the primary dilution and a 1/10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1/100 and 1/1000, respectively (to prevent homologous interference). ~~The ratio between inoculum size and volume of cell culture medium should be about 1:10.~~ For each dilution and each cell line, a minimum of about 2 $cm^2$ cell area, corresponding to one well in a 24-well cell culture plate, has to be used. Use of 24-well cell culture plates is recommended, but other units of a similar or larger growth area are also acceptable.

#### 2.4.3. Incubation of cell cultures

Inoculated cell cultures are incubated at the pathogen-specific temperature for 7–14 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution, or equivalent substances, has to be performed to ensure cell susceptibility to virus infection.

#### 2.4.4. Microscopy

Using ×40–150 magnification, inoculated cell cultures must be inspected regularly (at least ~~three~~ two times a week) for the occurrence of cytopathic effect (CPE). The use of a phase-contrast microscope is recommended. If obvious CPE is observed, virus identification procedures must be initiated immediately.

#### 2.4.5. Sub-cultivation

If no CPE has developed after the primary incubation for 7–14 days, sub-cultivation is performed with fresh cell cultures using a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to the cell line 7–14 days after inoculation. The pools are then inoculated onto homologous cell cultures undiluted and diluted 1/10 (resulting in final dilutions of 1/10 and 1/100, respectively, of the supernatant) as described above (Section B.2.4.2 *Inoculation of cell monolayers*). For SAV, and other non-

or slow CPE-forming viruses that are cell-bound, it is recommended that a freeze–thaw cycle or sonication step be included prior to passage.

Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into a well with a fresh cell culture (well-to-well sub-cultivation). In the case of salmonid samples, inoculation may be preceded by preincubation of the dilutions with an anti-IPNV antiserum at an appropriate dilution, as described above (see Section A.2.3.3 *Treatment to neutralise enzootic viruses*). The inoculated cultures are then incubated for 7–14 days at the appropriate temperature, with observation, as described above (see Section B.2.4.4 *Microscopy*).

If nonspecific cytotoxicity occurs within the first 3 days of incubation, sub-cultivation may be performed at that stage, but the cells must then be incubated for 7 days and sub-cultivated again with a further 7 days of incubation. When nonspecific cytotoxicity develops after 3 days, the cells may be passed once and incubated to achieve a total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final 7 days of incubation.

If bacterial contamination occurs despite treatment with antibiotics, sub-cultivation must be preceded by centrifugation at 2000–4000 ***g*** for 15–30 minutes at 2–5°C, or filtration of the supernatant through a 0.45 µm filter (low protein-binding membrane). In addition to this, sub-cultivation procedures are the same as for nonspecific cytotoxicity.

If no CPE occurs, the test may be declared negative~~, however, increased confidence of a negative result can be achieved by testing for the presence of virus using antibody-based or nucleic acid-based (PCR) methods~~. For SAV2/SAV3 no apparent CPE is common from field isolates. An IFAT for the detection of SAV antigen is routinely performed.

Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.) ~~that make~~ making it impossible to inoculate cells within 48 hours ~~of collection of the tissue samples~~ after tissue sampling, it is ~~acceptable~~ possible to ~~freeze~~ store the supernatants at –80°C and carry out virological examination within 14 days. ~~If the collected~~ Once supernatants ~~is~~ are stored at –80°C, thawing is recommended only once~~ within 48 hours of sampling it may be reused only once for virological examination~~. Another freeze–thaw cycle will substantially reduce virus titres. It is recommended to aliquot the homogenised sample material to avoid repeated freeze–thawing of the material. This also ensures reproducibility and comparability of the results.

#### 2.4.6. Virus identification

Infected cell cultures are used for virus identification by IFAT. Supernatant from cultures demonstrating CPE is used for virus identification by ~~either antibody-based and/or~~ nucleic acid-based techniques. The preferred method for confirmatory identification is by sequence analysis of PCR amplicons (see *Aquatic Manual* chapters on individual pathogens for details).

### 2.5. Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis

Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional ~~and the polymerase chain reaction (~~PCR~~)~~ and real-time PCR, have been developed for the identification of many pathogens of aquatic animals. ~~However, as is the case with several other diagnostic techniques, an advantage in sensitivity is frequently offset by problems in interpretation or susceptibility to technical problems.~~ Real-time PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared from fish tissue. The technique can be used in direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well as in the diagnosis of clinically affected animals (e.g. Garver *et al.,* 2011; Jonstrup *et al.,* 2013). Duplicates of unfixed samples testing positive using real-time PCR can be processed for virus isolation to confirm presence of infectious virus.

When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative controls, as well as validation of the PCR method chosen are important. ~~PCR can be quite dependent on the conditions under which it is run and can be highly subject to laboratory contamination by previous PCR products, yielding false-positive results. Nevertheless,~~ Real-time PCR is a powerful technique particularly for analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. ~~Thus, while~~ Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening, diagnostic or confirmatory methods for fish~~, where possible well-established techniques (e.g. virus isolation) are should~~ and

can be undertaken as ~~specified as~~ the standard ~~screening~~ method~~s~~. However, following PCR-positive results, where possible, virus isolation should be undertaken to confirm the presence of infectious virus. Conventional PCR with sequencing of PCR products should be used for confirmation of the cultured pathogen identity. ~~Whenever these newer molecular techniques are used, they should be performed with caution and with special attention to the inclusion of adequate positive and negative controls.~~

As with all PCR protocols, optimisation may be necessary depending on the reagents, equipment and the plasticware. PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction), may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. Therefore, each assay and tissue extraction should include a negative control to rule out contamination. False-negative results (positive samples giving a negative result), may lead to unwanted transmission of pathogens and biosecurity failure.

To minimise the risk of contamination, aerosol-preventing pipette tips should be used for all sample and PCR preparation steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the amplifications and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes and paper (e.g. workbooks). Also, ensure all work-tops and air-flow hoods used for the extractions and PCR set up are regularly cleaned and decontaminated. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location away from the molecular biology laboratory and reagents.

### 2.5.1. Sample preparation and types

~~For these techniques,~~ Samples should be prepared to preserve the nucleic acid of the pathogen~~. Likewise, samples intended for testing with antibody-based methods should be preserved to retain the reactive antigenic sites for the antibodies used. Samples selected for nucleic acid-based or antibody-based diagnostic tests~~ and should be handled and packaged with the greatest care to minimise the potential for cross-contamination among the samples or target degradation before the assay can be performed. To prevent contamination, new disposable containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular ~~or antibody-based~~ tests are:

- *Live iced specimens or chilled specimens:* For specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of ~~wet~~ ice packs ~~around the bagged samples~~ or ice bricks in an insulated box and ship to the laboratory.
- *Frozen whole specimens:* Select live specimens according to the purpose of sampling, euthanase fish humanely and quick-freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry ice in an insulated box, and ship to the laboratory. Freezing samples for histological analysis should be avoided.
- *Alcohol-preserved samples:* In regions where the storage and shipment of fresh (0–4°C) and frozen samples is problematic, ~~90 –95~~ 80–90% (v/v) ethanol (analytical grade) or ~~RNAlater~~ RNA preservative should be used to preserve, store, and transport ~~certain types of~~ samples for PCR analysis. Pack for shipment according to the methods described above.
- *Fixed tissues for* in-situ *hybridisation~~ and immuno-histochemistry:~~* For this purpose, classic methods for preservation of the tissues are adequate. Neutral-buffered formalin is usually a good choice~~. for later use of molecular probes. For DNA, specifically, over-~~Fixation for ~~(~~over 24–48 hours~~)~~ should be avoided; samples should be transferred to ethanol following the formalin treatment.

### 2.5.2. Preservation of RNA and DNA in tissues

Tissue is cut to be less than 0.5 cm in one dimension and submerged in 10 volumes of a suitable nucleic acid preservative (e.g. a 0.5 g sample requires about 5 ml of ~~RNAlater~~ RNA preservative or 80–90% ethanol). Smaller organs such as kidney, liver and spleen can be stored whole in ~~RNAlater~~ RNA preservative or 80–90% ethanol. ~~These~~ Samples preserved in this way can be stored at 4°C for 1 month, at 25°C for 1 week or indefinitely at –20°C or below. ~~Archive RNAlater-treated tissues at –20°C or below.~~

### 2.5.3. ~~DNA~~ Nucleic acid extraction

~~For DNA extraction, grind the sample in 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, and sodium dodecyl sulphate [SDS, 0.5%]) supplemented with proteinase K (100 µg ml$^{-1}$). Following overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol.~~ To isolate ~~DNA~~ nucleic acids from tissues preserved in ethanol or ~~RNAlater~~ an RNA preservative, simply remove the tissue from ~~ethanol or RNAlater~~ the fixative or preservative and treat it as though it was just harvested. Most fresh and ~~RNAlater/ethanol-~~preserved or fixed tissues can be homogenised (e.g. with a mortar and pestle or in bead-beating tubes) directly in the lysis or extraction buffer provided with commercially available DNA and RNA extraction kits. Commercial kits should be validated or undergo equivalence testing with current validated extraction procedures prior to routine use.

~~Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternatives. Use of commercial kits should be validated by comparison with a standard phenol/chloroform protocol prior to their routine use in diagnostic laboratories.~~

### ~~2.5.4. RNA extraction~~

~~To isolate RNA from tissues preserved in RNAlater, simply remove the tissue from RNAlater and treat it as though it was just harvested. Most tissues can be homogenised directly in lysis or extraction buffer.~~

~~Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternatives. Use of commercial kits should be validated by comparison with a standard phenol/chloroform protocol prior to their routine use in diagnostic laboratories.~~

### 2.5.4. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation ~~(ISH)~~, fish tissues should be fixed in neutral-buffered formalin for approximately 24 hours and then embedded in paraffin according to standard histological methods~~, as described under section 3.3~~. Sections are cut at a thickness of 5 µm and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg ml$^{-1}$) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For ~~ISH~~ *in-situ* hybridisation tests (see individual chapters for details), it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol (Qadiri *et al.,* 2019; Valverde *et al.,* 2017).

## 3. Additional information to be collected

Sample information should include the collector's name, organisation, date, time, and description of the geographical location. The geographical origin of samples may be described as the name or location of the sampling site or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the sample site to the storage facility or laboratory and within those facilities.

~~Storage facilities should record~~ Information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable) should be collected. This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

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CHAPTER 2.4.3.

# INFECTION WITH *BONAMIA OSTREAE*

**EU position**

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

. . .

## 2.2. Host factors

### 2.2.1. Susceptible host species

~~Natural host: European flat oysters, *Ostrea edulis*.~~

~~Oyster species infected when moved into *B. ostreae* endemic zones: *Ostrea puelchana, O. angasi, O. chilensis* (= *Tiostrea chilensis, T. lutaria*) (Carnegie & Cochennec-Laureau, 2004). However, the parasite was not identified to the species level in these hosts.~~

~~Experimental assays have indicated a low infectivity of *B. ostreae* to *Crasssotrea ariakensis* (Audemard *et al.*, 2005)~~

~~It has been speculated that *Ostrea conchaphila* (= *O. lurida*) and *Crassostrea angulata* have been infected with *B. ostreae* (Carnegie & Cochennec-Laureau, 2004), but confirmatory diagnosis has not been achieved.~~

~~Experimental work showed that the following species are not susceptible to *B. ostreae*: *C. gigas*, *Ruditapes decussatus*, *R. philippinarum*, *Mytilus edulis*, *M. galloprovincialis* (Culloty *et al*., 1999).~~

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia ostrea* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: European flat oyster (*Ostrea edulis*), Chilean flat oyster (*Ostrea chilensis*), and Suminoe oyster (*Crassostrea ariakensis*).

### 2.2.2. ~~Susceptible stages of the host~~ Species with incomplete evidence for susceptibility

~~Both 0+ and 1+ year-old *O. edulis* are susceptible to infection and can develop a high prevalence and high intensity of infection and even mortality over a 6-month period (Lynch *et al.*, 2005). However, individuals older than 2 years appear to be more susceptible to the disease (Culloty & Mulcahy, 1996; Grizel, 1985; Engelsma *et al.*, 2010). Seed from natural settlements appear to be significantly more parasitised than oyster seed from hatcheries (Conchas *et al*., 2003).~~

~~It has recently been shown that larvae can be infected with *B. ostreae* (Arzul *et al.*, 2010).~~

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *B. ostreae* according to Chapter 1.5 of the *Aquatic Code* are: Argentinean flat oyster (*Ostrea puelchana*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: beadlet anemone (*Actina equina*), brittle star (*Ophiothrix fragilis*), European sea squirt (*Ascidiella aspersa*), grouped zooplankton andPacific cupped oyster (*Crassostrea gigas*) .

### ~~2.2.3. Non-susceptible species~~

~~Species that fulfil the criteria for listing as non-susceptible to infection with *Bonamia ostreae* according to Chapter 1.5 of the *Aquatic Code* include: blue mussel (*Mytilus edulis*), European clam (*Ruditapes decussatus*), Manila clam (*Ruditapes philippinarum*) and Mediterranean mussel (*Mytilus galloprovincialis*).~~

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CHAPTER 2.1.3.

# INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

**EU position**

**The EU thanks the OIE and in general supports the adoption of this revised chapter. A comment is inserted in the text below.**

## 1. Scope

Infection with *Batrachochytrium salamandrivorans* ~~(Bsal)~~ means infection of amphibians with the pathogenic agent *Batrachochytrium salamandrivorans*, of the Division Chytridiomycota and Order Rhizophydiales ~~Genus *Batrachochytrium* and Family *Incertae sedis*~~.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

The type strain of the pathogenic chytrid fungal agent *Batrachochytrium salamandrivorans* (Bsal) ~~type strain~~ is AMFP13/1. Three more isolates have been described (Martel *et al*., 2014) but no information is available on genetic structuring or phenotypic variation. Phylogenetic analyses show that Bsal forms a clade with its sister species *B. dendrobatidis* (Martel *et al*., 2013). The genome size of the type strain was determined at 32.6 Mb with 10,138 protein-coding genes predicted (Farrer *et al*., 2017). The contribution of these proteins to virulence is currently not clear.

#### 2.1.2. Survival and stability inside the host tissues ~~in processed or stored samples~~

Bsal is an intracellular pathogen that develops inside epidermal cells. The presence of Bsal could be demonstrated using real-time polymerase chain reaction (~~q~~PCR) on dorsal skin swabs up to 7 days on average post-mortem and using histopathology of dorsal skin tissue up to 3 days on average post-mortem (Thomas *et al*., 2018). It is not clear how long Bsal can survive inside tissues of a dead host and how long a dead host remains infectious. ~~Storage of tissues or skin swabs in 70% ethanol or at –20°C allows detection of Bsal using qPCR for more than 150 years as demonstrated by analysis of museum specimens (Martel *et al*., 2014).~~

#### 2.1.3. Survival and stability outside the host

Encysted spores have been shown to remain infectious in pond water for up to at least 31 days (Stegen *et al*., 2017) and are considered more ~~environmentally~~ resistant in the environment compared with zoospores. Experimentally inoculated forest soil was demonstrated to remain infectious to fire salamanders for 48 hours (Stegen *et al.,* 2017). However, Bsal DNA was detected ~~up to 28 weeks~~ in contaminated forest soil for up to 28 weeks (Stegen *et al*., 2017). ~~However,~~ Whether this reflects the presence of viable Bsal organisms is not clear. The effect of ~~dessication~~ desiccation on Bsal survival has not been studied.

For inactivation methods, see Section 2.4.5.

### 2.2. Host factors

#### 2.2.1. Susceptible host species [under study]

Species that fulfil the criteria for listing as susceptible to infection with Bsal according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) ~~include~~ are: ~~[alpine newt (*Ichthyosaura alpestris*), blue-tailed fire-bellied newt (*Cynops cyanurus*), fire salamander (*Salamandra salamandra*), eastern newt (*Nothophthalmus viridescens*), French cave salamander (*Hydromantes strinatii*), Italian newt (*Lissotriton italicus*), yellow-spotted newt (*Neurergus crocatus*), Japanese fire-bellied newt (*Cynops pyrrhogaster*), northern spectacle salamander (*Salamandrina perspicillata*), Tam Dao salamander (*Paramesotriton*~~

~~*deloustali*), rough-skinned newt (*Taricha granulosa*), sardinian brook salamander (*Euproctus platycephalus*) and Spanish ribbed newt (*Pleurodeles waltl*)]~~ ~~(~~under study and may include:~~).~~

| Family | Scientific name | Common name |
|---|---|---|
| Plethodontidae | *Hydromantes strinatii* | French cave salamander |
| Salamandridae | *Cynops cyanurus* | blue-tailed fire-bellied newt |
| | *Cynops pyrrhogaster* | Japanese fire-bellied newt |
| | *Euproctus platycephalus* | sardinian brook salamander |
| | *Ichthyosaura alpestris* | Alpine newt |
| | *Lissotriton italicus* | Italian newt |
| | *Neurergus crocatus* | yellow spotted newt |
| | *Nothophthalmus viridescens* | eastern newt |
| | *Paramesotriton deloustali* | Tam Dao salamander |
| | *Pleurodeles waltl* | Spanish ribbed newt |
| | *Salamandrina perspicillata* | northern spectacle salamander |
| | *Salamandra salamandra* | fire salamander |
| | *Taricha granulosa* | rough-skinned newt |

### 2.2.2. Species with incomplete evidence for susceptibility [under study]

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: [under study]

~~**2.2.3. Non-susceptible species**~~

~~Species that have been found non-susceptible to infection with Bsal according to Chapter 1.5. of the *Aquatic Code* are: [under study]~~

### 2.2.4 3. Likelihood of infection by species, host life stage, population or sub-populations

Bsal is a pathogenic agent that mainly affects urodeles. Evidence from experimental infections and disease outbreaks in the wild and in captivity show that at least most, if not all, species of the family Salamandridae, as well as species of the family Hynobiidae are likely to become infected when exposed to Bsal. However, differences in susceptibility to infection between species do exist: for example, for fire salamanders ~~(*Salamandra salamandra*)~~, the infectious dose of Bsal was determined to be a theoretical one zoospore, whereas a significantly higher dose was necessary to infect Alpine newts ~~(*Ichthyosaura alpestris*)~~; Stegen *et al*., 2017) and one western Palearctic species (*Lissotriton helveticus*) may be more resistant to infection (Martel *et al*., 2014). For the largest family of salamanders (Plethodontidae), little information is currently available; at least one European species (*Speleomantes strinatii*) can be infected but other, North American species (*Gyrinophilus porphyriticus*, *Plethodon glutinosus,* Ambystomatidae) seem less susceptible to infection (Martel *et al*., 2014). Susceptibility of the family of *Cryptobranchidae* is not clear, with a single infection found in a farmed Chinese giant salamander (*Andrias davidianus*; Zhiyong *et al*., 2018). No information is available on the urodele families *Proteidae, Rhyacotritonidae* and *Amphiumidae*. Bsal infection in anurans has only been detected in two species, in captivity, the wild and in lab trials (Nguyen *et al*., 2017; Stegen *et al*., 2017).

Thus far, infections with Bsal have been demonstrated only in amphibians post-metamorphosis. In one experimental infection trial, larvae of fire salamanders were exposed to Bsal, but did not become ~~were not~~ infected (Van Rooij *et al*., 2015). The extent to which factors such as ~~like~~ age and sex affect susceptibility to infection post-metamorphosis is unknown.

In Europe, Bsal has been detected in captive collections of urodeles (Fitzpatrick *et al*., 2018, Sabino-Pinto *et al*., 2015) and the pet trade in salamanders and newts has been hypothesised to play a central role in the distribution of this fungus (Fitzpatrick *et al*., 2018; Yap *et al*., 2015; Zhiyong *et al*., 2018). Hence, urodeles that come into contact with traded urodeles, either directly (~~by~~ via co-housing or contact ~~of~~ with wild animals ~~with~~ or released or captive animals) or indirectly (via materials, contaminated water or soil) ~~come in contact with traded urodeles~~, may have a high likelihood of exposure to infection with ~~be more likely to contract~~ Bsal ~~infection~~.

For the purposes of Table 4.1, salamander larvae with gill buds may be considered early life stages, larvae with developed or developing gills and limbs are juveniles and salamander with full developed gills and limbs are adults.

#### 2.2.~~5~~ 4. Distribution of the pathogen in the host

Bsal only infects the skin, where it remains limited to the epidermis.

#### 2.2.~~6~~ 5. Aquatic animal reservoirs of ~~Persistent~~ infection

A large number of salamanders, mainly belonging to the families Salamandridae and Hynobiidae, may survive episodes of infection (for example Alpine newts) or be considered tolerant, resulting in persistent subclinical infections. Although persistent infection has not been demonstrated for all species, in the native Bsal range in east Asia, Bsal infection and disease dynamics appear to be consistent for all species examined and appear capable of long-term persistent infections (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018).

In its invasive range, persistent infections (e.g. in Alpine newts) have been implicated in the ~~extirpation~~ local extinction of a highly susceptible species (fire salamanders). It is currently not clear which of the species, mentioned in Section 2.2.1 may sustain persistent infections in the invasive Bsal range. At least some species (the best-known example is the fire salamander) are highly susceptible and invariably die shortly ~~briefly~~ after exposure (Martel *et al*., 2014; Stegen *et al*., 2017), making ~~which would make~~ them unlikely to sustain persistent infections.

It is not known whether other, biotic reservoirs of Bsal exist.

#### 2.2.~~7~~ 6. Vectors

There is evidence that birds may carry zoospores attached to their ~~the~~ feet ~~of birds~~ (Stegen *et al*., 2017)~~, which may~~ and thus may act as vectors for Bsal.

### 2.3. Disease pattern

#### 2.3.1. Mortality, morbidity and prevalence

In its native range in east Asia, Bsal has been demonstrated to be present in the wild at a prevalence of between 2 and 4% on average (data from China [People's Rep. of], Japan, Thailand, and Vietnam; Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018), but in the absence of any observed morbidity or mortality under natural conditions. In some populations (*Paramesotriton hongkongensis*), prevalence may reach 50% (Zhiyong *et al*., 2018). In its invasive range in Europe, Bsal was present in a population of fire salamanders at a prevalence of between 25 and 63% (Stegen *et al*., 2017). ~~In captive collections of urodeles in Europe,~~ Bsal occurrence and associated mortality has been ~~were~~ detected in captive collections of urodeles in Europe, including Germany (1), the United Kingdom (4), Belgium (1), the Netherlands (2) and Spain (1) (number in brackets indicates number of collections). When left untreated, morbidity and mortality can reach 100%, at least in members of the genus *Salamandra*.

Morbidity, mortality and minimum infectious dose vary considerably between species (Martel *et al*., 2014; Stegen *et al*., 2017). Based on natural outbreaks in captivity and in the wild and in ~~on~~ infection trials, the case morbidity and case mortality rate in fire salamanders can reach 100%, independent of the initial level of Bsal exposure. This has resulted in the loss of over 99.9% of the fire salamander population at the Bsal index outbreak site in the Netherlands (Spitzen-van der Sluijs *et al*., 2016). All tested western Palearctic urodeles, except for *Lissotriton helveticus* and *Salamandrella keyserlingii*, showed 100% morbidity and mortality when exposed to a single, high dose of Bsal (Martel *et al*., 2014). However, at least for Alpine newts, the case morbidity and case fatality rates depend on the Bsal dose that the animal is exposed to: a high dose resulting in the highest mortality, while a low dose does not necessarily result in morbidity or mortality.

~~It is important to mention that~~ Morbidity and mortality also depend on environmental temperature. For the Bsal type strain, temperatures above 20°C reduces the level of ~~tempers~~ infection and temperatures above

25°C eventually result in killing of Bsal and elimination of infection (Blooi *et al*., 2015b ~~2015a~~). Exposure of infected animals to conditions that inhibit Bsal growth may thus result in non-clinical or sub-clinical infections in susceptible species.

Co-occurrence of highly susceptible species such as fire salamanders with less susceptible species, such as Alpine newts may facilitate density independent disease dynamics that lead to the local extinction of the highly susceptible species (Stegen *et al*., 2017).

### 2.3.2. Clinical signs, including behavioural changes

Chytridiomycosis caused by Bsal may be accompanied by a combination of the following signs: epidermal ulcerations (ranging from discrete ~~tiny~~ to extensive), excessive skin shedding, skin haemorrhages and/or fluid loss, anorexia, apathy, abnormal body postures and convulsions ~~and death~~ (Martel *et al.,* 2013).

### 2.3.3 Gross pathology

Skin anomalies (haemorrhages, ulcerations, presence of sloughed skin) are the main pathological findings (Martel *et al.,* 2013).

### 2.3.4. Modes of transmission and life cycle

Colonial or monocentric thalli of this fungus develop inside host epidermal cells and produce motile zoospores or walled, encysted spores, both of which are infectious stages. Zoospores are released through one or several discharge tubes. While motile spores actively swim towards a suitable substrate (e.g. a host), the encysted spores float at the water–air interface and passively adhere to a passing host (Stegen *et al.,* 2017). *In vitro*, developing thalli form fine rhizoids. Mature thalli *in vitro* are between 16 and 50 µm in diameter, *in vivo* between 7 and 17 µm; zoospores are approximately 5 µm in diameter. Motile zoospores are roughly spherical, the nucleus is located outside of the ribosomal mass, with aggregated ribosomes, multiple mitochondria and numerous lipid globules. The position of the non-flagellated centriole in free swimming zoospores varies from angled to parallel to the kinetosome (Martel *et al.,* 2013).

There are no indications of vertical transmission. However, this cannot be excluded in species giving birth to metamorphosed offspring (e.g. *Salamandra atra*, *Salamandra lanzai*, *Lyciasalamandra helverseni*). Horizontal transmission occurs through direct contact or contact with contaminated soil or water (Stegen *et al*., 2017). Infectious stages include the motile zoospore and the environmentally resistant encysted spores (Stegen *et al*., 2017). Infections can be reproduced under experimental conditions by topically applying a Bsal inoculum on the dorsum of amphibians and housing the exposed animals at 15°C (Martel *et al*., 2013; 2014; Stegen *et al*., 2017). This inoculum can either contain motile zoospores or the immobile, encysted spores.

Pathways of Bsal dispersal within Europe are poorly understood but may be anthropogenic (e.g. through contaminated material). Zoospores attach to bird feet, suggesting birds may spread Bsal over larger distances (Stegen *et al*., 2017). Direct animal-to-animal contact is necessary for transmission of Bsal: salamanders only separated by 1 cm from infected conspecifics were not infected in laboratory trials, in contrast to co-housed animals (Spitzen-van der Sluijs *et al*., 2018). Overall, dispersal ability of Bsal in Europe currently seems limited: Bsal was found not to be transmitted to a neighbouring site in the Netherlands, despite being downstream of a small stream, and the current distribution of Bsal in Europe is probably not continuous (Spitzen-van der Sluijs *et al*., 2018).

Although Bsal dispersal between populations is now hypothesised to be mainly human mediated, other factors (e.g. wildlife, water) may play key roles and critical knowledge about Bsal dispersal is currently lacking.

### 2.3.5. Environmental ~~and management~~ factors

The Bsal type strain AMFP13/1 tolerates temperatures up to 25°C but is killed at higher temperatures (Blooi *et al*., 2015b ~~2015a~~). As Bsal infections have been demonstrated in aquatic newts at water temperatures above 25°C (Laking *et al*., 2017; Zhiyong *et al*., 2018), it is likely~~, however,~~ that thermal tolerance may be Bsal lineage dependent. A temperature of 4°C results in slower progression ~~build-up~~ of infection but does not reduce morbidity or mortality (Stegen *et al*., 2017). Desiccation is likely to be poorly

tolerated by Bsal, although data are currently lacking, and the encysted spore may be resistant to drying (Stegen *et al*., 2017; Van Rooij *et al*., 2015). It is not known to what extent Bsal tolerates freezing.

~~Co-occurrence of highly susceptible species such as fire salamanders with less susceptible species, such as Alpine newts may facilitate density independent disease dynamics that lead to the extirpation local extinction of the highly susceptible species (Stegen *et al*., 2017).~~

~~Barriers to pathogen dispersal, for example those preventing migration of infected hosts such as amphibian fences or roads, or those preventing transmission by potential Bsal vectors including humans, fomites and wildlife, may prevent transmission at small spatial scales (Spitzen-van der Sluijs *et al*., 2018).~~

#### 2.3.6. Geographical distribution

Asia is currently considered the region of origin of Bsal (Martel *et al*., 2014), where the infection appears to be endemic in amphibian communities across a wide taxonomic, geographical and environmental range, albeit at a low prevalence between 2 and 4% (Zhiyong *et al.,* 2018). In Asia, Bsal was shown to be widely present in urodele populations in China (People's Rep. of), Japan, Thailand and Vietnam. East Asia is presumed to be the native range of the fungus (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al.,* 2018).

Europe is considered the invasive range of the fungus where Bsal was first identified during a mortality event in fire salamanders ~~(*Salamandra salamandra*)~~ in Bunderbos, the Netherlands (Martel *et al.,* 2013). In Europe, Bsal was detected by surveys of wild susceptible species in Belgium, Germany and the Netherlands (Martel *et al.*, 2014; Spitzen-van der Sluijs *et al.,* 2016), and in captive urodele populations in Belgium, Germany, the Netherlands, Spain, and the United Kingdom (Fitzpatrick *et al.,* 2018; Sabino-Pinto *et al.,* 2015).

Bsal has not been reported in Africa or the Americas.

### 2.4. Biosecurity and disease control strategies

#### 2.4.1. Vaccination

Not available.

#### 2.4.2. Chemotherapy including blocking agents

A combined treatment using Polymyxin E, voriconazole and a temperature regime of 20°C has been shown to be effective in eradicating Bsal from infected hosts (Blooi *et al*., 2015c ~~2015b~~). If the treatment is not performed properly and does not achieve eradication, low level carriers are created and the likelihood of Bsal detection, is reduced.

#### 2.4.3. Immunostimulation

Not available.

#### 2.4.4. Breeding resistant strains

~~Breeding resistant strains is one of the few options for long term sustainable disease mitigation.~~

No information available.

#### 2.4.5. Inactivation methods

Bsal is sensitive to a wide variety of disinfectants (Van Rooij *et al*., 2015). Inactivation using formalin has been shown to hamper DNA detection using real-time PCR ~~qPCR~~. Bsal is killed within 30 seconds in 70% ethanol (Van Rooij *et al.,* 2017). Inactivation in 70% ethanol allows for subsequent molecular tests yet is less suitable for histopathology. The Bsal type strain AMFP 13/1 is killed at temperatures exceeding 25°C;

consequently, inactivation of this fungus can be achieved through heat treatment by autoclaving (Martel *et al.*, 2013).

### 2.4.6. Disinfection of eggs and larvae

No information available.

### 2.4.7. General husbandry

In captivity, pathogen detection is difficult due to low prevalence in subclinically infected animals that often carry Bsal at low intensities (Martel *et al*., 2014; Zhiyong *et al*., 2018). These subclinically infected animals often belong to (but are not restricted to) taxa of Asian urodeles. Highly susceptible species (such as fire salamanders ~~*Salamandra salamandra*~~) may serve a sentinel function. Temperature regimes in captivity may strongly interfere with pathogen detection. Temperatures higher than 20°C (and below 25°C) severely impair pathogen proliferation in the host skin (Blooi *et al*., 2015b ~~2015a~~) and may result in infections that cannot be detected.

Heat treatment can be used to clear infection with Bsal in thermotolerant salamander species (Blooi *et al*., 2015a).

Barriers to pathogen dispersal, for example those preventing migration of infected hosts such as amphibian fences or roads, or those preventing transmission by potential Bsal vectors including humans, fomites and wildlife, may prevent transmission at small spatial scales (Spitzen-van der Sluijs *et al*., 2018).

## 3. Specimen selection, sample collection, transportation and handling

This Section draws on information from Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

### 3.1. Selection of populations and individual specimens

In cases of disease or mortality in urodeles in captivity, sampling should be focused primarily on diseased or moribund animals (i.e. those showing skin lesions and abnormal behaviour). In a population with ongoing disease and mortality, live but diseased animals are preferentially sampled. The second choice is dead animals. Only freshly dead animals should be sampled as detectability of Bsal deteriorates post-mortem (Thomas *et al*., 2018). However, in the absence of diseased or freshly dead animals, apparently healthy animals can be sampled.

Similarly, in wild populations, samples should be taken preferentially from diseased ~~or~~, moribund or freshly dead animals ~~should preferentially be sampled, but~~; however, as these may quickly be removed (i.e. through predation, scavenging) only healthy animals may ~~only~~ be available. Populations which have declined or where dead animals have been observed should be targeted.

### 3.2. Selection of organs or tissues

The only relevant tissue is skin tissue and probably only from amphibians post-metamorphosis. Both invasive (skin biopsies) and non-invasive (~~cotton tipped~~ medical swabs) samples ~~sampling~~ are appropriate, given the apical shedding of Bsal spores. In dead animals, dorsal skin is the preferred tissue, given its slower post-mortem decay (Thomas *et al*., 2018).

### 3.3. Samples or tissues not suitable for pathogen detection

~~Any other t~~Tissues other than skin ~~is~~ are not suitable for the detection of Bsal in amphibians.

### 3.4. Non-lethal sampling

Non-lethal sampling is possible, either by collecting skin biopsies (toeclips or tailclips) or by non-invasively collecting samples using ~~cotton tipped~~ medical swabs. The latter is preferred given its minimal impact on animal welfare ~~well being~~. As Bsal is limited to the superficial skin layers of the amphibian host, non-lethal sampling results are equivalent to lethal sampling results. ~~In the absence of other, Bsal specific diagnostic tests (other than the laborious isolation of the fungus),~~ Large numbers of animals can be sampled using skin

swabs with minimal effects on animal welfare. ~~Cotton-tipped~~ Medical swabs should be rubbed firmly over the abdomen (10 times), the underside of a foot (10 times) and the ventral tail (10 times) using the tip of the swab. The use of disposable gloves for manipulating amphibians is highly recommended. Swabs should be transported immediately to the diagnostic laboratory or should be frozen until transfer.

### 3.5. Preservation of samples for submission

#### 3.5.1. Samples for pathogen isolation

Bsal isolation is a ~~very~~ laborious procedure, requiring up to two months to obtain ~~for obtaining~~ a pure culture from a clinical sample. Isolation from animals that died due to Bsal infection is hampered by bacterial overgrowth. The best sample for Bsal isolation is a diseased, living animal, which is euthanised just prior to an isolation attempt. Before sampling, diseased animals should be kept at temperatures between 5 and 15°C to avoid clearance of infection (Blooi *et al*., 2015b ~~2015a~~).

#### 3.5.2. Preservation of ~~Fixed~~ samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Skin swabs should be stored dry and preferably frozen.

#### 3.5.3. ~~Fixed~~ Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Skin samples for histopathology should be fixed immediately after collection. The recommended ratio of formalin (10%) to tissue is 10:1.

~~**3.5.4. Fixed Samples for electron microscopy**~~

~~For transmission electron microscopy, skin samples can be fixed in glutaraldehyde in 0.05 M sodium cacodylate buffer and 1% osmium tetroxide post-fixation (Martel *et al*., 2013).~~

#### 3.5.~~5~~ 4. Samples for other tests

Not applicable.

### 3.6. Pooling of samples

Pooling of up to four ~~five~~ skin swab samples appears to allow reliable detection of Bsal in clinically affected animals (Sabino-Pinto *et al*., ~~2018~~ 2019a; 2019b) but estimates of ~~on~~ the impact on diagnostic performance of the test ~~characteristics~~ have not been determined. Given low infection intensities in subclinically infected animals, sampling and testing of individual animals is recommended.

## 4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

| | |
|---|---|
| +++ = | Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; |
| ++ = | Suitable method(s) but may need further validation; |
| + = | May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application; |
| Shaded boxes = | Not appropriate for this purpose. |

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

***Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals***

| **Method** [amend or delete as relevant] | **A. Surveillance of apparently healthy animals** | | | | **B. Presumptive diagnosis of clinically affected animals** | | | | **C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Early life stages[2]** | **Juveniles[2]** | **Adults** | **LV** | **Early life stages[2]** | **Juveniles[2]** | **Adults** | **LV** | **Early life stages[2]** | **Juveniles[2]** | **Adults** | **LV** |
| **Wet mounts** | + | + | + | 1 | + | + | + | 1 | | | | 1 |
| **Histopathology[3]** | + | + | + | 1 | ++ | ++ | ++ | 1 | | | | 1 |
| **Cell or artificial media culture** | | | | | | | | | + | + | + | 1 |
| **Real-time PCR** | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 |
| **Conventional PCR** | | | | | | | | | | | | |
| **Amplicon sequencing[4]** | | | | | | | | | | | | |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Lateral flow assay** | + | + | + | 1 | ++ | ++ | ++ | 1 | | | | 1 |
| **Immunohistochemistry** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2.); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3. [3]Cytopathology and histopathology can be validated if the results from different operators has been statistically compared. [4]Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

**EU comment**

**The EU suggests reflecting the change in Section 4.4.1. as regards the level of validation of the real-time PCR test also in Table 4.1., i.e. to change "2" into "3" in the relevant "LV" columns.**

### 4.1. Wet mounts

Wet mounts of skin scraping or pieces of shed skin can be examined at magnification 10× using light microscopy. The presence of motile spores of approximately 5 µm are indicative of amphibian chytrid infection.

### 4.2. Histopathology and cytopathology

~~No reports are available on the use of cytology.~~ Histopathology of skin in amphibians post-metamorphosis may provide strong indications of Bsal infection. In ~~a~~ haematoxylin/eosin ~~staining of skin~~ stained sections, histopathological evidence suggestive of Bsal infections of skin, ~~is~~ multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular chytrid-type fungal thalli provides histopathological evidence of Bsal infection (Martel *et al*., 2013; White *et al*., 2016). Using immunohistochemistry, Bsal thalli can be stained, which aids in detecting low level infections (Thomas *et al*., 2018). Histopathology is highly indicative, yet does not allow ~~specific~~ definitive identification of Bsal, which needs further confirmation. In randomly collected skin samples from experimentally infected salamanders, histopathology was capable of detecting Bsal in only a minority of the samples (Thomas *et al*., 2018). In dead animals, post-mortem decay of the epidermis may mask the lesions (Thomas *et al*., 2018). Lesions can be so extensive, that the epidermis is entirely eroded and no fungal thalli can be observed. Mild infections can be missed due to the multifocal and small lesions (Thomas *et al*., 2018). ~~For asymptomatically~~ In subclinically infected animals, diagnostic sensitivity should be rated low. ~~Sensitivity,~~ In clinically affected animals, sensitivity and specificity of histopathology and immunohistochemistry have not been quantified.

No reports are available on the use of cytopathology.

### 4.3. Cell ~~or artificial media~~ culture for isolation

Bsal can be isolated and cultured on artificial media, yet this is a laborious and difficult procedure, typically requiring between 4 weeks and 2 months. There is a significant probability of bacterial overgrowth, which hampers fungal isolation, resulting in poor sensitivity. The protocol of Fisher *et al*. (2018) can be used. Small (approximately 1 $mm^2$) pieces of skin from an infected, diseased animal should first be thoroughly cleaned by wiping through agar plates. The cleaned pieces of skin can then each be transferred to a well of a 96-well plate, containing tryptone-gelatin hydrolysate lactose broth (TGhL) containing penicillin/streptomycin (200 mg/litre) and incubated at 15°C. Wells showing chytrid growth without bacterial contamination can be used for subculturing (Martel *et al*., 2013). Chytrid growth can be visualised by examining the wells under an inverted microscope (10–40 × magnification).

Given the difficulties to isolate Bsal from infected animals and the high uncertainty to obtain a viable culture, this method is not appropriate as ~~first diagnostic approach~~ a routine diagnostic method, but ~~(~~in rare cases~~)~~ may be useful to confirm infection ~~and for~~ or to obtain~~ing~~ isolates for research ~~(for example for epidemiological tracing)~~.

### 4.4. Nucleic acid amplification

#### 4.4.1. Real-time PCR

The following information is derived from Blooi *et al*. (2013), Thomas *et al*. (2018) and Sabino Pinto *et al*. (2018). DNA from skin swabs can be extracted using commercial DNA extraction kits. ~~in 100 µl Prepman Ultra Reagent (Applied Biosystems, Foster City, CA) or by using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The latter follows the animal tissues protocol (Qiagen DNeasy Blood and Tissue kit) with pre-treatment for Gram-positive bacteria and expanded initial incubation for 1 hour. DNA from skin tissue can be extracted using proteinase K digestion or DNA Easy Tissue Kit.~~ Extracted DNA is diluted tenfold to minimise possible PCR inhibition. Controls should be run with each assay: at least a

negative extraction control and a positive control; preferably, an internal PCR control is included. Positive control consists of DNA extracts of a tenfold dilution series of BsaI zoospores from 1 to 100,000 to allow quantification.

A TaqMan PCR has been partially validated to level ~~2~~ 3 without however, stating its intended purpose (Thomas *et al*., 2018). SYBR green real-time PCR, may be used as well but needs further validation to determine specificity and sensitivity (Martel *et al*., 2013). The TaqMan PCR can either be used as simplex PCR or in combination with primers to detect *B. dendrobatidis* in a duplex PCR (Blooi *et al*., 2013) and uses the forward primer STerF (5'-TGC-TCC-ATC-TCC-CCC-TCT-TCA-3'), reverse primer STerR (5'-TGA-ACG-CAC-ATT-GCA-CTC-TAC-3') and Cy5 labelled probe STerC (5'-ACA-AGA-AAA-TAC-TAT-TGA-TTC-TCA-AAC-AGG-CA-3') to detect the presence of the 5.8S rRNA gene of BsaI. Intra- and interassay efficiency were 95.7 and 96% ~~94 and 99%~~, respectively (Blooi *et al*., 2013). This TaqMan duplex PCR does not decrease detectability of both Bd and BsaI, except in case of mixed infections (Thomas *et al*., 2018). The use of simplex BsaI-specific PCR is therefore recommended in case Bd has been detected in the sample. The sensitivity of this real-time ~~q~~PCR is between 96 and 100% and diagnostic specificity 100% (95% CI: 73–100%; Thomas *et al*., 2018) when used in clinically affected animals. Although DNA quantities as low as 0.1 genomic equivalent can be detected (Blooi *et al*., 2013), Thomas *et al*. (2018) recommend a threshold of 1 genomic equivalent per reaction to reduce the likelihood of false positive results. Borderline results (≤ 1 GE per reaction) should be classified as suspect and need confirmation by sequencing (or isolation).

Samples are preferably run in duplicate. A sample is considered positive based on the combination of (1) the shape of the amplification curves (2) positive results in both duplications, (3) returning GE values above the detection threshold (1 GE per reaction) (4) low variability between duplicates (< 0.3 Ct value).

#### 4.4.2. Conventional PCR (PCR)

~~The use of real-time PCR is recommended.~~ No conventional PCR protocol has been validated.

#### 4.4.3. Other nucleic acid amplification methods

None validated.

### 4.5. Amplicon sequencing

~~For confirmation of suspect samples, amplified products can be sequenced with the primers as described in 4.4.1.~~

No conventional PCR protocol has been validated.

### 4.6. *In-situ* hybridisation ~~(and histoimmunochemistry)~~

No *In-situ* hybridisation~~:~~ ~~no~~ validated protocols are available.

### 4.7. Immunohistochemistry

Immunohistochemistry is currently not BsaI specific, due to the lack of BsaI specific antibodies (Dillon *et al*., 2017; Thomas *et al*., 2018). ~~Sensitivity of immunohistochemistry in diseased or dead animals can be estimated to be high if clinically affected skin regions have been selected.~~

### 4.8. Bioassay

Not available.

### 4.9. Antibody- or antigen-based detection methods

A lateral flow assay (LFA) using an IgM monoclonal antibody (MAb) was developed to detect infection in amphibian skin samples. This MAb does not discriminate between *B. salamandrivorans, B. dendrobatidis* and *Homolaphlyctis polyrhiza* (Dillon *et al.*, 2017 ~~2016~~). The sensitivity of this test is likely to be lower than that of the real-time ~~q~~PCR (Dillon *et al.*, 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using real-time ~~q~~PCR. This would make this technique most useful in

animals with high infection loads. Such techniques may be useful for point-of-care testing if specificity is increased and provided thorough validation.

### 4.10. Other ~~serological~~ methods

Not applicable

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The use of real-time PCR on skin swabs is recommended for surveillance.

## 6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the ~~presence~~ absence (Section 6.1) or in the presence ~~absence~~ of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

### 6.1. Apparently healthy animals or animals of unknown health status[13]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

Such surveys typically consist of non-invasive sampling using skin swabs that are examined for the presence of Bsal using real-time PCR. When applied to animals in the wild, confirmation by using a complementary technique, other than sequencing the PCR product, is often not feasible.

#### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with Bsal shall be suspected if ~~a positive result has been obtained on at least one animal from at least~~ one of the following ~~diagnostic tests~~ criteria is met:

i) Positive result by real-time PCR;

ii) Histopathological changes ~~(including immunohistochemistry)~~ consistent with the presence of the pathogen or the disease;

iii) The presence of motile spores, compatible with chytrid zoospores, in wet mount of urodele skin.

~~iv) Positive result from lateral flow assay (LFA).~~

#### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with Bsal is confirmed if ~~positive results have been obtained on at least~~, in addition to the criteria in Section 6.1.1, one ~~animal from two tests used in~~ of the following ~~combination~~ criteria is met:

i) Positive result by real-time PCR ~~on skin swab or skin tissue, and by histopathology or immunohistochemistry on skin tissue~~;

ii) ~~Positive result by real-time PCR on skin swab or skin tissue, and~~ Pathogenic agent isolation from the skin in culture and ~~confirmation~~ identification by real-time PCR.

13 For example transboundary *commodities*.

## 6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

#### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with Bsal shall be suspected if at least one of the following criteria is met:

i) Clinical signs (haemorrhages, ulcerations, presence of sloughed skin, see Section 2.3.2), notably the presence of skin ulcers and/or disecdysis;

ii) Positive result by real-time PCR ~~on at least one swab or skin tissue~~;

iii) Histopathological changes consistent with the presence of the pathogenic agent or the disease;

iv) Visual observation (by microscopy) of motile spores, compatible with amphibian chytrid zoospores, in a wet mount of the skin of at least one diseased urodele;

v) Positive result of antigen detection ~~technique such as~~ by LFA.

~~vi) Positive result from immunohistochemistry.~~

#### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with Bsal is confirmed if, in addition to the criteria in Section 6.2.1, ~~positive results have been obtained on at least one animal from two tests used in~~ one of the following ~~combination diagnostic tests~~ criteria is met:

i) Positive result by real-time PCR ~~on skin swab or skin tissue and by histopathology~~;

ii) ~~Positive result by real-time PCR on skin swab or skin tissue, and~~ Pathogenic agent isolation from the skin in culture and identification by real-time PCR ~~and confirmation by real-time PCR~~.

~~Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.~~

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with Bsal is provided in Table 6.3. This information can be used for the design of surveys for infection with Bsal, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3. Diagnostic performance of tests recommended for surveillance or diagnosis***

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Real-time PCR | Diagnosis | Experimentally infected salamanders (clinical and subclinical infection) | Skin swabs | *Salamandra salamandra* | 96–100 (26) | 100 (12) | Droplet digital PCR | Thomas *et al.* (2018) |

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study.

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**NB:** There are currently no OIE Reference Laboratories for infection with *Batrachochytrium salamandrivorans*
**NB:** First adopted in 2020.

Back to Agenda

CHAPTER 2.3.9.

# INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

**EU position**

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

## 1. Scope

Infection with spring viraemia of carp virus means infection with the pathogenic agent *Carp sprivivirus* (commonly known as spring viraemia of carp virus [SVCV]), of the Genus *Sprivivirus* and the Family *Rhabdoviridae.*

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

The virus genome is a non-segmented, negative-sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (Ahne *et al.,* 2002). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Bjorklund *et al.* [1996] and Genbank accession AJ318079 by Hoffmann *et al.* [2002]). The complete genome sequence of isolates from China (People's Rep. of) has also been deposited in Genbank (Genbank accession DQ097384 by Teng *et al.* [2007] and Genbank accession EU177782 by Zhang *et al.* [2009]).

Stone *et al.* (2003) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 isolates from different fish species and geographical locations that were previously identified by serology as SVCV or pike fry rhabdovirus (PFRV) ~~by serology~~. The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to genogroup I, sharing <61% nucleotide identity with viruses in the other three genogroups. Re-analysis of the sequence data generated for viruses assigned to Genogroup I identified four subgroups (Ia–d). Those viruses originating in Asia were assigned to Subgroup Ia, those from Moldova, the Ukraine and Russia to Subgroups Ib and Ic, and those from the UK to Subgroup Id.

#### 2.1.2. Survival and stability in processed or stored samples

There are limited published data on the stability of the pathogen in host tissues. There is also limited information on the stability of the virus in the tissues after death of a diseased animal. Detection of SVCV in the tissues of recently dead animals by either ~~both reverse transcription polymerase chain reaction (~~RT-PCR~~)~~ or ~~and~~ culture may be possible ~~should not be ruled out~~, and therefore, dead fish ~~as well as moribund should~~ may be taken for analysis if moribund fish are not available.

The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre ~~for~~ when stored ~~for 1 month~~ at –20°C for 1 month, or ~~for 6 months~~ at –30 or –74°C for 6 months (Ahne, 1976; de Kinkelin & Le Berre, 1974). The virus ~~is~~ remains stable over four freeze (–30°C)–thaw cycles in medium containing 2% serum (de Kinkelin & Le Berre, 1974).

#### 2.1.3. Survival and stability outside the host

The virus ~~has been shown to~~ can remain ~~viable~~ infectious outside the host for 5 weeks in river water at 10°C and for more than 6 weeks in pond mud at 4°C, reducing to 4 days in pond mud at 10°C (Ahne, 1976).

For inactivation methods, see Section 2.4.5.

## 2.2. Host factors

### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: ~~all varieties and subspecies of common carp (*Cyprinus carpio*), bighead carp (*Aristichthys nobilis*), bream (*Abramis brama*), Caspian white fish (*Rutilus kutum*), fathead minnow (*Pimephales promelas*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon Idella*), roach (*Rutilus rutilus*) and sheatfish (also known as European or wels catfish) (*Silurus glanis*).~~

| Family | Scientific name | Common name |
|---|---|---|
| Cyprinidae | *Abramis brama* | Bream |
| | *Aristichthys nobilis* | Bighead carp |
| | *Carassius auratus* | Goldfish |
| | *Ctenopharyngodon idella* | Grass carp |
| | *Cyprinus carpio* | Common carp (all varieties and subspecies) |
| | *Danio rerio* | Zebrafish |
| | *Notemigonus crysoleucas* | Golden shiner |
| | *Pimephales promelas* | Fathead minnow |
| | *Rutilus kutum* | Caspian white fish |
| | *Rutilus rutilus* | Roach |
| Siluridae | *Silurus glanis* | ~~Sheatfish (also known as European or wels catfish)~~ Wels catfish |

### 2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: Crucian carp (*Carassius carassius*), pike (*Esox lucius*), firebelly newt (*Cynops orientalis*), silver carp (*Hypophthalmichthys molitrix*), and yellow perch (*Perca flavescens*) ~~and zebrafish (*Danio rerio*)~~.

~~Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is SVCV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.~~

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
|---|---|---|
| ~~Cyprinidae~~ | ~~*Carassius carassius*~~ | ~~Crucian carp~~ |
| | ~~*Hypophthalmichthys molitrix*~~ | ~~Silver carp~~ |
| ~~Esocidae~~ | ~~*Esox lucius*~~ | ~~Northern pike~~ |
| ~~Percidae~~ | ~~*Perca flavescens*~~ | ~~Yellow perch~~ |
| ~~Salamandridae~~ | ~~*Cynops orientalis*~~ | ~~Firebelly newt~~ |

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated:

| Family | Scientific name | Common name |
|---|---|---|
| Catostomidae | *Catostomus commersonii* | White sucker |
| Cichlidae | ~~*Sarotherodon niloticus*~~ *Oreochromis niloticus* | Nile tilapia |
| Cyprinidae | *Notropis atherinoides* | Emerald shiner |
| | *Cirrhinus mrigala* | Mrigal carp |
| | *Labeo rohita* | Rohu |
| | *Tinca tinca* | Tench |

| | | |
|---|---|---|
| Penaeidae | *Litopenaeus vannamei* | Pacific white shrimp |
| Salmonidae | *Oncorhynchus tshawytscha* | Chinook salmon |
| | *Oncorhynchus nerka* | Sockeye salmon |
| | *Oncorhynchus mykiss* | Rainbow trout |
| | ~~Oncorhynchus mykiss~~ | ~~Steelhead trout~~ |

~~**2.2.3. Non-susceptible species**~~

~~Species that have been found non-susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Code* are: Largemouth bass (*Micropterus salmoides*), Muskellunge (*Esox masquinongy*), and Walleye (*Sander vitreus*).~~

| ~~**Family**~~ | ~~**Scientific name**~~ | ~~**Common name**~~ |
|---|---|---|
| ~~Centrarchidae~~ | ~~*Micropterus salmoides*~~ | ~~Largemouth bass~~ |
| ~~Esocidae~~ | ~~*Esox masquinongy*~~ | ~~Muskellunge~~ |
| ~~Percidae~~ | ~~*Sander vitreus*~~ | ~~Walleye~~ |

### 2.2.~~4~~ 3. Likelihood of infection by species, host life stage, population or sub-populations

Common carp varieties are the principal hosts for SVCV and are considered to be most likely ~~susceptible~~ to be infected ~~infection~~ with SVCV followed~~, in order of susceptibility,~~ by other carp species (including hybrids), other susceptible cyprinid species and finally ~~susceptible~~ non-cyprinid fish species. ~~When sampling during surveillance programmes for SVCV, common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp), then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Should these species not be available then other known susceptible species should be sampled. Cyprinid species may be are increasingly mixed together in polyculture systems and the risk of transmission of SVCV between species during disease outbreaks is high (Billard & Berni, 2004).~~

Generally, young fish up to one-year old are most ~~susceptible to~~ likely to demonstrate clinical signs of disease, but all age groups can be affected. Moreover, there is a high variability in the degree of susceptibility to infection with SVCV among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age or the age-related status of innate immunity appears to be extremely important to the manifestation of clinical disease: ~~the~~ younger ~~the~~ fish~~, the higher the susceptibility~~ are more likely to show signs of overt disease~~, although even adult broodfish can be susceptible to infection~~.

Fish that have separated from the shoal and found at the water inlet or sides of a pond are more likely to be infected.

For the purposes of Table 4.1 carp larvae and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, carp may be considered juveniles (i.e. fingerlings and grower fish) up to 250 g, and adults are above 250 g.

### 2.2.~~5~~ 4. Distribution of the pathogen in the host

~~The transmission of SVCV is horizontal (Fijan, 1988).~~ SVCV appears to enter via the gills and then spread~~s~~ to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks high titres of virus occur in the liver and kidney of infected fish, but much lower titres occur in the spleen, gills and brain (Dixon, 2008). The virus has been detected in ovarian fluid (Bekesi & Csontos, 1985)~~, but vertical transmission has yet to be demonstrated~~.

### 2.2.~~6~~ 5. Aquatic animal reservoirs of infection

Liu *et al.* (2004) isolated SVCV in China (People's Rep. of) from common and koi carp exhibiting no external or internal signs of disease, and similarly, the virus was isolated from apparently healthy wild carp in Canada (Garver *et al*., 2007). Thus fish ~~surviving infection~~ with ~~SVCV~~ long-term subclinical infections may act as reservoirs of infection.

### 2.2.~~7~~ 6. Vectors

The parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola geometra* (Annelida, Hirudinea) have been demonstrated to transfer SVCV from diseased to healthy fish under experimental conditions and the virus has been isolated from *A. foliaceus* removed from infected carp (Ahne *et al.*, 2002; Dixon, 2008). It has been demonstrated experimentally that virus can be isolated from fish tissues regurgitated by herons (*Ardea cinerea*) 120 minutes after being fed with SVCV-infected carp, suggesting a potential route for SVCV transmission, but is not known whether such transmission has occurred in nature (Peters & Neukirch, 1986).

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

A noticeable increase in mortality will occur in the population during an outbreak of infection with SVCV ~~there will be a noticeable increase in mortality in the population. Co-infections with koi herpesvirus or carp oedema virus can increase levels of mortality.~~ Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both nonspecific (e.g. interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Poor physiological condition of over-wintered fish may be a contributory factor to the onset of clinical disease in infected animals ~~susceptibility~~. In European aquaculture, losses can be up to 70% in young carp (Ahne *et al.*, 2002), but are usually from 1 to 40%.

In one survey from Serbia, the virus was isolated by culture in samples collected from 12 of the 38 hatcheries screened over the 10-year period (1992–2002) (Svetlana *et al.,* 2004). The virus occurred sporadically in different ponds on one site, and sporadically from year to year at different sites (Svetlana *et al.,* 2004). In another study, 18 of 30 tissue pools (five fish/pool) of wild, clinically health, common carp sampled in Canada in 2006 were positive for SVCV by culture (Garver *et al.,* 2007). ~~The isolation of SVCV in the latter case was from asymptomatic common carp which correlates with~~ This observation suggests that SVCV infection may ~~can often~~ be clinically inapparent (Fijan, 1999).

### 2.3.2. Clinical signs, including behavioural changes

Fish can become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Clinical signs of infection with SVCV are nonspecific and not all fish will exhibit all of the signs. Two of the most obvious and consistent features are abdominal distension and haemorrhages, which ~~. The latter~~ may be pale and occur on the skin, fin bases, eyes and gills ~~, which may be pale~~. The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. ~~During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Diseased fish usually appear darker in colour.~~ There may be no clinical signs in cases with a sudden onset of mortality.

### 2.3.3 Gross pathology

There are no pathognomonic gross lesions. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucous instead of food. Oedema and haemorrhage of the visceral organs is commonly observed (the spleen is often enlarged), and organs adhere to each other and to the peritoneum. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder (see Dixon, 2008). However, petechial haemorrhages are infrequent ~~uncommon~~ in cases caused by Asian strains of SVCV (Dikkeboom *et al*., 2004).

### 2.3.4. Modes of transmission and life cycle

The transmission of SVCV is horizontal (Fijan, 1988). Horizontal transmission may be direct, or via water, fomites or vectors (Section 2.2.7) (Fijan, 1988). The virus appears to enter the host via the gills. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine (Ahne, 1982).

Vertical ~~or~~ and 'egg-associated' transmission cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid, although there have been no further reports (Bekesi & Csontos, 1985).

~~Horizontal transmission may be direct or vectorial, water being the major abiotic vector (Fijan, 1988). Animate vectors (Section 2.2.6.) and fomites may also be involved in transmission of SVCV (Fijan, 1988).~~ ~~Once SVCV~~

~~is established in populations, it may be very difficult to eradicate without destroying all susceptible species and vectors types of life at the site.~~

### 2.3.5. Environmental ~~and management~~ factors

Disease outbreaks in carp generally occur between 11 and 17°C. They rarely occur below 10°C, and mortalities, particularly in older fish, decline as the temperature exceeds 22°C (Fijan, 1988). However, the virus was isolated from apparently healthy fish from a lake in Canada that had been sampled over a 13-day period during which the water temperature varied between 24.2°C and 27.3°C (Garver *et al,* 2007). These fish may have been more susceptible to infection as they were penned and detection was during spawning. Secondary and concomitant bacterial ~~and/~~or parasitic infections can affect the mortality rate and ~~display~~ the appearance of clinical signs. In carp, the disease is often observed during ~~in~~ spring~~time~~ (hence the common name for the disease), particularly in countries having cold winters. It is believed that the poor condition of the over-wintered fish may be a contributory factor in the ~~disease~~ occurrence of clinical disease. Clinical ~~The~~ disease can occur in fish in quarantine following the stress of transportation, even though there has been no evidence of infection prior to transportation.

### 2.3.6. Geographical distribution

For a long time, the geographical range of SVCV was limited to countries of the European continent that experience low water temperatures during winter. ~~Consequently~~ The disease has been recorded from most European countries. ~~and from certain of the western Independent States of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine) (see Dixon 2008 for references to these and the following locations).~~ However, in 1998, the disease was recorded in South America ~~(in goldfish in a lake in Brazil)~~ and in 2002 in ~~the USA,~~ North America. ~~and in 2006 in Canada~~ The virus was first detected in Asia ~~Detection of the virus in carp in China (People's Rep. of) was confirmed~~ in 2004.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

A safe and effective vaccine is not currently available; however, ~~a number~~ the efficacy of an experimental DNA vaccine has been investigated ~~inactivated preparations, live attenuated vaccines and DNA vaccines have given encouraging results (Dixon, 2008, (~~Emmenegger & Kurath, 2008). ~~The use of live attenuated vaccines or the DNA vaccines might affect diagnostic performance.~~

### 2.4.2. Chemotherapy including blocking agents

Methisoprinol inhibits the replication of SVCV *in vitro*, but has not been tested under carp culture conditions (Siwicki *et al.,* 2002).

### 2.4.3. Immunostimulation

Injection into carp of single-stranded and double-stranded RNA (which is an interferon inducer) protected carp for longer than 3 weeks, but the treatment is not effective by bath administration (Alikin *et al.,* 1996).

### 2.4.4. Breeding resistant strains

The "Krasnodar" strain of common carp has been bred for increased resistance to SVCV (Kirpichnikov *et al*., 1993).

### 2.4.5. Inactivation methods

The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants ~~are also effective for inactivation~~ inactivate the virus: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre$^{-1}$ chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al.,* 2007).

### 2.4.6. Disinfection of eggs and larvae

Eggs can be disinfected by iodophor treatment (Ahne & Held, 1980).

### 2.4.7. General husbandry

Methods to control ~~of~~ infection with SVCV rely ~~relies~~ on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. ~~Hygiene measures should include disinfection of eggs by iodophor treatment (Ahne & Held, 1980), until it has been confirmed unequivocally that vertical transmission does not occur, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish.~~ Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C may stop or prevent outbreaks of infection with SVCV.

## 3. Specimen selection, sample collection, transportation and handling

This Section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

### 3.1. Selection of populations and individual specimens

~~Sampling samples, target comprise of susceptible species on the site with each group being represented in the sample. A group is defined as a. The population to be sampled may be stratified into groups of the same fish species that shares a common water supply and originate from the same broodfish or spawning population. Generally young Moribund fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Any moribund fish present in the fish population to be sampled should be sampled selected first for sample collection and the remainder of the samples should comprise randomly selected live fish from all groups of susceptible species rearing units that represent the lot being examined.~~

Clinical inspections should be carried out during a period when the water temperature is between 11°C and 17°C. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention should be paid to the water outlet area where weak fish tend to accumulate due to the water current.

For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) Common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp), then other ~~carp~~ cyprinid species such as ~~crucian carp,~~ goldfish, grass carp, bighead carp, bream and roach ~~silver carp~~. Susceptible species should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).

ii) If more than one water source is used for fish production, fish from the highest risk water source should be targeted. If all water sources are of equal risk, all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with SVCV should be collected. Ideally fish should be collected while alive, however, recently dead fish can also be selected for diagnostic testing ~~purposes~~. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no clinical signs or gross pathognomonic lesions ~~and no clinical signs~~ in cases of sudden mortality ~~(see Section 4.1.1.)~~.

### 3.2. Selection of organs or tissues

Kidney, spleen, gill and encephalon should be selected from ~~subclinically infected fish~~ (apparently healthy fish~~)~~.

For clinically affected fish: whole fry ~~alevin~~ (body length ≤ 4 cm), entire viscera including kidney and ~~encephalon~~ brain (> 4 cm body length ≤ 6 cm) or, for larger ~~sized~~ fish, liver, kidney, spleen and encephalon should be selected.

### 3.3. Samples or tissues not suitable for pathogen detection

~~Virus isolation may also not be possible from~~ Decomposed clinical samples ~~A number of studies in which attempts were made to isolate virus from reproductive fluids were unsuccessful, although~~ and seminal fluid samples are not suitable. While the virus has been isolated at low frequency from ovarian~~, but not seminal,~~ fluids, the suitability of these tissues for detection of SVCV ~~samples~~ has not been substantiated (Bekesi & Csontos, 1985).

### 3.4. Non-lethal sampling

Serological assays for antibodies can be undertaken on blood samples~~; the~~ and can indicate possible exposure to SVCV, however, serology is not a suitable test for making a suspect diagnosis. ~~can only be used for a presumptive diagnosis given cross reactivity of anti-SVCV antibodies with viruses of the species pike fry sprivivirus allows for a presumptive indication of infection with SVCV.~~

### 3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter ~~2.2.0 or~~ 2.3.0 ~~or 2.4.0~~.

#### 3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (diseases of fish).

~~Samples for virus isolation (Section 3.2.) should be transported to the laboratory at 4°C using refrigerated containers or on ice, preferably in virus transport medium and tested within 24 hours or, in exceptional circumstances, 48 hours. The shipment of organ samples is preferred, but live or whole dead fish can be submitted to the testing laboratory if necessary. If this is not possible, samples can be frozen, but there may be loss of virus viability on thawing the samples. Repeated freeze–thawing of the sample must be avoided.~~

#### 3.5.2. Preservation of ~~Fixed~~ samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. [Alternatives to ethanol can be mentioned if they can be referenced.]~~

The material collected for virus culture is generally used for the molecular diagnostic assays, but additional tissue samples for RT-PCR can be preserved in commercially available RNA preservation solutions according to the manufacturers' recommendations, or, alternatively, samples can be preserved in 80–90% (v/v) analytical grade (absolute) ethanol at the recommended ratio of ethanol to tissue of 10:1.

#### 3.5.3. ~~Fixed~~ Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

**EU comment**

**The EU suggests deleting the words "or in-situ hybridisation" from the title above, as that method is neither recommended in Table 4.1. nor is it mentioned in the text below.**

~~Histology samples from each individual fish must be taken~~ placed into 10% neutral buffered formalin (NBF) ~~immediately after collection to prevent sample deterioration. The recommended ratio of fixative to tissue is 10:1 and each sample should be cut cleanly and be no thicker than approximately 4 mm to allow the fixative to penetrate the material and should be cut cleanly.~~ Standard methods for histopathology can be found in Chapter 2.3.0 *General information* (*diseases of fish*).

~~**3.5.4. Fixed Samples for electron microscopy**~~

~~EM sampling is~~ ~~Samples for electron microscopy are not routinely required as standard, and the material is and are collected only when where it is considered beneficial to facilitate potential further diagnostic investigation work. From each fish sampled, a 2 mm cubed (approximately) sample section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.~~

### 3.5.~~5~~ 4. Samples for other tests

Tubes for the separation of serum are available commercially. After collection, the blood is allowed to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Serum is clarified by centrifuging at 1000–2000 ***g*** for 10 minutes ~~in a refrigerated centrifuge~~ at 4–8°C.

~~It is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette and maintain the samples at 2–8°C while handling. If the serum is not analysed immediately, it should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze thaw cycles because this is detrimental to many serum components. Samples that are haemolysed, icteric or lipaemic can invalidate certain tests.~~

## 3.6. Pooling of samples

~~Traditionally pools of five animals have been used and more recently this has been increased to pools of ten animals for virus culture. However, no published data on the effect of pooling on test characteristics has been published.~~

Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

# 4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

| | |
|---|---|
| +++ = | Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; |
| ++ = | Suitable method(s) but may need further validation; |
| + = | May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application; |
| Shaded boxes = | Not appropriate for this purpose. |

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

***Table 4.1.*** *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology[3]** | | | | | | | | | | | | |
| **Cytopathology[3]** | | | | | | | | | | | | |
| **Cell ~~or artificial media~~ culture** | | ++ | ++ | 1~~-3~~ | | ++ | ++ | 1~~-3~~ | | ++ | ++ | 1~~-3~~ |
| **Real-time PCR** | | | | | | | | | | | | |
| **Conventional PCR** | | ++ | ++ | 1~~-2~~ | | ++ | ++ | 1~~-2~~ | | ++ | ++ | 1~~-2~~ |
| **Amplicon sequencing[4]** | | | | | | | | | | +++ | +++ | 1~~-3~~ |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Immunohistochemistry** | | | | | | ++ | ++ | 1 | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | ++ | ++ | 1 | | | | |
| **IFAT ~~Other antigen detection methods~~** | | | | | | ++ | ++ | 1 | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

[2]Early and juvenile life stages have been defined in Section 2.2.3.

[3]Histopathology and cytopathology can be validated if the results from different operators have been statistically compared.

[4]Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Not applicable.

## 4.2. Histo–pathology and cytopathology

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivasculitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necrosis and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres, and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed, and lymph vessels are filled with detritus and macrophages. In the swim bladder, the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the submucosa are dilated with nearby lymphocyte infiltration.

As the histopathological presentation ~~picture~~ is not specific for the disease, and not all fish will exhibit each feature (Misk *et al.,* 2016), microscopic methods by themselves are not recommended for diagnosis of SVCV ~~as the histopathological picture is not specific for the disease~~. They may, however, provide supporting evidence, particularly, when immunohistochemistry ~~immunohistological (IHC)~~ or nucleic acid ~~DNA~~ based *in-situ* hybridisation methods are used (see the relevant Sections below).

~~Fixed sections can also be used for histoimmunochemistry (but see caveats in Section 4.6.).~~

## 4.3. Cell ~~or artificial media~~ culture for isolation

### 4.3.1. Cell lines

~~If culturing viruses~~ The recommended cell lines for SVCV detection are EPC, FHM or GCO. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

EPC, FHM and GCO cells are grown at 20–30°C in suitable medium, e.g. Eagle's minimal essential medium (MEM or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2. Cell culture plates should be seeded 4–48 hours and not 100% confluent prior to inoculation. 15–30 minutes prior to sample inoculation, cells should be pre-treated with 7% (w/v) PEG-20,000 solution (10–15 µl/cm$^2$) (Batts & Winton, 1989; Wang *et al.,* 2016 ).

### 4.3.2. Sample preparation and inoculation

~~*Cell culture*~~

~~Cell line to be used: EPC, FHM or GCO.~~

Virus isolation ~~extraction~~: Use the procedure described in Section ~~A.~~2.2.2 of Chapter 2.3.0.

Inoculation of cell monolayers: make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium (i.e. the homogenate supernatants will be 1/100 and 1/1000 dilutions of the original organ material) and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers drained of their culture medium. Alternatively, make a single tenfold dilution of the 1/10 organ homogenate (i.e. a 1/100 dilution of the original organ material) and add an appropriate volume of both the 1/10 and 1/100 dilutions directly to undrained 24 hour-old cell monolayers, to effect 1/100 and 1/1000 final dilutions of the organ homogenate. Should toxicity of the sample be a problem, make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium as described above and inoculate at least 2 cm$^2$ of drained cell monolayer with 100 µl of each dilution. Allow to adsorb for 0.5–1 hour at 10–15°C, withdraw the inoculum and add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml well$^{-1}$ for 24-well cell culture plates). Incubate at 20°C.

Monitoring incubation: Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

Maintain the pH of the cell culture medium at between 7.3. and 7.6. during incubation. This can be achieved by the addition to the inoculated medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris (Tris [hydroxymethy]) aminomethane)/HCl buffer solution (for cell culture plates).

The cytopathic effect (CPE) is characterised by rounding, detachment and lysis of cells (Fijan, 1999). If a CPE appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately ~~(see Section 4.6.2.)~~.

Subcultivation procedures: Using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24 hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above. ~~If no CPE occurs, the test may be declared negative.~~

If no CPE occurs the test may be declared negative. However, if undertaking surveillance to demonstrate freedom from SVCV it would be advisable to screen the cells at the end of the 14 days using an SVCV-specific RT-PCR ~~or real-time RT-PCR~~ (Section 4.4). Following a positive result culture should be re-attempted.

Following isolation, the virus must be identified, and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods are generally regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur. Commercially available kits using polyclonal antibodies may also lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al*., 2007) to confirm the identity of the virus.

## 4.4. Nucleic acid amplification

### 4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control if available and validated.

Real-time RT-PCR assays are available to detect and confirm ~~infection with~~ SVCV (Yue *et al*., 2008; Zhang *et al*., 2009), however, they are not currently recommended as they have not been sufficiently validated.

### 4.4.2. Conventional RT-PCR

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~ Positive and negative controls should be run with each stage of the assays: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

*Nested reverse-transcription polymerase chain reaction* (*RT-PCR*) (*confirmation of virus identity from cell culture isolation or directly from fish tissue extracts*)

The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).

i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 50 µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.

   A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR. ~~Examples are Trizol ReagentT (RL, Life Technologies, Paisley, UK), SV Total RNA isolation system (Promega) and Nucleospin® RNA (AB gene), EZ virus mini kit, Ez RNA tissue mini kit (Qiagen).~~

ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or an equivalent reverse transcriptase system and 1/10 of the total RNA extracted above.

iii) RT-PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.

iv) If the CPE in culture is not extensive it is possible that a visible product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).

v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.

vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.

vii) In cases where the CPE is extensive and the virus replicates to a high titre, or where a semi-nested RT-PCR assay was used, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

~~**NOTE:** The appropriate IUB codes have been used where appropriate and are indicated by an asterisk (*).~~

*Reverse-transcription polymerase chain reaction* (*RT-PCR*) (*confirmation of virus identity*)

Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutna *et al*., 2003; Shimahara *et al*., 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al*., 2014). With the exception of the conventional RT-PCR assay developed by Shimahara *et al*. (2016) the other assays were not sufficiently ~~fully~~ validated against representatives from each of the recognised SVCV genogroups and they may fail to detect the full range of SVCV genotypes.

A summary of the Shimahara *et al.* (2016) RT-PCR method follows. Amplification of a 369 bp fragment of SVCV glycoprotein gene is performed using primers as follows: SVCV-G1: 5'-TGA-AGA-YTG-TGT-CAA-TCA-AGTC-3' and SVCV-G2: 5'-GCG-ART-GCA-GAG-AAA-AAG-TG-3'. Preparation of RNA template is the same as nested RT-PCR above. Reverse transcription of SVCV RNA and amplification of cDNA are carried out using SuperScript III one-step RT-PCR with PlatinumR Taq (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction mixture contained 10 pmol of each primer, 12.5 µl of 2× reaction mix, 1 µl of SuperScript III RT/Platinum *Taq* Mix and 2.5 µl template. After reverse transcription at 50°C for 30 minutes and 94°C for 2 minutes, 40 amplification cycles of 94°C for 15 seconds, 56°C for 30 seconds and 68°C for 1 minute followed by a final extension step at 68°C for 7 minutes is performed. All amplified products are confirmed as SVCV in origin by sequencing.

### 4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification assays are available to detect and confirm SVCV infections (Shivappa *et al.,* 2008), however, they are currently not recommended as they are not sufficiently validated.

~~Infection with~~ SVCV has also been ~~confirmed~~ detected using RT-PCR and hybridisation with non-radioactive probes to determine the genotype (~~Oreshkova *et al.,* 1999;~~ Sheppard *et al*., 2007), however, it is currently not recommended as it is not sufficiently validated.

## 4.5. Amplicon sequencing

~~See above (Section 4.4.2). All~~ Nucleotide sequencing of all RT-PCR amplicons ~~should be sequenced to confirm that they are SVCV in origin~~ (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. SVCV-specific ~~products~~ sequences will share a higher degree of nucleotide ~~identity~~ similarity to one of the published reference sequences for SVCV (Genbank accession U18101, AJ318079, DQ097384 and EU177782) compared to the published reference sequences for the *Pike sprivivirus*es (GenBank FJ872827, KC113518 and KC113517).

## 4.6. *In-situ* hybridisation ~~(and histoimmunochemistry)~~

Although *in-situ* hybridisation can be used to locate SVCV ~~the virus~~ in different tissues ~~on~~ in known positive animals, ~~but~~ this assay is currently not recommended as it has not been ~~well~~ validated as a diagnostic tool for the detection of SVCV ~~as a diagnostic tool~~.

## 4.7. Immunohistochemistry

SVCV can be detected by immunohistochemistry, however, care must be taken with interpreting the results of ~~serological~~ these tests for SVCV, and positive results ~~from antibody-based assays~~ should be confirmed by RT-PCR and sequencing ~~(see Section 4.8.)~~.

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store and transport the kidney pieces as indicated in Section 2.2.1 of Chapter 2.3.0. together with the other organs required for virus isolation.

iv) Allow the imprint to air-dry for 20 minutes.

v) Fix with cold acetone (stored at –20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBS containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

x) Rinse four times with PBST.

xi) Incubate the imprints with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xii) Rinse four times with PBST.

xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.

xiv) Examine under ~~incident ultraviolet (UV) light using~~ a fluorescence microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical aperture of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

## 4.8. Bioassay

Not available.

## 4.9. Antibody-~~based~~ or antigen-based detection methods ~~(ELISA, etc.)~~

~~Serological~~ Antibody- or antigen-based methods that detect SVCV must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other ~~viruses~~ closely related spriviviruses (PFRV, GrCRV and TenRV) may occur. Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). These techniques should not be used as a screening method.

### 4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)

~~*Virus identification by enzyme-linked immunosorbent assay* (*ELISA*)~~

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well$^{-1}$). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well$^{-1}$).

v) Rinse four times with PBST.

vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.

vii) Dispense 100 µl well$^{-1}$ of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control). Also include SVCV positive control virus. Incubate for 1 hour at 37°C.

viii) Rinse four times with PBST.

ix) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. An MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

x) Rinse four times with PBST.

xi) If HRPO-conjugated antibody has been used, go to step xiii. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xii) Rinse four times with PBST.

xiii) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

*Enzyme-linked immunosorbent assay* (*ELISA*) *using tissue homogenates*

See Section ~~A.~~2.2.2 of Chapter 2.3.0, for obtaining organ homogenates.

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well$^{-1}$). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well$^{-1}$).

v) Rinse four times with PBST.

vi) Store a 1/4 aliquot of each homogenate at 4°C, in case the test is negative and virus isolation in cell culture is required.

vii) Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulphonide fluoride; mix gently.

viii) Dispense 100 µl well–1 of two- or four-step dilutions of the sample to be identified, and of negative control tissues. Also include an SVCV positive control virus. Incubate for 1 hour at 37°C.

ix) Rinse four times with PBST.

x) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. A MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) If HRPO-conjugated antibody has been used, go to step xiv. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xiii) Rinse four times with PBST.

xiv) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

xv) If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 4.3.

### 4.9.2. Indirect fluorescent antibody test (IFAT)

*Virus identification Confirmation of virus identity by the indirect fluorescent antibody test (IFAT)*

i) Prepare monolayers of cells in 2 cm$^2$ wells of plastic cell culture plates, flasks or on cover-slips or glass slides in order to reach approximately 80% confluency within 24 hours of incubation at 25°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. For tests using cells cultured on glass cover-slips or slides, the dilutions are made in sterile containers and then used to inoculate the cells.

iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5000–10,000 PFU ml$^{-1}$ in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for slides or cover-slips or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for cells on plastic substrates. Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 $cm^2$ of cell monolayer.

vi) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vii) Rehydrate the dried cell monolayers, if they have been stored frozen, by four rinsing steps with ~~PBS containing 0.05% Tween 20~~ PBST and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

viii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

ix) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

x) Incubate the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

xi) Rinse four times with PBST.

xii) Incubate the cell monolayers with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) View the treated cell monolayers on plastic substrates immediately, or mount the slides or cover-slips using glycerol saline at pH 8.5, or a commercially available mountant.

xv) Examine under ~~incident ultraviolet (UV) light using~~ a fluorescence microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

### 4.10. Other ~~serological~~ methods

Not applicable

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The method for surveillance of apparently healthy populations ~~susceptible fish populations~~ for declaration of freedom from infection with SVCV is inoculation of cell culture with tissue homogenates ~~extracts~~ (as described in Section 4.3 ~~4.5~~) ~~to demonstrate absence of the virus~~. Cell culture is considered the most suitable method despite the lack of validation data for diagnostic methods for SVCV.

## 6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the ~~presence~~ absence (Section 6.1) or in the presence ~~absence~~ of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

## 6.1. Apparently healthy animals or animals of unknown health status[14]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link~~(s)~~ to an infected population. Geographical proximity to, or movement of animals or animal products or equipment~~, etc.,~~ from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with SVCV shall be suspected if~~: a positive result has been obtained on at least one animal from~~ at least one of the following ~~diagnostic tests~~ criteria is met:

i) Positive result by conventional RT-PCR~~ a recommended molecular or antigen or antibody detection test~~;

ii) SVCV-typical CPE. ~~Cytopathic effect~~ in cell culture ~~(viruses)~~.

### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SVCV is considered to ~~shall~~ be confirmed if, in addition to the criteria in Section 6.1.1, ~~positive results has been obtained on at least one animal from two test used in the following combination~~ the following criterion is met:

i) ~~Pathogen isolation AND Conventional~~ SVCV-typical CPE in cell culture followed by virus identification by conventional RT-PCR test ~~followed by~~ and amplicon sequencing.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2. Clinically affected animals

Clinical signs are not pathognomonic for infection with SVCV~~ a single disease~~; however they may narrow the range of possible diagnoses. ~~[For many diseases, especially those affecting mollusc, 'clinical signs' are extremely limited and mortality may be the only or most dominant observation.]~~

### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with SVCV shall be suspected if at least one of the following criteria is ~~are~~ met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality;

ii) Positive result by conventional RT-PCR ~~a recommended molecular or antigen or antibody detection test on at least one animal~~;

iii) Positive~~s~~ result by antigen ELISA~~ or IFAT or immunohistochemistry~~;

iv) Positive result by IFAT;

v) Positive result by immunohistochemistry;

vi) SVCV-typical CPE ~~Cytopathic effect~~ in cell culture.

[14] For example transboundary *commodities*.

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with SVCV is considered to ~~shall~~ be confirmed if, in addition to the criteria in Section 6.2.1, ~~positive results has been obtained on at least one animal from two test used in the following combination~~ the following criterion is met:

i) ~~Pathogen isolation AND Conventional~~ SVCV-typical CPE in cell culture followed by virus identification by conventional RT-PCR test ~~followed by~~ and amplicon sequencing.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with SVCV is provided in Table 6.3. (note: no data are currently available). This information can be used for the design of surveys for infection with SVCV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3.*** *Diagnostic performance of tests recommended for surveillance or diagnosis*

| Test type | Test purpose | Source population | Tissue/ sample type | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Cell culture | Surveillance, diagnosis | = | Tissue homogenates | = | Not yet available | Not yet available | = | = |
| RT-PCR | Surveillance, diagnosis | = | Tissue homogenates | = | Not yet available | Not yet available | = | = |
| RT-PCR | Surveillance, diagnosis | = | Cell culture | = | Not yet available | Not yet available | = | = |
| ~~RT-LAMP*~~ | ~~Surveillance~~ | ~~Live imported fish~~ | ~~Spleen, kidney and brain homogenate~~ | ~~Common carp, koi, goldfish~~ | ~~92.6 (27)~~ | ~~98.2 (445)~~ | ~~Virus isolation~~ | ~~Liu *et al.*, 2008~~ |

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study;
~~RT-LAMP: = real-time loop mediated isothermal amplification. *Listed as suitable test~~

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* *

**NB:** There are OIE Reference Laboratories for Spring viraemia of carp
(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/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
Spring viraemia of carp

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CHAPTER 2.3.4.

# INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

**EU position**

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

## 1. Scope

Infection with infectious haematopoietic necrosis virus means infection with the pathogenic agent Salmonid novirhabdovirus (commonly known as infectious haematopoietic necrosis virus [IHNV]) of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

IHNV consists of a bullet-shaped particle of approximately 150–190 nm in length and 65–75 nm in diameter that encapsulates a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides. The viral genome codes ~~for~~ six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L). Due to the primary position of the nucleoprotein gene on the IHNV genome, nucleoprotein transcripts and protein are the first and most abundant during viral infection and is typically the preferred target of diagnostic tests. The glycoprotein forms spike-like projections on the surface of the mature virion and is the primary antigenic component of the virus such that anti-glycoprotein serum is sufficient to neutralise ~~infections~~ IHNV.

The type strain of IHNV is the Western Regional Aquaculture Center (WRAC) strain available from the American Type Culture Collection (ATCC VR-1392). The GenBank accession number of the genomic sequence of the WRAC strain is L40883 (Morzunov *et al.,* 1995; Winton & Einer-Jensen, 2002).

Phylogenetic analyses based on G-gene nucleotide sequences have classified IHNV isolates into five major genogroups denoted U, M, L, E, and J that correspond to geographical location rather than host species (Cieslak *et al.,* 2017; Enzmann *et al.,* 2005; 2010; Johansson *et al.,* 2009; Kim *et al.,* 1999; Kolodziejek *et al.,* 2008; Kurath *et al.,* 2003; Nishizawa *et al.,* 2006). Nevertheless, IHNV displays a strong phylogeographic signature reflecting the host species from which the virus is most commonly isolated in various geographical areas (e.g. sockeye salmon [*Oncorhynchus nerka*] in the Northeast Pacific – U genogroup; Chinook salmon [*Oncorhynchus tshawytscha*] in California, USA – L genogroup; and rainbow trout [*Oncorhynchus mykiss*] in Europe, Asia, and Africa (Mulei *et al.,* 2019) and ~~Idaho,~~ USA – E, J and M genogroups, respectively). Additionally, experimental infections demonstrating that U and M genogroup viruses had higher virulence in sockeye salmon and rainbow trout, respectively, and L genogroup showed medium virulence to both sockeye salmon and rainbow trout (Garver *et al*., 2006), supports the ~~observation~~ finding that virulence depends on viral strain and species infected, and IHNV strains isolated from its historical phylogeographic host tends to be more virulent for the same species in comparison to other species.

#### 2.1.2. Survival and stability in processed or stored samples

IHNV stability in host tissues during storage and processing is largely influenced by temperature. The virus is more stable at lower temperature and remained infectious for at least 3 days at 4°C in naturally infected or IHNV-seeded tissue (Burke & Mulcahy, 1983; Gosting & Gould, 1981; Hostnik *et al.,* 2002; Pietsch *et al.*, 1977). For long-term survival of infectious virus, tissues should be stored at temperatures below –20°C (Burke & Mulcahy, 1983; McClure *et al.,* 2008). The preferred method for retaining infectious virus is to maintain the IHNV sample on ice with rapid processing and inoculation of cell cultures as soon as possible

due to the progressive reduction in titre with increasing temperature (Barlic-Maganja *et al.,* 2002; Gosting & Gould, 1981).

### 2.1.3. Survival and stability outside the host

IHNV can survive outside the host tissue in fresh water and sea water, but is ~~impacted~~ affected by temperature, ultraviolet (UV) exposure, microbial community and suspended sediments. ~~At 4°C–15°C, $10^5$ pfu/ml of IHNV remained detectable via cell culture after 1 week in either fresh or salt water (Kell *et al.,* 2014).~~ For all genotypes, inactivation rates are reduced at lower water temperatures and virions remain infectious for longer in freshwater compared with seawater (Kell *et al.,* 2014). However, when exposed to sunlight (UV-A and UV-B), IHNV at the water surface is rapidly inactivated with six orders of magnitude of virus rendered non-infectious within 3 hours (Garver *et al.,* 2013). In addition, infectious virus is inactivated by the microbial community within the water source and with increased amounts of suspended sediments (Garver *et al.,* 2013; Kamei *et al.,* 1987).

For inactivation methods, see Section 2.4.6.

## 2.2. Host factors

### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are:

| Family | Scientific name | Common name |
|---|---|---|
| Esocidae | *Esox lucius* | Northern pike |
| Salmonidae | *Salmo marmoratus* | Marble trout |
| | *Salmo salar* | Atlantic salmon |
| | *Salmo trutta* | Brown trout |
| | *Salvelinus alpinus* | Arctic char |
| | *Salvelinus fontinalis* | Brook trout |
| | *Salvelinus namaycush* | Lake trout |
| | *Oncorhynchus clarkii* | Cutthroat trout |
| | *Oncorhynchus tshawytscha* | Chinook salmon |
| | *Oncorhynchus keta* | Chum salmon |
| | *Oncorhynchus kisutch* | Coho salmon |
| | *Oncorhynchus masou* | Masou salmon |
| | *Oncorhynchus mykiss* | Rainbow trout |
| | *Oncorhynchus nerka* | Sockeye salmon |

### 2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5 of the *Aquatic Code* are: White sturgeon (*Acipenser transmontanus*), European eel (*Anguilla anguilla*), Tube-snout (*Aulorhynchus flavidus*), Pacific herring (*Clupea pallasii*), Shiner perch (*Cymatogaster aggregate*) and Turbot (*Scophthalmus* ~~*maxima*~~ *maximus*).

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
|---|---|---|
| ~~Acipenseridae~~ | ~~*Acipenser transmontanus*~~ | ~~White sturgeon~~ |
| ~~Anguillidae~~ | ~~*Anguilla anguilla*~~ | ~~European eel~~ |
| ~~Aulorhynchidae~~ | ~~*Aulorhynchus flavidus*~~ | ~~Tube-snout~~ |
| ~~Clupeidae~~ | ~~*Clupea pallasii*~~ | ~~Pacific herring~~ |
| ~~Embiotocidae~~ | ~~*Cymatogaster aggregate*~~ | ~~Shiner perch~~ |
| ~~Schophthalmidae~~ | ~~*Scophthalmus maxima*~~ | ~~Turbot~~ |

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: Common carp (*Cyprinus carpio*) and American yellow perch (*Perca flavescens*).

| ~~Family~~ | ~~Scientific name~~ | ~~Common name~~ |
|---|---|---|
| ~~Cyprinidae~~ | ~~*Cyprinus carpio*~~ | ~~Common carp~~ |
| ~~Percidae~~ | ~~*Perca flavescens*~~ | ~~American yellow perch~~ |

~~**2.2.3. Non-susceptible species**~~

~~None known.~~

### 2.2.~~4~~ 3. Likelihood of infection by species, host life stage, population or sub-populations

IHNV predominantly infects ~~salmon and trout~~ salmonid species with fry being the most highly susceptible stage (LaPatra, 1998). Resistance to infection typically increases with fish age until the spawning stage. Returning adult spawning salmon, can be highly infected and shed large amounts of virus in ovarian fluid and milt despite ~~a lack~~ the absence of clinical disease (Dixon *et al.,* 2016).

For the purposes of Table 4.1 rainbow trout alevin and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, fingerlings and ongrowing fish up to 50 g be considered as juveniles and fish over 50 g adults.

### 2.2.~~5~~ 4. Distribution of the pathogen in the host

IHNV targets the haematopoietic tissue and is most commonly isolated from kidney and spleen tissues. The virus has also been isolated from gill, oral region, pharynx, oesophagus, intestine, stomach, pyloric caeca, liver, brain, heart, thymus, pancreas, adipose tissue, muscle, cartilage, skin, fin and mucous (Brudeseth *et al.*,2002; Dixon *et al.,* 2016; Drolet *et al.,* 1994; Harmache *et al.,* 2006; LaPatra *et al.,* 1989; Yamamoto *et al.,* 1990a). ~~In spawning fish~~ IHNV has also been isolated ~~in~~ from the ovarian fluid and milt of spawning fish (Mulcahy *et al*., 1982).

### 2.2.~~6~~ 5. Aquatic animal reservoirs of infection

Field surveillance programmes and experimental infection trials have documented subclinical IHNV infections in various salmon and trout species (Knusel *et al.,* 2007; Mulcahy *et al.*, 1984; Pascoli *et al.,* 2015; St-Hilaire *et al.,* 2001; Traxler *et al.,* 1997). Survivors of laboratory exposures have demonstrated IHNV persistence for months to over one-year post-exposure (Drolet *et al.,* 1995; Foott *et al.,* 2006; Kim *et al.,* 1999; Muller *et al.,* 2015). With the exception of high viral load occurring in subclinically infected spawning adult salmon, the IHNV levels associated with subclinical infections tend to be lower than in fish undergoing clinical disease.

### 2.2.~~7~~ 6. Vectors

A single study has demonstrated that adult salmon lice, *Lepeophtheirus salmonis* are capable of acquiring and transmitting IHNV to naïve Atlantic salmon through parasitism (Jakob *et al.,* 2011). Regardless of whether salmon lice acquired IHNV through water bath exposure or after parasitising IHNV-infected fish, the duration of virus association with salmon lice diminished rapidly with infectious virus levels falling below cell culture detection limits within hours. IHNV has also been isolated from freshwater invertebrates (e.g. leeches, copepods, and mayflies), however, their capacity to transmit virus is unknown (Dixon *et al.,* 2016; Garver & Wade, 2017).

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

Depending on the species of fish, rearing conditions, temperature, and virus strain, outbreaks of infection with IHNV may range from acute to chronic. An outbreak of infection with IHNV in farmed Atlantic salmon in British ~~Colombia~~ Columbia resulted in cumulative losses on affected farms of between 20 and 94% (Saksida, 2006). In chronic cases, losses are protracted and fish in various stages of disease can be observed in the pond. The prevalence of infection in chronic cases remains unknown. The limited available data indicated that prevalence of infection with IHNV can be high (59%) in endemically infected rainbow trout farms in Europe (reviewed by Dixon *et al*., 2016).

IHNV is endemic among populations of free-ranging salmonids throughout much of its historical range along the west coast of North America. Sockeye salmon have incurred losses of up to ~~99%~~ 36.9% at the fry stage (~~Kurath *et al*., 2003~~ Meyers *et al.*, 2003). ~~As the fish~~ With ages, the prevalence of infection decreases ~~with~~ in marine phase sockeye salmon smolts, and ~~the prevalence of infection~~ in adults is generally low (<15%) to undetectable. However, the prevalence of infection can again reach high levels in mature adult spawning sockeye salmon, with long-term studies revealing greater than 50% prevalence in wild populations (Meyers *et al.,* 2003).

### 2.3.2. Clinical signs, including behavioural changes

Fish with acute infection with IHNV can exhibit lethargy interspersed with bouts of frenzied, abnormal activity. During outbreaks, fish can display spiral swimming, flashing, and have trailing faecal casts. Fish may also show darkening of the skin, exophthalmia, distended abdomen and external haemorrhaging. In instances where fish survive an outbreak, spinal deformities may become evident (Bootland & Leong, 1999).

### 2.3.3 Gross pathology

Gross observations are non-pathognomonic and ~~can involve~~ may include ascites, pale gills, liver, kidney and spleen, petechial haemorrhaging, yellow mucous in the intestine and a lack of food in the stomach (Bootland & Leong, 1999; Traxler, 1986).

### 2.3.4. Modes of transmission and life cycle

The transmission of IHNV between fish is primarily horizontal through direct contact with virus contaminated water or via cohabitation with IHNV infected fish (Bootland & Leong, 1999). ~~However, cases of vertical or egg-associated transmission have been recorded (Mulcahy & Pascho, 1985).~~ There is insufficient evidence to demonstrate true vertical transmission. Outbreaks of IHNV as a result of egg movements likely occurred as a result of inadequate disinfection of eggs originating from moderaterly infected or untested broodstock (Dixon *et al*., 2016). ~~While egg-associated transmission is significantly reduced by the now common practice of surface disinfection of eggs with an iodophor solution, it is the only mechanism accounting for the appearance of infection with IHNV in new geographical locations among fry originating from eggs that were incubated and hatched in virus-free water (Dixon *et al.,* 2016; Winton, 1991).~~

### 2.3.5. Environmental ~~and management~~ factors

The most important environmental factor affecting the disease progression is water temperature. Experimental trials have demonstrated that IHNV can produce mortality in water temperatures from 3°C to 18°C; however, clinical disease typically occurs below 15°C under natural conditions (LaPatra, 1998).

### 2.3.6. Geographical distribution

Cases of infection with IHNV have been reported from Europe, Asia-Pacific, Africa and the Americas. For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

Plasmid DNA vaccines containing the gene for the IHNV glycoprotein have proven highly efficacious against infection with IHNV resulting in the licensing of one for commercial use in Atlantic salmon net-pen aquaculture on the west coast of North America (Alonso & Leong, 2013; Salonius *et al.,* 2007). Administered via intramuscular injection, an IHNV DNA vaccine was rapidly disseminated systemically followed by plasmid persistence in muscle at the injection site (Garver *et al.,* 2005); consequently, caution should be employed when testing fish vaccinated with the IHNV DNA vaccine as diagnostic methods targeting viral G-gene nucleotide sequence or protein have the potential to cross react with the vaccine.

#### 2.4.2. Chemotherapy including blocking agents

Chemotherapeutics, including natural compounds, have been identified to have anti-IHNV properties; however, these have not found commercial use in aquaculture against IHNV (Winton, 1991). Direct application of anti-IHNV compounds to cell cultures has caused growth inhibition and toxicity that could affect the sensitivity of detecting IHNV in affected cultures (Balmer *et al.,* 2017; Hasobe & Saneyoshi, 1985).

#### 2.4.3. Immunostimulation

Immunostimulants are not used commercially in aquaculture for IHNV (Ooi *et al.,* 2008).

#### 2.4.4. Breeding resistant strains

Experimental trials of triploid or inter-species hybrids have been conducted (Barroso *et al.,* 2008; Winton, 1991) with resistance typically determined early in the infection process and associated with lower early viral replication (Purcell *et al*., 2010). However, no resistant strains are commercially available.

#### 2.4.5. Inactivation methods

IHNV is readily inactivated by common disinfectants with active ingredients such as sodium hypochlorite, iodophor, benzalkonium chloride, saponated cresol, formaldehyde and potassium permanganate solution (Yoshimizu *et al.,* 2005). As these substances have virucidal properties any carry-over on sampling equipment or contact with samples may result in reduced viral titres.

#### 2.4.6. Disinfection of eggs and larvae

Iodophor disinfection of eggs is a common practice to effectively mitigate egg-associated transmission of IHNV (Bovo *et al.,* 2005). Chapter 4.4. of the *Aquatic Code* provides recommendations for surface disinfection of salmonid eggs. Iodine has been shown to inhibit PCRs (Auinger *et al.,* 2008) and could affect RT-PCR testing results of disinfected eggs.

#### 2.4.7. General husbandry

In addition to disinfection of eggs (according to Chapter 4.4 of the *Aquatic Code*), use of a virus-free water supply and decreasing rearing densities have significant positive effects in the management of IHNV. Transmission of IHNV increases with host density (Ogut & Reno, 2004).

## 3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

### 3.1. Selection of populations and individual specimens

Clinical inspections ~~are best~~ should be carried out during a period when~~ever~~ the water temperature is below 14°C, or whenever the water temperature is likely to reach its lowest annual point. All production units (ponds, tanks, net-cages, etc.) ~~must~~ should be inspected for the presence of dead, weak or abnormally behaving fish ~~of any susceptible species, and if they are present, such fish should be selected~~. Particular attention should be paid to the water outlet area, where weak fish tend to accumulate due to the water current.

For the purposes of disease surveillance, fish to be sampled are selected as follows: ~~If additional fish are required for the sample, healthy individuals should be selected as follows:~~

i) Species of the *Oncorhynchus* genus are the most susceptible and should be sampled in preference to species from other genera. ~~Rainbow trout and the~~ Other susceptible species listed in Section 2.2.1 should be sampled proportionally~~, or following~~. In addition, risk-based criteria should be employed to preferentially sample ~~for targeted selection of~~ lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status). ~~In farms with salmonids, if rainbow trout are present, only fish of that species should be selected for sampling. If rainbow trout are not present, the sample has to be obtained from fish of all other IHNV-susceptible species.~~

~~ii) Susceptible species should be sampled following risk-based criteria for targeted selection of populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown risk status).~~

ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with IHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no clinical signs or gross pathognomonic lesions in cases of sudden mortality.

### 3.2. Selection of organs or tissues

In populations with clinical disease, the optimal tissue is anterior kidney, spleen and heart or brain (Dixon *et al.,* 2016) However, IHNV can also be found in spleen, heart, liver, gastrointestinal track and brain (Drolet *et al.,* 1994).

In apparently healthy populations, the optimal tissues are anterior kidney and brain as IHNV can persist in tissues of the nervous system during the chronic phase of infection (LaPatra *et al.,* 1995; Muller *et al.,* 2015; Yamamoto *et al.,* 1990b).

When sampling fish of insufficient size to permit dissection of individual tissues, viscera including kidney should be collected or whole fish homogenised after removal of the body behind the anal pore. When sampling broodstock, ovarian fluid and milt can be taken.

~~The optimal tissues material to be examined is are spleen, anterior kidney, and either heart or brain. In the case of spawning fish, ovarian fluid and milt may be taken examined.~~

~~In the case of small fry, whole fish less than 4 cm long can be homogenised (using, for example, sterile scissors or a scapel) after removal of the body behind the anal pore gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. For larger size fish, kidney, spleen, heart, encephalon, and ovarian fluid from brood fish at the time of spawning, should be the tissues to be sampled. When possible, Samples should be taken in duplicate to permit retesting if needed.~~

### 3.3. Samples or tissues not suitable for pathogen detection

IHNV is very sensitive to enzymic degradation, therefore sampling tissues with high enzymatic activities or large numbers of contaminating bacteria, such as the intestine or skin, should be avoided when possible. Given the haematopoietic nature of IHNV, muscle tissue should be avoided as a target tissue. The yolk sac of fry has also shown toxicity to cell lines and should be removed before inoculating cells for virus isolation. Preservatives and fixatives, such as RNAlater and formaldehyde can be toxic to tissue culture cells such as epithelioma papulosum cyprini (EPC) and fathead minnow (FHM), and can impact molecular detection methods (Auinger *et al.,* 2008; Pham *et al.,* 2018).

### 3.4. Non-lethal sampling

Ovarian fluid and milt are suitable samples for detection of IHNV in spawning adult salmon and trout (Dixon *et al.,* 2016; Meyers *et al.,* 2003). There is evidence that IHNV may be isolated from gill, fin and mucous samples but detection may be impacted by the state of infection, time since exposure and sample size (Burbank *et al.,* 2017; LaPatra *et al.,* 1989).

## 3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

### 3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (diseases of fish).

~~The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should only be used after consultation with the receiving laboratory.~~

~~Before shipment or transfer to the laboratory, pieces of the organs to be examined should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficacy may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required.~~

~~Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C, and ice must still be present in the transport box at receipt or at least one or more freeze blocks must still be partly or completely frozen.~~

~~Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with present national and international transport regulations, as appropriate.~~

~~The virological examination on cell culture should be started as soon as possible, and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium, and that the temperature requirements during transportation can be fulfilled.~~

### 3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1, using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, samples may be placed in at least five volumes of RNA stabilisation reagents, according to the recommendation from the manufacturers. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

Whole fish may also be sent to the laboratory (see Section 3.5.1).

### 3.5.3. ~~Fixed~~ Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be immediately fixed at a fixative to tissue ratio of 10:1. A suitable fixative is 10% buffered formalin. To avoid excessive cross-linking, tissue should be transferred to ethanol after 24 hours if methods other than histopathology are used e.g. *in-situ* hybridisation.

~~**3.5.4. Fixed Samples for electron microscopy**~~

~~Not relevant. Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.~~

### 3.5.~~5~~ 4. Samples for other tests

Not relevant.

## 3.6. Pooling of samples

~~No data are currently available concerning the effect of pooling samples on the detection of IHNV. However, small life stages such as fry can be pooled to provide the minimum amount of material needed for testing.~~ Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

# 4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

| | |
|---|---|
| +++ = | Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; |
| ++ = | Suitable method(s) but may need further validation; |
| + = | May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application; |
| Shaded boxes = | Not appropriate for this purpose. |

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

*Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology[3]** | | | | | | ++ | ++ | 1 | | | | |
| **Cytopathology[3]** | | | | | | | | | | | | |
| **Cell ~~or artificial media~~ culture** | +++ | +++ | +++ | 3 | +++ | +++ | +++ | **3** | +++ | +++ | +++ | **3** |
| **Real-time PCR** | +++ | +++ | +++ | **3** | +++ | +++ | +++ | **3** | +++ | +++ | +++ | **3** |
| **Conventional PCR** | | | | | | ++ | ++ | ~~++~~ 2 | ++ | ++ | ++ | ~~1~~ 2 |
| **Amplicon sequencing[4]** | | | | | | | | | +++ | +++ | +++ | 3 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **IFAT** | | | | | | | | | ++ | ++ | ++ | 2 |
| **Ag-ELISA** | | | | | | | | | ++ | ++ | ++ | 2 |
| **Neutralisation test (antibody or antiserum)[5]** | | | | | | | | | ++ | ++ | ++ | 2 |

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification. IFAT = indirect fluorescent antibody test; Ag-ELISA = antigen enzyme-linked immunosorbent assay. [1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3.
[3]Cytopathology and histopathology can be validated if the results from different operators has been statistically compared.
[4]Sequencing of the PCR product.
[5]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Not relevant

## 4.2. Histopathology and cytopathology

Histopathological findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. Necrosis of eosinophilic granular cells in the intestinal wall is pathognomonic of IHNV infection (Bootland & Leong, 1999).

The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leucocytes and thrombocytes, and large amounts of cellular debris. As with other haemorrhagic viraemias of fish, blood chemistry is altered in severe cases (Bootland & Leong, 1999).

Electron microscopy of virus-infected cells reveals bullet-shaped virions of approximately 150–190 nm in length and 65–75 nm in width (Wolf, 1988). ~~The~~ Virions are visible at the cell surface or within vacuoles or intracellular spaces after budding through cellular membranes. The virion possesses an outer envelope containing host lipids and the viral glycoprotein spikes that react with immunogold staining to decorate the virion surface

Smears are not appropriate for detection or identification of IHNV.

## 4.3. Cell ~~or artificial media~~ culture for isolation

### 4.3.1. Cell lines

The recommended cell lines for IHNV detection are EPC or FHM. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

EPC or FHM cells are grown at 20–30°C in suitable medium, e.g. Eagle's minimal essential medium (MEM; or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2. Cell culture plates should be seeded 4–48 hours and not 100% confluent prior to inoculation. 15–30 minutes prior to sample inoculation, cells should be pre-treated with 7% (w/v) PEG-20,000 solution (10–15 µl/cm$^2$) (Batts & Winton, 1989; Wang *et al.*, 2016).

### 4.3.2. Sample preparation and inoculation

Note: Tissue and fluid samples should be kept cool throughout sample preparation procedures.

i) Homogenise tissue samples using mortar and pestle or a tissue homogeniser~~, stomacher, polytron or equivalent~~. A small volume of media (MEM-4 or Hank's balanced salt solution with antibiotics) may be needed to achieve complete homogenisation.

ii) Adjust the volume of media to a final ratio of 10:1 (media:tissue) and mix thoroughly. For fluid samples adjust the volume of media to a final ratio of 1:1.

iii) Centrifuge the homogenate or fluid samples at 2000–4000 ***g*** for 15 minutes at 2–5°C.

iv) Remove the supernatant and pass through a 0.45 µM membrane filter (if available).

v) If the sample cannot be inoculated within 48 hours after collection, the supernatant may be stored at –80°C provided virological examination is carried out within 14 days.

vi) If samples originate from an area where infectious pancreatic necrosis virus (IPNV) is present, supernatants may be treated with IPNV antiserum. Mix the supernatant with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubate for a minimum of 1 hour at 15°C or up to 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

vii) Samples are inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively. The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 $cm^2$ cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are acceptable as well.

viii) Inoculated cell cultures are incubated at 15°C for 7–10 days. Using a microscope with 40–150× magnification, cultures should be inspected for toxicity the day after inoculation, particularly if supernatant was not filtered in step iv. The use of a phase-contrast microscope is recommended.

ix) ~~Monitor t~~The cells are monitored regularly (2–3 times a week) for the presence of cytopathic effect (CPE).

~~*Interpretation of results*~~

If CPE is observed, virus identification ~~confirmatory testing~~ is required ~~to identify IHNV~~ using tests recommended in Section 6. If no CPE is observed ~~in~~ after the primary incubation period, ~~culture or~~ subcultivation~~, the sample is negative~~ is performed.

*Subcultivation*

i) Remove cell culture supernatant from the primary culture and inoculate a newly (<48 hours) seeded cell culture plate.

ii) Incubate inoculated plates at 15°C and monitor for 7–10 days as described above.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period and subcultivation, the sample is negative

## 4.4. Nucleic acid amplification

### 4.4.1. Real-time PCR

There are several ~~reverse-transcription~~ real-time reverse-transcription (RT) PCR assays available for the detection of IHNV. Two assays are described, a two-step real-time PCR and a one-step real-time PCR.

The first assay described is a stage 3 validated two-step real-time TaqMan PCR assay that amplifies a region of the nucleoprotein gene of all known IHNV genogroups with some E-genogroup isolates (D332-92, FV23, and FV91-40) having reduced amplification efficiency due to single nucleotide polymorphism within the probe sequence (Hoferer *et al.,* 2019; Purcell *et al.,* 2013).

Positive and negative controls should be run with each stage of the assay: extraction, reverse transcription and real-time PCR. Due to the sensitive nature of PCR-based assays, it is important to be able to distinguish a true positive from the positive control material. This may be achieved using an artificial positive control as employed by Purcell *et al.* (2013). It is also highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

*RNA extraction and reverse-transcription* (*RT*)

i) Total RNA from infected cells and/or tissues is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially available RNA isolation kit used according to the manufacturer's instructions.

ii) Extracted RNA is reverse transcribed non-discriminately into cDNA using random primers. The cDNA synthesis reactions and cycling conditions are best performed using the manufacturer's instructions for commercially available kits which have been extensively tested with a variety of RNA templates, including GC- and AU-rich targets and RNAse expressed at low levels.

*Real-time PCR*

The TaqMan real-time PCR assay uses forward primer IHNv N 796F (5'-AGA-GCC-AAG-GCA-CTG-TGC-G-3'), reverse primer IHNv N 875R (5'-TTC-TTT-GCG-GCT-TG-GTT-GA-3') and FAM-labelled probe, IHNv N 818T (5'-6FAM-TGA-GAC-TGA-GCG-GGA-CA-MGBNFQ-3'). Primers are used at a final concentration of 900 nM each and the final probe concentration is 250 nM. 2.5 µl cDNA product is added to each 25 µl rPCR reaction. Thermal cycling conditions are 50°C 2 minutes, 95°C 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The sample is negative if no Ct (threshold cycle) is recorded, while samples with a Ct are considered positive for IHNV.

*One step real-time RT-PCR*

The one step real-time RT-PCR is performed using the SuperScript III Platinum One - Step qRT - PCR Kit (Thermo Fisher Scientific, Schwerte, Germany) or the AgPath - ID One - Step RT - PCR Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For all quantitative assays, the following unique parameters were used: (a) total volume of 25 µl consisting of 20 µl mastermix and 5 µl of RNA; (b) 900 nM of each primer; (c) 200 nM of IHNV probe and 250 nM of VHSV probe, respectively; (d) hard - shell 96 - well skirted plates with white shell (Bio - Rad, Munich, Germany, cat. No HSP9601); (e) Microseal B adhesive optical clear seals (Bio - Rad, cat. no MSB 1001); (f) run on a C1000TM Thermal Cycler controlled by the CFX96TM Real - Time PCR Detection System (Bio - Rad); and (g) use of the CFX Manager software (Bio - Rad) for data analysis. The threshold was set automatically (Hoferer *et al.*, 2019).

The one-step real-time RT-PCR test does not differ significantly in performance to the two-step test (Cuenca *et al.*, 2020).

### 4.4.2. Conventional RT-PCR

Several conventional RT-PCR assays are available with limited validation data.

The RT-PCR assay described recognises a broad range of genotypes by targeting a central region of the IHNV G gene (Emmenegger *et al.,* 2000), and produces a PCR amplicon that is used for identification of genetic strains and for epidemiological tracing of virus movements (Kurath *et al.,* 2003).

Positive and negative controls should be run with each stage of the assay: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

*RNA extraction*

Total RNA may be prepared as described in Section 4.4.1.

*Conventional RT-PCR* (*Round 1*)

The first round RT-PCR combines cDNA synthesis and PCR amplification into one step by using an IHNV-specific primer set that generates the first-strand synthesis of IHNV RNA and subsequent PCR amplification through 30 cycles. The first round PCR produces a 693 bp PCR amplicon using forward primer (5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3') and reverse primer (5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3') at a final concentration of 200 nM each. The thermal cycling conditions are one cycle of 50°C for 30 minutes; one cycle of 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; one cycle of 72°C for 7 minutes and 4°C hold.

A sample is IHNV positive if a 693 bp PCR amplicon is observed and no bands were observed in the negative controls. If no band is observed for a sample and the positive controls passed proceed to the second round nested PCR.

*Second round* (*nested PCR*)

Due to the sensitivity of the test along with the need for repetitive handling of tubes, nested PCR is prone to contamination and good sterile technique must be practiced.

The first round positive and negative controls are carried over and included with the nested PCR assay. In addition, a separate negative and positive control specific to the nested assay are required.

The second round PCR produces a 483 bp PCR amplicon using forward primer (5'-TCA-CCC-TGC-CAG-ACT-CAT-TGG-3') and reverse primer (5'-ATA-GAT-GGA-GCC-TTT-GTG-CAT-3') at a final concentration of 200 nM each. The thermal cycling conditions are: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; one cycle of 72°C for 7 minutes and 4°C hold.

A sample is IHNV positive if a 483 bp PCR amplicon is observed and no band(s) are observed in the negative controls. A sample is negative if no bands are observed and positive controls passed.

#### 4.4.3. Other nucleic acid amplification methods

To date, no other nucleic acid amplification method capable of universal IHNV detection has been sufficiently validated.

### 4.5. Amplicon sequencing

Nucleotide sequencing of the conventional PCR product (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. This central region of IHNV glycoprotein gene is used for identification of genetic strains and for epidemiological study (Kurath *et al.,* 2003). It is recommended to forward any sequence data obtained to the OIE Reference Laboratory, particularly in the event where isolate sequences differ from any of the target sequences of the recommended molecular assays.

### 4.6. *In-situ* hybridisation

Not relevant.

### 4.7. Immunohistochemistry

Not relevant.

### 4.8. Bioassay

Not relevant.

### 4.9. Antibody- or antigen-based detection methods

Antibody- and antigen-based detection methods may be used to confirm the presence of IHNV in cell culture. Kits and antibodies are commercially available and should be used according to manufacturer's instructions. Sensitivity, specificity and sample preparation can influence the results; a negative result should be viewed with caution. These techniques should not be used as a screening method.

#### 4.9.1. Neutralisation test (identification in cell culture)

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge an aliquot at 2000 g for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.

ii) Dilute virus-containing medium from $10^2$–$10^4$.

iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of an IHNV antibody solution. The neutralising antibody solution must have a 50% plaque reduction titre of at least 2000. Likewise, treat a set of aliquots of each virus dilution with cell culture medium to provide a non-neutralised control.

iv) In parallel, a neutralisation test must be performed against a homologous IHNV strain (positive neutralisation test) to confirm the reactivity of the antiserum.

v) Incubate all the mixtures at 15°C for 1 hour.

vi) Transfer aliquots of each of the above mixtures on to 24-hour-old monolayers overlaid with cell culture medium containing 10% FBS (inoculate two wells per dilution) and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

vii) Check the cell cultures for the onset of CPE and read the results for each suspect IHNV sample and compare to the occurrence of CPE of non-neutralised controls. Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

viii) The tested virus is identified as IHNV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IHNV-specific antibody, whereas CPE is evident in all other cell cultures.

Other neutralisation tests of demonstrated performance may be used instead.

#### 4.9.2. Indirect fluorescent antibody test (IFAT) (identification in cell culture)

i) Prepare monolayers of cells in 2 $cm^2$ wells of cell culture plastic plates or on cover slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at ~~22°C~~ the optimal temperature of the cell line in question (e.g. 26°C for EPC and 20°C for RTG) (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FBS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of black 96-well plates for immunofluorescence is recommended.

ii) When the cell monolayers are ready for infection (i.e. on the same day or on the day after seeding) inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of IHNV in a similar way, in order to obtain a virus titre of about 5,000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with a cold mixture of acetone 30%/ethanol 70% (v/v) (stored at –20°C).

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 $cm^2$ of cell monolayer.

vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

viii) Prepare a solution of purified IHNV antibody or serum in 0.01 M PBS, pH 7.2, containing 0.05% Tween-80 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution and remove this buffer completely after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur (e.g. by adding a piece of wet cotton to the humid chamber). The volume of solution to be used is 0.25 ml/2 $cm^2$ well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate- or tetramethylrhodamine-5-(and-6-) isothiocyanate-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover slips using, for example, glycerol saline, pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with × 10 eye pieces and × 20–40 objective lens having numerical aperture >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of demonstrated performance may be used instead.

#### 4.9.3. Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IHNV, in 0.01 M PBS, pH 7.2 (200 µl/well).

ii) Incubate overnight at 4°C.

iii) Rinse four times with 0.01 M PBS containing 0.05% Tween-20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Rinse four times with PBST.

vi) Add 2% Triton X-100 to the virus suspension to be identified.

vii) Dispense 100 µl/well of two- or four-step dilutions of the virus to be identified and of IHNV control virus, and a heterologous virus control (e.g. viral haemorrhagic septicaemia virus). Allow the samples to react with the coated antibody to IHNV for 1 hour at 20°C.

viii) Rinse four times with PBST.

ix) Add to the wells either biotinylated polyclonal IHNV antiserum or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.

xiii) Rinse four times with PBST. Add the substrate and chromogen. Stop the course of the test when positive controls react and read the results.

xiv) Interpretation of the results is according to the optical absorbencies achieved by negative and positive controls and must follow the guidelines for each test, e.g. absorbency at 450 nm of positive control must be minimum 5–10 × A450 of negative control.

The above biotin–avidin-based ELISA version is given as an example. Other ELISA versions of demonstrated performance may be used instead.

### 4.10. Other ~~serological~~ methods

Not applicable

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Virus isolation in cell culture or real-time RT-PCR are the recommended tests for surveillance to demonstrate freedom from infection with IHNV.

## 6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or in the presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

## 6.1. Apparently healthy animals or animals of unknown health status[15]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with IHNV shall be suspected if at least one of the following criteria is met:

i) Positive result by real-time RT-PCR;

ii) IHNV-typical CPE ~~Cytopathic effect~~ in cell culture.

### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with IHNV is considered to ~~shall~~ be confirmed if~~, in addition to the criteria in Section 6.1.1, positive results has been obtained on at least one animal from two test used in the following combination~~ one or more of the following criteria is met:

i) Positive result by real-time ~~RT-~~ RT-PCR ~~followed by~~ and detection of IHNV in a tissue sample by ~~a positive result from a~~ conventional RT-PCR targeting a non-overlapping region of the genome and amplicon sequencing;

ii) CPE ~~Isolation of virus~~ in cell culture ~~confirmed by~~ identified as IHNV by real-time RT-PCR, conventional RT-PCR, IFAT, ~~or~~ Ag-ELISA, or by a neutralisation test ~~and a positive result~~ ~~followed by~~ and detection of IHNV in a tissue sample by real-time RT-PCR;

iii) CPE ~~Isolation of virus~~ in cell culture ~~confirmed by~~ identified as IHNV by real-time RT-PCR, conventional RT-PCR, IFAT, ~~or~~ Ag-ELISA, or by a neutralisation test ~~and followed by~~ and detection of IHNV in a tissue sample by conventional RT-PCR and amplicon sequencing.

~~iv) Positive result by real-time RT-PCR followed by isolation of virus in cell culture confirmed by identified as IHNV by real-time RT-PCR, conventional PCR, IFAT, Ag-ELISA, or by a neutralisation test and amplicon sequencing.~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with IHNV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality;

[15] For example transboundary *commodities*.

~~ii) Positive result by real-time RT-PCR;~~

ii) Histopathological changes characteristic of infection with IHNV;

iii) IHNV-typical CPE ~~Cytopathic effect~~ in cell culture;

iv) Positive result by real-time RT-PCR;

v) Positive result by conventional RT-PCR.

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with IHNV is considered to ~~shall~~ be confirmed if~~, in addition to the criteria in Section 6.2.1, positive results has been obtained on at least one animal from two tests used in the following combination~~ one or more of the following criteria is met:

i) Positive result by real-time ~~RT-~~RT-PCR ~~followed by~~ and detection of IHNV in a tissue sample ~~a positive result from a~~ conventional RT-PCR targeting a non-overlapping region of the genome and amplicon sequencing;

ii) CPE ~~Isolation of virus~~ in cell culture ~~confirmed by~~ identified as IHNV by real-time RT-PCR, conventional RT-PCR, IFAT, ~~or~~ Ag-ELISA, or by a neutralisation test ~~and a positive result followed by~~and detection of IHNV in a tissue sample by real-time RT-PCR;

iii) CPE ~~Isolation of virus~~ in cell culture ~~confirmed by~~ identified as IHNV by real-time RT-PCR, conventional RT-PCR, IFAT, ~~or~~ Ag-ELISA, or by a neutralisation test ~~and followed by~~ and detection of IHNV in a tissue sample by conventional RT-PCR and amplicon sequencing.

~~iv) Positive result by real-time RT-PCR followed by isolation of virus in cell culture confirmed by identified as IHNV by real-time RT-PCR, conventional PCR, IFAT, Ag-ELISA, or by a neutralisation test and amplicon sequencing~~.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHNV is provided in Table 6.3. This information can be used for the design of surveys for infection with IHNV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3.*** *Diagnostic performance of tests recommended for surveillance or diagnosis*

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Real-time RT-PCR | Diagnosis | Experimentally infected salmon | Kidney | ~~Steelhead~~ Rainbow trout (*Oncorhynchus mykiss*) | 100 (50) | 100 (50) | Animals of known infection status | Purcell *et al.*, 2013 |
| RT-PCR (single step) | Diagnosis | Experimentally infected salmon | Kidney | ~~Steelhead~~ Rainbow trout (*Oncorhynchus mykiss*) | 58 (50) | 100 (50) | Animals of known infection status | Purcell *et al.*, 2013 |
| Virus Isolation | Diagnosis | Experimentally infected salmon | Kidney | ~~Steelhead~~ Rainbow trout (*Oncorhynchus mykiss*) | 84 (50) | 100 (50) | Animals of known infection status | Purcell *et al.*, 2013 |
| | | Field samples | Kidney and spleen | Atlantic salmon (*Salmo salar*) | 80-86 (50) | 100 (50) | Clinical signs – history | McClure *et al.*, 2008 |

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study;
~~RT-LAMP: = real-time loop mediated isothermal amplification. *Listed as suitable test~~

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* *

NB: There are OIE Reference Laboratories for Infection with infectious haematopoietic necrosis virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/ ).
Please contact the OIE Reference Laboratories for any further information on infection with viral haemorrhagic septicaemia virus

Back to Agenda

CHAPTER 2.3.10.

# INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

**EU position**

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

## 1. Scope

Infection with viral haemorrhagic septicaemia virus (VHSV) means infection with the pathogenic agent viral haemorrhagic septicaemia virus of the Genus *Novirhabdovirus* and Family *Rhabdoviridae.*

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

VHSV is a bullet-shaped particle, approximately 70 nm in diameter and 180 nm in length, that contains a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides, and possesses an envelope that contains the membrane glycoprotein, which is the neutralising surface antigen. The genome encodes six proteins: a nucleoprotein N; a phosphoprotein P (formerly designated M1); a matrix protein M (formerly designated M2); a glycoprotein G; a non-virion protein NV and a polymerase L (Walker *et al*., 2000).

G-gene nucleotide sequences have been used to classify VHSV isolates into four major genotypes (I, II, III and IV) and nine subtypes (Ia–Ie and IVa-IVd) with almost distinct geographical distributions (Einer-Jensen *et al*., 2004; Elsayed *et al*., 2006). The host range and the pathogenicity appear, at least to some extent, to be linked to the genotype of VHSV.

i) Genotype Ia

Almost all VHSV isolates causing outbreaks in European rainbow trout (*Oncorhynchus mykiss*) farms cluster in sub-lineage Ia, of which isolates have been reported from most continental European countries (Einer-Jensen *et al*., 2004; Kahns *et al*., 2012; Snow *et al*., 2004; Toplak *et al*., 2010). However, genotype Ia isolates have also been detected in other finfish species in Europe such as brown trout (*Salmo trutta*), pike (*Esox lucius*) and grayling (*Thymallus thymallus*) (de Kinkelin & Le Berre, 1977; Jonstrup *et al*., 2009). Genotype Ia isolates have generally caused outbreaks in freshwater-farmed rainbow trout ~~European freshwater farms~~, but isolates have also been obtained from sea-reared rainbow trout ~~in seawater net pens~~ and turbot (*Scophthalmus maximus* ~~syn. *Psetta maxima*~~) (Schlotfeldt *et al*., 1991; Snow *et al*., 2004). Genotype Ia can be further subdivided into two major subpopulations, Ia-1 and Ia-2, with a distinct geographic distribution within Europe (Kahns *et al*., 2012).

ii) Genotype Ib

The isolates included in this genotype ~~Viruses~~ have been ~~isolated~~ obtained from finfish in the marine environment in the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel (Einer-Jensen *et al*., 2004; Skall *et al*., 2005b; Snow *et al*., 2004) and as far north as latitude 70°N close to Nordkapp in Norway (Sandlund *et al.,* 2014). A single case was observed in Japan (Nishizawa *et al*., 2002). None of the isolations from wild fish has been associated with clinical disease outbreaks (Johansen *et al.,* 2013). Genotype Ib has been associated with evidence of transfer between wild fish and farmed rainbow trout in only two cases in pen-reared rainbow trout in Sweden in 1998 and 2000 (Nordblom, 1998; Nordblom & Norell, 2000; Skall *et al*., 2005a).

iii) Genotype Ic

This genotype consists of ~~is~~ a smaller group ~~consisting~~ of Danish isolates from freshwater farmed rainbow trout ~~isolates from earlier dates~~. Isolates of this genotype have also been ~~identified~~ detected in Germany and Austria (Jonstrup *et al*., 2009).

iv) Genotype Id

~~This group~~ The isolates included in this genotype consist~~s~~ of some old Scandinavian isolates from the 1960s and from ~~until the first~~ VHS outbreaks of infection with VHSV ~~occurred~~ in Finland in sea-reared rainbow trout in 2000. These outbreaks occurred in ~~at~~ two different areas ~~where~~ and all of the isolates sampled were ~~proved to~~ clustered in the Id genotype group. In infection trials, it was demonstrated that the isolates were pathogenic to rainbow trout, but less virulent than most Ia isolates (Raja-Halli *et al*., 2006).

v) Genotype Ie

The~~se~~ isolates included in this genotype have been obtained from both freshwater and marine (the Black Sea) environments in Georgia and Turkey. Isolations were from both farmed and wild turbot (Jonstrup *et al*., 2009; Kalayci *et al*., 2006; Nishizawa *et al*., 2006) and from rainbow trout (Einer-Jensen *et al*., 2004). VHSV Ie has also been ~~identified~~ isolated from ~~in~~ whiting (*Merlangius merlangus*) and sea bass (*Dicentrarchus labrax*) ~~from~~ in the Black Sea (Altuntas & Ogut, 2010).

vi) Genotype II

The ~~members~~ isolates included in ~~of~~ this ~~group~~ genotype ~~consist of~~ have been primarily detected in marine ~~isolates from~~ wild finfish, in particular ~~especially from~~ Atlantic herring (*Clupea harengus*), from ~~in~~ the Baltic Sea, including the Gulf of Bothnia and the Gulf of Finland, (Gadd *et al*., 2011; Snow *et al*., 2004). Genotype II isolates have also been detected in lamprey (*Lampetra fluviatilis*) caught in freshwater from the rivers Kalajoki and Lestijoki, which have~~ing~~ an outlet into the Gulf of Bothnia (Gadd *et al*., 2010).

vii) Genotype III

The~~se~~ isolates included in this genotype originate from wild and farmed finfish in the North Atlantic Sea from the Flemish Cap (Lopez-Vazquez *et al*., 2006b) to the Norwegian coast (Dale *et al*., 2009), the North Sea around the British Isles, Skagerrak and Kattegat. ~~VHS outbreaks~~ Outbreaks of infection with VHSV in sea-farmed turbot in the United Kingdom and Ireland in the 1990s were attributed ~~due~~ to infection with genotype III isolates, and in 2007 an outbreak in sea-reared rainbow trout at the Norwegian west coast was due to VHSV genotype III. ~~VHS~~ Outbreaks of infection with VHSV in five species of wrasse used as cleaner fish around the Shetland Islands were also due to this genotype (Munro *et al*., 2015).

viii) Genotype IVa

The isolates included in this genotype have been detected ~~originate~~ in finfish from the coastal environments of North America spanning from California to Alaska in the west and around the northeastern United States up through Newfoundland, Canada. This genotype has also been reported ~~both the east and west coasts of North America, as well as~~ from the Asian countries of South Korea and Japan. Genotype IVa isolates in North America have caused severe epidemics in numerous wild marine species such as Pacific herring (*Clupea pallasii pallasii*) (Meyers & Winton, 1995), which can serve as a reservoir of virus to sympatric sea-farmed ~~net-pen farmed~~ Atlantic salmon (*Salmo salar*) (Garver *et al*., 2013). In Asia, genotype IVa isolates have caused disease outbreaks in ~~olive flounder~~ bastard halibut (*Paralichthys olivaceus*) (Ogut & Altuntas, 2014).

ix) Genotype IVb

The isolates included in this genotype have been detected ~~originate~~ in finfish in fresh water ~~originate~~ from the North America Laurentian Great Lakes region (Gagne *et al*., 2007; Thompson *et al*., 2011; Winton *et al*., 2008) and ~~where they~~ have caused die-offs ~~events~~ in numerous fish species~~. and have been detected in a micro-invertebrate (*Diporeia* spp.)~~ (Faisal & Winters, 2011).

x) Genotype IVc

The isolates included in this genotype ~~originate~~ have been detected from finfish from the estuarine waters of New Brunswick and Nova Scotia, Canada (Gagne *et al*., 2007; Pierce & Stepien, 2012; Stepien *et al*., 2015).

xi) Genotype IVd

The isolates included in this genotype ~~originate~~ have been detected in ~~from~~ Iceland where they were identified in wild and sea-farmed lumpfish (*Cyclopterus lumpus*) (Gudmundsdottir *et al*., 2019).

### 2.1.2. Survival and stability in processed or stored samples

VHSV survival in host tissue is dependent on the conditions for storage. VHSV can remain~~s~~ infectious for long time periods while stored frozen in fish tissue. However, VHSV-infected fish subjected to the ~~at~~ commercial freezing process ~~temperatures~~ (core block temperature of –24°C) had a 90% reduction in viral titre after the tissue was thawed (Arkush *et al*., 2006). VHSV is sensitive to enzymatic degradation, environments with high bacterial load and high temperatures (above 28°C). Fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout could transmit ~~infection with~~ VHSV to naïve fish (Oidtmann *et al.,* 2011a). VHSV is ~~also~~ tolerant of high salt concentrations such as in brine-treated fish (Skall *et al.,* 2015) or while stored in concentrated ammonium sulphate solution (Pham *et al*., 2018). For optimal retention of VHSV in fish tissue, the sample should be placed in transport medium with antibiotics and kept on ice without freezing and processed within 24 hours after sampling.

### 2.1.3. Survival and stability outside the host

VHSV survival outside the host is dependent on the physico-chemical conditions of the aqueous medium (Ahne, 1982) and on temperature: the virus survives for longer periods at 4°C compared with 20°C (Parry & Dixon, 1997).

VHSV is significantly more stable in freshwater than seawater ~~saltwater~~. The virus has been documented to persist in freshwater for 28–35 days at 4°C (Parry & Dixon, 1997) and has been found to be infective for 1 year at 4°C in filtered freshwater (Hawley & Garver, 2008). In raw freshwater at 15°C, the 99.9% inactivation time was 13 days, but in seawater the virus was inactivated within 4 days (Hawley & Garver, 2008). In another study using seawater at 15°C, the infectivity of the virus was reduced by 50% after 10 hours, but could still be recovered after 40 hours (Kocan *et al.,* 2001). There appears to be no consistent correlation between the origin and stability of the virus isolates: freshwater isolates are not always the most stable in freshwater and seawater isolates are not consistently more stable in seawater (Hawley & Garver, 2008).

The virus remains stable for a longer time if sterile organic materials are added to the water, such as ovarian fluids or blood products, such as bovine serum (Kocan *et al.,* 2001). When the seawater was sterilised by autoclaving, or when passed through a 0.22 µm membrane, virus survival was prolonged significantly (60 days at 15°C and 32 days at 20°C), suggesting the bacterial load in the water is an important factor of viral decay.

## 2.2. Host factors

### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with VHSV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) ~~include~~ are:

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| Ammodytidae | *Ammodytes hexapterus* | Pacific sand lance | IVa |
| Aralichthyidae | *Paralichthys olivaceus* | Bastard halibut | IVa |
| Carangidae | *Trachurus mediterraneus* | Mediterranean horse mackerel | Ie |
| Centrarchidae | *Ambloplites rupestris* | Rock bass | IVb |
| | *Lepomis gibbosus* | Pumpkinseed | IVb |
| | *Lepomis macrochirus* | Bluegill | IV, IVb |
| | *Micropterus dolomieu* | Smallmouth bass | IVb |
| | *Micropterus salmoides* | Largemouth bass | IVb |
| | *Pomoxis nigromaculatus* | Black crappie | IVb |

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| Clupeidae | *Alosa immaculata* | Pontic shad | Ie |
| | *Sardina pilchardus* | Pilchard | ND |
| | *Clupea harengus* | Atlantic herring | Ib, III |
| | *Clupea pallasii pallasii* | Pacific herring | IVa |
| | *Dorosoma cepedianum* | American gizzard shad | IVb |
| | *Sardinops sagax* | South American pilchard | IVa |
| | *Sprattus sprattus* | European sprat | Ib |
| Cyclopteridae | *Cyclopterus lumpus* | Lumpfish | IVd |
| Cyprinidae | *Danio rerio* | Zebra fish | IVa |
| | *Notropis hudsonius* | Spottail shiner | IVb |
| | *Notropis atherinoides* | Emerald shiner | IVb |
| | *Pimephales notatus* | Bluntnose minnow | IVb |
| | *Pimephales promelas* | Fathead minnow | IVb |
| Embiotocidae | *Cymatogaster aggregata* | Shiner perch | IVa |
| Engraulidae | *Engraulis encrasicolus* | European anchovy | Ie |
| Esocidae | *Esox lucius* | Northern pike | Ia, IVb |
| | *Esox masquinongy* | Muskellunge | IVb |
| Fundulidae | *Fundulus heteroclitus* | Mummichog | IVc |
| Gadidae | *Gadus macrocephalus* | Pacific cod | IVa |
| | *Gadus morhua* | Atlantic cod | Ib, III |
| | *Merlangius merlangus* | Whiting | Ie |
| | *Micromesistius poutassou* | Blue whiting | Ib, III |
| | *Trisopterus esmarkii* | Norway pout | Ib, III |
| Gasterosteidae | *Gasterosteus aculeatus* | Three-spine stickleback | IVc |
| Gobiidae | *Neogobius melanostomus* | Round goby | IVb |
| | *Pomatoschistus minutus* | Sand goby | Ib |
| Ictaluridae | ~~*Ictalurus*~~ *Ameiurus nebulosus* | Brown bullhead | IVb |
| Labridae | *Centrolabrus exoletus* | Rock cook wrasse | III |
| | *Ctenolabrus rupestris* | Goldsinny wrasse | III |
| | *Labrus bergylta* | Ballan wrasse | III |
| | *Labrus mixtus* | Cuckoo wrasse | III |
| | *Symphodus melops* | Corkwing wrasse | III |
| Lotidae | *Gaidropsarus vulgaris* | Three-bearded rockling | Ie |
| Moronidae | *Morone americana* | White perch | IVb |
| | *Morone chrysops* | White bass | IVb |
| | *Morone saxatilis* | Striped bass | IVb, IVc |
| Mullidae | *Mullus barbatus* | Red mullet | Ie |
| Osmeridae | *Thaleichthys pacificus* | Eulachon | IVa |
| Percidae | *Sander vitreus* | Walleye | IVb |
| | *Perca flavescens* | Yellow perch | IVb |
| Petromyzontidae | *Lampetra fluviatilis* | River lamprey | II |
| Pleuronectidae | *Limanda limanda* | Common dab | Ib |
| | *Platichthys flesus* | European flounder | Ib |
| | *Pleuronectes platessus* | European plaice | III |
| Rajidae | *Raja clavata* | Thornback ray | Ie |
| Salmonidae | *Coregonus artedii* | Lake cisco | IVb |
| | *Coregonus clupeaformis* | Lake whitefish | IVb |
| | *Coregonus lavaretus* | Common whitefish | Ia |
| | *Oncorhynchus kisutch* | Coho salmon | IVa |
| | *Oncorhynchus mykiss* | Rainbow trout | Ia-e, III, IVb |
| | *Oncorhynchus mykiss* X *Oncorhynchus kisutch* hybrids | Rainbow trout X coho salmon hybrids | Ia |
| | *Oncorhynchus tshawytscha* | Chinook salmon | IVa, IVb |
| | *Salmo marmoratus* | Marble trout | Ia |
| | *Salmo salar* | Atlantic salmon | Ia, Ib, II, III, IVa |
| | *Salmo trutta* | Brown trout | Ia, Ib |
| | *Salvelinus namaycush* | Lake trout | Ia, IVa, IVb |

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| | *Thymallus thymallus* | Grayling | Ia |
| Scophthalmidae | *Scophthalmus maxima* | Turbot | Ib, III |
| Sciaenidae | *Aplodinotus grunniens* | Freshwater drum | IVb |
| Scombridae | *Scomber japonicus* | Pacific chub mackerel | IVa |
| Soleidae | *Solea senegalensis* | Senegalese sole | III |
| Uranoscopidae | *Uranoscopus scaber* | Atlantic stargazer | Ie |

**ND:** Not determined.

### 2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with VHSV according to Chapter 1.5 of the *Aquatic Code* ~~include~~ are:

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| Adrianichthydiae | *Oryzias latipes* | Japanese rice fish | IVb |
| | *Oryzias dancena* | Marine medaka | IVa |
| Ammodytidae | *Ammodytes personatus* | Sandeel | Ib |
| Anguillidae | *Anguilla anguilla* | European eel | III |
| Argentinidae | *Argentina sphyraena* | Lesser Argentine | Ib |
| Belonidae | *Belone belone* | Garfish | Ie |
| Carangidae | *Seriola dumerili* | Greater amberjack | IVa |
| Catostomidae | *Catostomus commersonii* | White sucker | IVb |
| | *Moxostoma anisurum* | Silver redhorse | IVb |
| | *Moxostoma macrolepidotum* | Shorthead redhorse | IVb |
| Centrarchidae | *Pomoxi annuluris* | White crappie | IVb |
| Clupeidae | *Alosa pseudoharengus* | Alewife | IVb |
| | ~~*Clupea harengus**~~ | ~~Atlantic herring*~~ | ~~IVa~~ |
| Cottidae | *Cottus pollux* | Japanese fluvial sculpin | IVb |
| Cyprinidae | *Semotilus corporalis* | Fallfish | IVb |
| | *Notemigonus crysoleucas* | Golden shiner | IVb |
| Esocidae | *Esox lucius* X *E. masquinongy* hybrids | Tiger muskellunge (*Esox masquinongy* X *E. lucius* or *E. lucius* X *E. masquinongy*) | IVb |
| Fundulidae | *Fundulus diaphanus* | Banded killifish | IVb |
| Gadidae | *Gadiculus argenteus* | Silvery pout | Ib |
| | *Melanogrammus aeglefinus* | Haddock | III |
| | *Theragra chalcogramma* | Alaska pollock | IVa |
| | *Trisopterus minutus* | Poor cod | III |
| Ictaluridae | *Ictalurus punctatus* | Channel catfish | IVb |
| Liparidae | *Liparis tessellatus* | Cubed snailfish | IV |
| Lotidae | *Lota lota* | Burbot | IVb |
| | *Enchelyopus cimbrius* | Fourbeard rockling | Ib |
| Merlucciidae | *Merluccius productus* | North Pacific hake | IVa |
| Moronidae | *Dicentrarchus labrax* | European sea bass | Ia |
| Mugilidae | *Mugil cephalus* | Flathead grey mullet | IV |
| Ophidiidae | *Hoplobrotula armata* | Armoured cusk | IV |
| Osmeridae | *Hypomesus pretiosus* | Surf smelt | ND |
| Oxudercidae | *Rhinogobius* sp. (undescribed species) | Yoshinobori | IVb |
| Percopsidae | *Percopsis omiscomaycus* | Trout perch | IVb |
| Petromyzontinae | *Petromyzon marinus* | Sea lamprey | IVb |
| Pleuronectidae | *Glyptocephalus stelleri* | Blackfin flounder | IVa |
| | *Hippoglossus hippoglossus* | Atlantic halibut | III |
| | *Reinhardtius hippoglossoides* | Greenland halibut | III |
| Salmonidae | *Oncorhynchus mykiss* X *Salvelinus alpinus* hybrids | Rainbow trout X Arctic charr hybrids | Ia |
| | *Oncorhynchus mykiss* X *Salvelinus namaycush* | Rainbow trout X lake trout hybrids | Ia |

| Family | Scientific name | Common name | Genotype |
|---|---|---|---|
| | hybrids | | |
| | *Oncorhynchus mykiss* X *Salmo trutta hybrids* | Rainbow trout X brown trout hybrids | Ia |
| | *Salvelinus alpinus* | Arctic charr | Ia |
| | *Salvelinus fontinalis* | Brook trout | Ie |
| Sciaenidae | *Larimichthys polyactis* | Yellow croaker | IV |
| Scorpaenidae | *Scorpaena porcus* | Black scorpionfish | Ie |
| | *Scorpaena izensis* | Izu scorpionfish | IV |
| Scyliorhinidae | *Scyliorhinus torazame* | Claudy catshark | IV |
| Stromateidae | *Pampus argenteus* | Silver pomfret | IV |
| Trichiuridae | *Trichiums lepturus* | Largehead hairtail | IV |
| Triglidae | *Eutrigla gurnardus* | Gray gurnard | III |

**ND:** Not determined.

In addition, pathogen-specific positive reverse-transcription polymerase chain reaction (RT-PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: Sablefish (*Anoplopoma fimbria*).

~~**2.2.3. Non-susceptible species**~~

~~None known.~~

### 2.2.4 ~~3~~. Likelihood of infection by species, host life stage, population or sub-populations

Rainbow trout is the most susceptible species to VHSV infection with genotype Ia. For VHSV genotypes Ib, II and III, shoaling wild-living species such as Atlantic herring and European sprat ~~(*Sprattus sprattus*)~~ are likely to be the natural hosts, while for genotype IVa, Pacific herring is the natural host. VHSV genotype III has caused disease in farmed turbot and wrasse and genotype IVa in sea-farmed Atlantic salmon, turbot, and ~~olive flounder~~ bastard halibut.

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. VHSV does not infect fish eggs (Munro & Gregory, 2010).

In surveys of wild marine fish, VHSV has been isolated from most year classes. Few fry have been tested however, as they are usually not caught during the surveys. The highest prevalence of virus in sampled wild populations was found in shoaling fish, such as Atlantic herring, European sprat and Norway pout ~~(*Sprattus sprattus*)~~ (Skall *et al*., 2005a).

For the purposes of Table 4.1 rainbow trout alevin and fry (e.g. up to approximately 1 g in weight) may be considered early life stages, fingerlings and ongrowing fish up to 50 g be considered as juveniles and fish over 50 g adults.

### 2.2.~~5~~ 4. Distribution of the pathogen in the host

In fish showing clinical signs, the virus is abundant in all tissues including gill, skin and muscles (Sandlund *et al.,* 2014). Target organs are anterior kidney, heart and spleen, as these are the sites in which virus is most abundant. In chronic stages, virus titres can become high in the brain (Smail & Snow, 2011; Wolf, 1988).

### 2.2.~~6~~ 5. Aquatic animal reservoirs of infection

Some survivors of epizootics will become long-term carriers of the virus. Pacific herring surviving infection with VHSV genotype IVa have transmitted disease to naïve cohabitants (Gross *et al*., 2019). Almost all isolations of VHSV genotype Ib, II and III from wild ~~free-living~~ fish species are from individuals with no clinical signs of infection with VHSV and with low virus titres (Skall *et al.,* 2005a).

### 2.2.~~7~~ 6. Vectors

VHSV has been detected in numerous species of animals, which are not susceptible species and may therefore ~~may~~ act as vectors. However, ~~there is no demonstrated~~ transmission of VHSV by vectors has not

been demonstrated. VHSV has been isolated from common snapping turtle (*Chelra serpentina*), leech

(*Myzobdella lugubris*), northern map turtle (*Grapetemys geographicas*) and water flea (*Moina macrocopa*) and these species ~~are considered~~ may be potential ~~to be~~ vectors for transmission of VHSV ~~rather than true susceptible species~~ (Faisal & Schultz, 2009; Goodwin & Merry, 2011; Ito & Olesen, 2017). VHSV has also been isolated from the amphipods *Hyalellea* spp. and *Diporeia spp*., suggesting that benthic macroinvertebrates may be vectors for VHSV IVb in endemically affected systems. In contrast VHSV was not detected in mussels or sediments in the same water environment (Faisal & Winters 2011; Throckmorton *et al*., 2017). VHSV has also been isolated from leech, *Myzobdella lugubris,* in the Great Lakes ~~but whether the leech or amphipods can transmit VHSV from one fish to another is unknown~~ (Faisal & Schulz, 2009; Faisal & Winters, 2011).

Piscivorous birds may act as VHSV vectors by carrying the virus, for example, on their beaks and feet (Olesen & Jorgensen, 1982), or through regurgitation of infected fish (Peters & Neukirch, 1986).

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

Mortality varies, depending on many environmental and physiological conditions, most of which have not been fully determined. The disease is, in general, a cool or cold water disease with highest mortality at temperatures around 9–12°C. Small rainbow trout fry (0.3–3 g) are most susceptible to genotype Ia with mortalities close to 100%, but all sizes of rainbow trout can be affected with mortalities ranging from 5 to 90% (Skall *et al.*, 2004). Immersion infection trials also induced up to 100% mortality in Pacific herring when challenged with genotype IVa (Hershberger *et al.,* 2010a). Mortality in ~~free living~~ wild finfish also varies from no observable deaths to severe die-offs. The prevalence of VHSV genotype Ib, II and III varies from 0 to 16.7% in Northern European waters (Skall *et al.*, 2005b).

### 2.3.2. Clinical signs, including behavioural changes

The occurrence of the following clinical signs is characteristic of infection with VHSV: rapid onset of mortality, lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins or in the gills, eyes or skin, abnormal swimming such as flashing and spiralling, and a distended abdomen due to oedema in the peritoneal cavity. In rainbow trout, the clinical appearance is typically lethargic dark fish with exophthalmia at the pond shores and the outlet. Characteristically, diseased fish will not attempt to escape when netted.

Infection with some genotypes of VHSV results in ~~have specific~~ predominant clinical signs ~~of infection with VHSV~~ in some susceptible species. Skin lesions in cod and herring from the Pacific and Atlantic Oceans (including the North Sea), and in haddock from the North Sea, have been described frequently (Jensen & Larsen, 1979; Meyers *et al.,* 1992; Meyers & Winton, 1995; Smail, 2000; Vestergard Jorgensen & Olesen, 1987). In farmed ~~Japanese flounder~~ bastard halibut, an 'anaemic' form (pale gills) of infection with VHSV has also been described (Isshiki *et al.,* 2001).

### 2.3.3 Gross pathology

Gross pathology includes generalised petechial haemorrhaging in the skin, muscle tissue (especially in dorsal muscles) and internal organs. It is important to examine the dorsal musculature for the presence of petechial bleeding, which is a very common sign of infection with VHSV. The kidney is dark red in the acute phase and can demonstrate severe necrosis in moribund fish. The spleen is moderately swollen. The liver is often pale and mottled. The gastrointestinal tract, especially the hindgut, is pale and devoid of food.

### 2.3.4. Modes of transmission and life cycle

Transmission primarily occurs horizontally through water, with excretion of virus in the urine, and directly from the skin (Smail & Snow, 2011). Oral transmission was also demonstrated indicating that preying on infected fish and vectors may transfer the disease (Schonherz *et al.* 2012).

Experimentally it has been demonstrated that feeding fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout can transmit VHSV to naïve fish (Oidtmann *et al.,* 2011a).

There are no indications or evidence of true vertical transmission of VHSV (Bovo *et al*., 2005a; Munro & Gregory, 2010).

### 2.3.5. Environmental ~~and management~~ factors

Disease generally occurs at temperatures between 4°C and 14°C. At water temperatures between 15°C and 18°C, the disease generally takes a short course with low levels of mortality.

Low water temperatures (1–5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. Outbreaks of infection with VHSV occur during all seasons but are most common in spring when water temperatures are rising or fluctuating.

Field observations and experimental studies suggest that warmer water temperatures greatly reduce or inhibit transmission. Natural outbreaks of infection with VHSV are not observed at water temperatures greater than 18°C. In challenge trials, fish exposed to VHSV and reared at temperatures below 15°C displayed high mortality whereas those infected and reared at 20°C did not (Arkush *et al.,* 2006; Castric & de Kinkelin, 1984). For more detailed reviews, see Wolf (1988) and Smail & Snow (2011).

### 2.3.6. Geographical distribution

~~Until the late 1980s, VHSV was considered to be restricted to farmed rainbow trout in continental Europe, with the occasional isolation from a restricted number of other freshwater fish species (e.g. brown trout, pike [Meier & Jorgensen, 1980; Schlotfeldt & Ahne, 1988]). With the detection and isolation of VHSV from Pacific salmon off the Pacific North American coast in the late 1980s, subsequent studies have demonstrated that infection with VHSV occurs in numerous farmed and wild fish species along the Pacific and Atlantic North American coast (Skall *et al*., 2005), in the Great Lakes area of North America (Thompson *et al*., 2011), the seas around the UK (Skall *et al*., 2005), the Baltic Sea, Skagerrak and Kattegat (Skall *et al*., 2005), in the waters around Japan (Skall *et al*., 2005), and in the Black Sea area, with the distinct genotype Ie (Nishizawa *et al*., 2006).~~

Infection with VHSV ~~in farmed rainbow trout~~ has been reported from countries in Europe, North Amercia and North Asia. Some countries in these regions have declared freedom from infection with VHSV. ~~almost all European and Middle East countries and from China (People's Rep. of) and Russia. However, a number of countries in Europe, such as Denmark, Ireland, Norway, Sweden and UK, are officially declared free of infection with VHSV. Infection with VHSV~~ The disease has never been reported from the Southern Hemisphere.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

Although research on vaccine development for VHSV has been ongoing for more than four decades, ~~the only commercial vaccine available is against VHSV genotype IVa for bastard halibut in Korea~~ a commercial vaccine is not yet available. Candidate vaccines have included killed vaccines, attenuated live vaccines, a recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines. For a review see Lorenzen & LaPatra (2005). No vaccines currently affect the diagnostic sensitivity and specificity of tests for infection with VHSV ~~diagnostics~~.

### 2.4.2. Chemotherapy including blocking agents

No therapies are currently available.

### 2.4.3. Immunostimulation

Several immunostimulants, such as yeast-derived beta-glucans, IL-1β-derived peptides, and probiotics have been assessed for enhancing protection against infection with VHSV (Peddie *et al*., 2003). Several researchers report positive effects, but no immunostimulant directed specifically at enhanced resistance to infection with VHSV is available. Furthermore, it remains unknown as to whether their use can affect sensitivity and specificity of ~~infection with~~ VHSV assays~~diagnostics~~.

#### 2.4.4. Breeding resistant strains

Additive genetic variation in rainbow trout ~~has been detected~~ for resistance to infection with VHSV has been demonstrated (Dorson *et al*., 1995; Henryon *et al*., 2002a; 2002b). In a study by Henryon *et al*. (2005), the heritability of resistance to VHSV was 0.11 for time to death on a logarithmic timescale. Identification of a major quantitative trait loci (QTL) for VHSV resistance in rainbow trout may pave the way for genetic selection for VHSV resistant fish (Verrier *et al.,* 2013), however, no resistant rainbow trout strains are ~~yet~~ commercially available.

#### 2.4.5. Inactivation methods

VHSV is sensitive to a number of common disinfectants (e.g. UV light, chlorine, iodophore, sodium hypochlorite), to temperatures above 30°C, to bacterial degradation in sediments and enzymatic activity in decomposing fish. For a review see Bovo *et al*., 2005b.

#### 2.4.6. Disinfection of eggs and larvae

Disinfection of newly fertilised or eyed ~~and green~~ eggs is an efficient and cost-effective preven~~ta~~tive measure for stopping the spread of the disease in salmonids (for the recommended protocol see Chapter 4.4. of the *Aquatic Code*).

#### 2.4.7. General husbandry

Poor water quality, high fish density, high feeding rate, infection with other diseases such as proliferative kidney disease, ichthyophthiriasis, bacterial kidney disease, etc. can influence the course and severity of infection with VHSV. In general, an increase in temperature, restricted feeding, reduced fish density and restricted handling may reduce mortality. In endemically infected farms, stocking with naïve fry is usually done ~~at as high~~ when the water temperature~~s~~ is at near maximum levels ~~as possible~~.

## 3. Specimen selection, sample collection, transportation and handling

### 3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is below 14°C or whenever the water temperature is likely to reach its lowest annual point. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention should be paid to the water outlet area where weak fish tend to accumulate due to the water current.

Fish to be sampled are selected as follows:

i) For genotype I, in farms where rainbow trout are present, ~~only~~ fish of that species should be selected for sampling. If rainbow trout are not present, the sample should be obtained from fish of all other VHSV-susceptible species present (as listed in Table~~s~~ 2.1) ~~and~~ or from species with incomplete evidence for susceptibility (as listed in Table 2.2). However, the species should be proportionally represented in the sample. For other genotypes (II, III, and IV), species of known susceptibility to the genotype in question should be sampled.

ii) Susceptible species should be sampled following risk-based criteria for targeted selection of populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or introduction of stocks of unknown risk status).

iii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

### 3.2. Selection of organs or tissues

~~The optimal tissues material to be examined is are spleen, anterior kidney, heart and encephalon. When sampling broodstock, In some cases, ovarian fluid and milt can be taken must be examined.~~

~~In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the anal pore gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. For larger size fish, kidney, spleen, heart and encephalon, and ovarian fluid from brood fish at the time of spawning should be the tissues to be sampled.~~

In populations with clinical disease, the optimal tissues are anterior kidney, spleen and heart (Lovy *et al.,* 2012; Oidtmann *et al.,* 2011).

In apparently healthy populations, the optimal tissues are anterior kidney and heart and, during the chronic phase of infection, brain, as VHSV can persist in tissues of the nervous system (Hershberger, 2010b; Lovy *et al.,* 2012; Oidtmann *et al.,* 2011b).

When sampling fish too small in size to permit dissection of individual tissues, viscera including kidney should be collected or whole fish homogenised after removal of the body behind the anal pore. When sampling broodstock, ovarian fluid and milt can be taken.

## 3.3. Samples or tissues not suitable for pathogen detection

When possible, tissues with high enzymatic activity such as liver and viscera should be avoided as VHSV is very sensitive to enzymatic degradation~~, therefore, sampling tissues with high enzymatic activities, such as viscera and liver, or large numbers of contaminating bacteria, such as the intestine or skin, should be avoided~~ When performing cell culture assays tissues containing high bacteria counts, such as the intestine or skin, should be avoided to minimise risk of bacterial contamination of tissue culture cells. Preservatives and fixatives, such as RNAlater and formaldehyde can be toxic to tissue culture cells such as epithelioma papulosum cyprini (EPC) and fathead minnow (FHM), and can impact molecular detection methods (Auinger *et al.,* 2008; Pham *et al.,* 2018).

## 3.4. Non-lethal sampling

Fin and gill biopsies were shown to be effective nonlethal samples for detection of VHSV genotype IVb (Cornwell *et al.,* 2013) in clinically diseased fish and nested RT-PCR on blood samples from infected fish was also shown to be effective~~ efficient~~ for VHSV detection (Lopez-Vazquez *et al.,* 2006a). In the case of brood fish, ovarian fluid and milt can be used for testing as an alternative to lethal testing. However, ~~no~~ non-lethal sampling~~s~~ methods have not been fully validated for detection of all VHSV genotypes~~ and are therefore not prescribed in this chapter~~.

## 3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

### 3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (diseases of fish).

~~The success of pathogen isolation and results of bioassay depend strongly heavily on the quality of samples (level of autolysis of fish samples, time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should be used only after consultation with the receiving laboratory.~~

~~Before transfer to the laboratory, pieces of the organs to be examined should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required.~~

~~Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C and ice must still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen.~~

~~Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with present current national and international transport regulations, as appropriate.~~

~~The virological examination for isolation in cell culture should be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.~~

#### 3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1, using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, samples may be placed in at least five volumes of RNA stabilisation reagents according to the recommendation from the manufacturers. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

Whole fish may also be sent to the laboratory (see Section 3.5.1).

Samples may also be frozen at –80°C and kept frozen until assayed (Siah *et al.*, 2014).

#### 3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be fixed in 10% neutral buffered formalin immediately after collection. The recommended ratio of fixative to tissue is 10:1. To avoid excessive cross-linking, tissue should be transferred to ethanol after 24 hours if methods other than histopathology are used e.g. *in-situ* hybridisation.

#### ~~3.5.4. Fixed Samples samples for electron microscopy~~

~~Sampling for electron microscopy should be done according to standard procedures (for an example, see Chapter 2.2.9 *Infection with yellow head virus genotype 1*). Sampling for electron microscopy is not relevant for diagnostic purposes. Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.~~

#### 3.5.~~5~~ 4. Samples for other tests

If samples are processed for ELISA or other immunochemical assays, the procedures described in Section 3.5.1 for pathogen isolation should be followed.

### 3.6. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore, larger fish should be processed and tested individually. However, samples, especially fry or specimens up to 0.5 g, can be pooled to obtain enough material for virus isolation or molecular detection.

## 4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;

++ = Suitable method(s) but may need further validation;

| | |
|---|---|
| + = | May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application; |

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, and repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

*Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals*

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **~~Immunohistopathology~~[3] Immunohistochemistry[3]** | | | | | | | | | ++ | ~~2~~ ++ | ~~2~~ ++ | 2 |
| **Histopathology[3]** | | | | | | ++ | ++ | 1 | | | | |
| **Cell culture** | +++ | +++ | +++ | 3 | +++ | +++ | +++ | 3 | +++ | +++ | +++ | 3 |
| **Real-time PCR** | +++ | +++ | +++ | 3 | +++ | +++ | +++ | 3 | +++ | +++ | +++ | 3 |
| **Conventional RT-PCR** | ++ | ++ | ++ | 3 | +++ | ++ | +++ | 3 | +++ | ++ | +++ | 3 |
| **Amplicon sequencing[4]** | | | | | | | | | +++ | +++ | +++ | 3 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | ~~+~~ | ~~++~~ | 2 | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | +[5] | ++[5] | ++[5] | 1 |
| **IFAT** | | | | | ++ | ++ | ++ | 2 | ++[5] | ++[5] | ++[5] | 2 |
| **Serum neutralisation for Ab detection** | | ~~+~~ | ~~++~~ | 2 | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); RT-PCR = reverse-transcription polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test;
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3. [3]Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. [4]Sequencing of the PCR product. [5]only for identification of cultured pathogen. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Not relevant.

## 4.2. Histopathology and cytopathology

The kidney, liver and spleen show extensive focal necrosis and degeneration – cytoplasmic vacuoles, pyknosis, karyolysis, and lymphocytic invasion. While the skeletal muscle does not appear to be a primary site of infection, erythrocytes can accumulate in the skeletal muscle bundles and fibres without causing damage to the muscle *per se* (Evensen *et al.,* 1994).

## 4.3. Cell ~~or artificial media~~ culture for isolation

The recommended cell lines for VHSV detection are bluegill fry (BF-2), Chinook salmon embryo (CHSE-214), epithelioma papulosum cyprini (EPC) ~~or~~ fathead minnow (FHM) or rainbow trout gonad (RTG-2). Susceptibility of a cell line to VHSV infection will depend on a range of parameters, including cell-line lineage or viral strain differences. Generally, VHSV isolates belonging to either genotypes I, II, or III culture best on BF-2 (Lorenzen *et al*., 1999), while genotype IV isolates culture best on the EPC cell line (US Department of the Interior, 2007).

### 4.3.1. Cell lines

Cell lines should be monitored regularly (e.g. every 6 months) to ensure that susceptibility to targeted pathogens has not changed.

Cells are grown at 20–24°C in a suitable medium, e.g. Eagle's minimal essential medium (MEM) (or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris/HCl (23 mM) and Na-bicarbonate (6 mM), or with HEPES-buffered medium (HEPES=N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid). The pH must be maintained at 7.6 ± 0.2. Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively growing (not confluent) at inoculation. Cell susceptibility can be enhanced by reducing the amount of FBS to 2%. Pre-treatment of cells with 7% (w/v) PEG-20,000 solution (10–15 µl/cm$^2$) 15–30 minutes prior to sample inoculation has also been shown to increase detection of VHSV in culture (Batts *et al.,* 1991).

### 4.3.2. Sample preparation and inoculation

i) **Note:** Tissue and fluid samples should be kept cool throughout sample preparation procedures. Homogenise tissue samples using mortar and pestle~~, stomacher, polytron or equivalent~~ or a tissue homogeniser. A small volume of medium (MEM-4 or HBSS [Hank's balanced salt solution] + antibiotics) may be needed to achieve complete homogenisation.

ii) Adjust the volume of medium to a final ratio of 10:1 (medium:tissue) and mix thoroughly. For fluid samples adjust the volume of medium to a final ratio of 1:1.

iii) Centrifuge the homogenate or fluid samples at 2000–4000 ***g*** for 15 minutes at 2–5°C.

iv) Remove the supernatant and pass through a 0.45 µM membrane filter (if available) or treat for either 4 hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml$^{-1}$.

If the sample cannot be inoculated within 48 hours after collection, the supernatant may be stored at –80°C provided virological examination is carried out within 14 days.

v) If samples originate from an area where infectious pancreatic necrosis virus (IPNV) is present, supernatants may be treated with IPNV antiserum. Mix the supernatant with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubate for a minimum of one hour at 15°C or up to 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

Treatment of all inocula with antiserum to IPNV ~~(a virus that in some parts of Europe occurs in 50% of fish samples)~~ aims at preventing cytopathic effect (CPE) caused by IPNV from developing in inoculated cell cultures. This will reduce the duration of the virological examination as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of VHSV. When samples come from production units that are considered free from infection with IPNV, treatment of inocula with antiserum to IPNV may be omitted.

vi) Samples are inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively. The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 $cm^2$ cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are also acceptable.

vii) Inoculated cell cultures are incubated at 15°C for 7–10 days. Using a microscope with 40–150× magnification, cultures should be inspected for toxicity the day after inoculation, particularly if supernatant was not filtered in step iv. The use of a phase-contrast microscope is recommended.

viii) Monitor the cells regularly (2–3 times a week) for the presence of CPE.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period, subcultivation is performed.

*Subcultivation*

i) Remove cell culture supernatant from the primary culture and inoculate a newly (<48 hours) seeded cell culture plate.

ii) Incubate inoculated plates at 15°C and monitor for 7–10 days as described above.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period or subcultivation, the sample is negative.

### 4.4. Nucleic acid amplification

Use of molecular tests (conventional RT-PCR and real-time RT-PCR) is common because of their rapidity, sensitivity and specificity. Real-time RT-PCR tests are generally more sensitive than conventional RT-PCR tests. The use of these tests for virus detection and identification during the acute stage of disease has been justified for a number of years. ~~At~~ In the acute stage of infection, the sensitivity of some conventional RT-PCR (Kim *et al.,* 2018) and real-time RT-PCR tests (Garver *et al.,* 2011; Jonstrup *et al.,* 2013) is comparable to detection by cell culture and subsequent identification. The molecular methods described in this chapter are all targeting the ~~N~~nucleoprotein gene, as it is the highest transcribed gene in the VHSV genome (Chico *et al.,* 2006).

Recently, a novel one-step RT-PCR test was developed and validated (Kim *et al.,* 2018) to be used instead of the previously recommended conventional RT-PCR for detecting VHSV. This novel assay has a higher sensitivity detecting all VHSV genotypes, and outperforms the old method, particularly in detecting genotype IV.

For detecting all genotypes of VHSV with real-time RT-PCR, the ~~one-step~~ methods of Jonstrup *et al*. (2013) and Garver *et al.* (2011) have ~~has~~ been validated to stage 3 ~~validated~~, showing a sensitivity similar to detection by cell culture. These~~This~~ methods~~,~~ have ~~having~~ high analytical and diagnostic sensitivity and specificity, and ~~has been shown to be highly~~ are robust across laboratories (Garver *et al.,* 2011; Jonstrup *et al.,* 2013; Warg *et al.,* 2014a; 2014b).

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

### 4.4.1. Real-time RT-PCR

Total RNA can be purified from: aliquots of cell culture medium from infected monolayer cells; or tissue/organs homogenised in MEM specified in Section 4.3.1, tissue samples in RNA stabilising reagent, fresh or frozen tissue samples, ovarian fluid.

In the case of culture medium from infected monolayer cells, or in tissue homogenised in MEM, aliquots should be centrifuged at 1000 ***g*** for 5 minutes to remove cell debris.

One-step (Jonstrup *et al.,* 2013) and two-step (Garver *et al.,* 2011) real-time RT-PCR assays targeting the nucleoprotein gene of VHSV have been stage 3 validated and are described herein.

Positive and negative controls should be included with each stage of the assay: extraction, reverse-transcription (two-step assay only) and real-time RT-PCR. An internal (endogenous) PCR control can be included however given the large number of fish species susceptible to infection with VHSV, the selection of an internal control is not trivial. If an endogenous control is to be used, primers and probes have to be designed, optimised and validated for each fish species to be tested.

Total RNA from infected cells and/or tissues is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially available RNA isolation kit used according to the manufacturer's instructions.

*One-step real-time RT-PCR*

In one-step RT-PCR gene-specific primers are used both to generate a cDNA transcript and for real-time RT-PCR. Both reactions occur in the same tube, which minimises the risk ~~probability~~ of contamination. The one-step real-time RT-PCR amplification can be performed using forward primer 5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3', reverse primer: 5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3', and FAM-labelled probe: 6'-FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1. Primers are used at a final concentration of 900 nM and the final probe concentration is 250 nM. 5 µl of extracted RNA (50 ng–2 ug) is added to each 25 µl RT-PCR reaction. The assay was validated using Quantitect Probe RT-PCR kit (Qiagen, Germany) following the manufacturer's instructions and is recommended as other one-step kits have demonstrated reduced sensitivity (Jonstrup *et al.,* 2013). Thermal cycling conditions are 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 40 seconds, 72°C for 20 seconds.

*Two-step real-time RT-PCR*

i) Step 1: Reverse-transcription

Extracted RNA is reverse transcribed non-discriminately into cDNA using random primers. The cDNA synthesis reactions and cycling conditions are best performed using manufacturer's instructions for commercially available kits which have been extensively tested with a variety of RNA templates, including GC- and AU-rich targets and RNAse expressed at low levels.

ii) Step 2: Real-time PCR

The TaqMan real-time PCR assay uses forward primer 5'-ATG-AGG-CAG-GTG-TCG-GAG-G-3', reverse primer 5'-TGT-AGT-AGG-ACT-CTC-CCA-GCA-TCC and FAM-labelled probe 5'-6FAM-TAC-GCC-ATC-ATG-ATG-AGT-MGBNFQ-3'. Primers are used at a final concentration of 600 nM, and the final concentration of the probe is 200 nM. 2.5 µl of cDNA product is added to each 25 µl PCR reaction. Thermal cycling conditions are 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Garver *et al.,* 2011).

A sample is negative if no Ct (threshold cycle) is recorded, while samples with a Ct are considered positive for VHSV. Cut-off value depends on the set-up in each laboratory ~~but is usually set at Ct ≥ 40~~).

### 4.4.2. Conventional RT-PCR

RNA isolation is done as in Section 4.4.1. Positive and negative controls should be run with each stage of the assays: extraction and RT-PCR ~~and second round PCR~~. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and RT-PCR amplification occur in designated hoods or spatially separated areas.

A one-step RT-PCR should be performed as described by Kim *et al.* (2018) with 3F2R primer set: forward primers (3F, 5'-(GGG-ACA-GGA-ATG-ACC-ATG-AT-3') and reverse primer (2R, (5'-TCT-GTC-ACC-TTG-ATC–CCC-TCC-AG-3') targeting a 319 nt region in the nucleoprotein gene (positions 658–977).

The RT-PCR can be performed using, e.g. Qiagen OneStep RT-PCR System (Qiagen, Germany) or similar kit, according to the manufacturer's instructions. Briefly, the reaction mixture is adjusted to a final volume of 25 µl including 5 µl of extracted viral RNA, 5 µl 5 × One Step RT-PCR Buffer containing 12.5 mM $MgCl_2$ (final concentration 2.5 mM), 10 pM of each primer, and 1 µl of enzyme mix.

The following cycles are recommended: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

#### 4.4.3. Other nucleic acid amplification methods

To date, no other nucleic acid amplification method capable of universal VHSV detection has been sufficiently validated.

### 4.5. Amplicon sequencing

The VHSV genotype can be identified by sequencing the amplicon generated by the conventional RT-PCR using the 3F2R primer set (Kim *et al.,* 2018). Nucleotide sequencing of the glycoprotein gene is commonly used for identification of genetic strains and for epidemiological study and is recommended as one of the final steps for confirmatory diagnosis. There are several conventional RT-PCR assays available that amplify the central (669 nt) or full (1524 nt) glycoprotein gene coding sequence, but there are limited validation data. The glycoprotein gene can be amplified by conventional RT-PCR using the primer sets and concentrations listed in Table 4.2. The reverse transcription and subsequent PCR amplification can be done using a kit designed for that purpose according to the manufacturer's instructions.

***Table 4.2.*** *Primer sets for the conventional RT-PCR, sequencing and phylogenetic analysis*

| Primer | Sequence (5'–3') | Product size (bp) | Final primer concentration | Reference |
|---|---|---|---|---|
| GB+ | GTC-GAA-GAA-GAG-ATA-GGC | 1757 | 0.6 µM | Einer-Jensen *et al.,* 2004<br>Gudmundsdottir *et al.,* 2019 |
| GB- | GTT-GGG-TCG-CCA-TGT-TTC-T | | 0.6 µM | |
| G330+ | ACT-ACC-TAC-ACA-GAG-TGA-C | 914 | 0.2 µM | Garver *et al*., 2013 |
| G1243- | CAA-TTT-GTC-CCC-GAA-TAT-CAT | | 0.2 µM | |
| G422+ | TCC-CGT-CAA-GAG-GCC-AC | 669 | 0.2 µM | |
| G1179- | TTC-CAG-GTG-TTG-TTT-ACC-G | | 0.2 µM | |

### 4.6. *In-situ* hybridisation

Not relevant in relation to primary diagnosis and surveillance of infection with VHSV.

### 4.7. Immunohistochemistry

Immunohistochemistry reveals VHSV-positive endothelial cells, primarily in the vascular system (Evensen *et al*., 1994). Specific polyclonal and monoclonal antibodies for immunohistochemistry are commercially available.

### 4.8. Bioassay

Not relevant in relation to primary diagnostics and surveillance of infection with VHSV.

## 4.9. Antibody- or antigen-based detection methods

Antibody- and antigen-based detection methods should not be used as a method of screening healthy populations ~~method~~.

### 4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of protein-A purified immunoglobulins (Ig) from rabbit anti sera against VHSV in carbonate buffer, pH 9.6 (50 µl well$^{-1}$).

ii) Incubate overnight at 4°C.

iii) Rinse in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST).

iv) Add 1% Triton X-100 to the virus suspension to be identified.

v) Dispense 50 µl well$^{-1}$ of two- or four-step dilutions (in PBST containing 1% bovine serum albumin) of the virus to be identified and of VHSV control virus, as well as a negative control (e.g. infectious haematopoietic necrosis virus [IHNV]), and allow to react with the coated antibody to VHSV for 1 hour at 37°C.

vi) Rinse in PBST.

vii) Add to the wells monoclonal antibodies to VHSV N protein (IP5B11) 50 µl well$^{-1}$.

viii) Incubate for 1 hour at 37°C.

ix) Rinse in PBST.

x) Add to the wells (50 µl well$^{-1}$) horseradish peroxidase (HRP)-conjugated monoclonal anti-mouse antibodies.

xi) Incubate for 1 hour at 37°C.

xii) Rinse in PBST.

xiii) Visualise the reaction using TMB (3,3',5,5'-tetramethylbenzidine) and measure the absorbance at a wavelength of 450 nm.

The above ELISA version is given as an example. Other ELISA versions of demonstrated performance may be used instead.

For positive controls, use cell culture supernatant from cultures inoculated with known VHSV isolate.

For negative controls, use cell culture supernatant from same cell line inoculated with heterologous virus (e.g. IHNV) or from non-infected culture.

### 4.9.2. Indirect fluorescent antibody test (IFAT)

i) Prepare monolayers of cells in 2 cm$^2$ wells of cell culture plastic plates or on cover-slips to reach around 80% confluence, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of VHSV in a similar way, in order to obtain a virus titre of about 5000–10,000 plaque-forming units (PFU) $ml^{-1}$ in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M PBS, pH 7.2, then three times briefly with a cold mixture of acetone 30% and ethanol 70% (v/v) (stored at –20°C).

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 $cm^2$ of cell monolayer.

vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

viii) Prepare a solution of purified VHSV antibody or serum in 0.01 M PBST, pH 7.2, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by using four rinsing steps with the PBST solution and remove this buffer completely after the last rinse.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur, e.g. by adding a piece of wet cotton in the humid chamber. The volume of solution to be used is 0.25 ml per ~~/~~ 2 $cm^2$ $well^{-1}$.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)- or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibody to the immunoglobulin used as the primary antibody and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using, for example glycerol saline, pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must yield the expected results prior to any other observation.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of demonstrated performance may be used instead.

Always include positive control such as wells or coverslip with cells infected with a known VHSV isolate.

## 4.10. Other ~~serological~~ methods

### 4.10.1. Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2000 ***g*** for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.

ii) Dilute virus-containing medium from $10^{-2}$ to $10^{-4}$.

iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of a VHSV antibody solution and, likewise, treat aliquots of each virus dilution with cell culture medium. The neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.

iv) In parallel, another neutralisation test must be performed against a homologous virus strain (positive neutralisation test).

v) If required, a similar neutralisation test may be performed using antibodies to IPNV.

vi) Incubate all the mixtures at 15°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures on to 24–48 hour-old monolayers, overlaid with cell culture medium containing 10% FCS (inoculate two wells per dilution), and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

viii) Check the cell cultures for the onset of CPE and read the result as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

ix) The tested virus is identified as VHSV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the VHSV-specific antibody, whereas CPE is evident in all other cell cultures.

x) In the absence of any neutralisation by NAb to VHSV, it is mandatory to conduct an RT-PCR, an ELISA or IFAT, using the suspect sample. Some cases of antigenic drift of surface antigen have been observed, resulting in occasional failure of the neutralisation test using NAb to VHSV.

Other neutralisation tests of demonstrated performance may be used instead.

## 5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Virus isolation, real-time RT-PCR and conventional RT-PCR are the recommended tests for surveillance to demonstrate freedom of disease in apparently healthy population.

## 6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or in the presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

### 6.1. Apparently healthy animals or animals of unknown health status[16]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. Geographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

#### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with VHSV shall be suspected if at least one of the following criteria is met:

i) VHSV-typical CPE in cell cultures ~~before confirmation~~;

[16] For example transboundary *commodities.*

ii) A positive result from a real-time RT-PCR assay;

iii) A positive result from a conventional RT-PCR assay.

~~iv) Detection of antibodies (by Ab-ELISA or serum neutralisation in adults only).~~

#### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with VHSV is considered to be confirmed if~~, in addition to the criteria in Section 6.1.1.,~~ one or more of the following criteria is met:

~~i) VHSV isolation in cell culture followed by virus identification by conventional RT-PCR, and by sequencing of the amplicon;~~

i) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, ~~or~~ IFAT ~~and detection of VHSV in tissue preparations~~ or by conventional RT-PCR and sequencing of the amplicon;

~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;~~

ii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon.

iii) Detection of VHSV in tissue preparations by immunohistochemistry, and by a conventional RT-PCR and sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

### 6.2 Clinically affected animals

No clinical signs are pathognomonic for infection with VHSV however, they may narrow the range of possible diagnoses.

#### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with VHSV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with VHSV as described in this chapter, with or without elevated mortality;

ii) Histopathological changes consistent with infection with VHSV as described in this chapter;

iii) A positive result from real-time RT-PCR~~, conventional PCR, or IFAT~~;

iv) A positive result from a conventional RT-PCR;

v) A positive result by IFAT;

vi) VHSV-typical CPE ~~Cytopathic effect~~ in cell culture.

#### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with VHSV shall be confirmed if~~, in addition to the criteria in Section 6.2.1., positive results has been obtained on at least one animal from two tests used in the following combination~~ one or more of the following criteria is met:

i) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, ~~or~~ IFAT ~~and detection of VHSV in tissue preparations~~ or by conventional RT-PCR and sequencing of the amplicon;

~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;~~

~~i~~ii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon.

iii) Detection of VHSV in tissue preparations by immunohistochemistry, and by a conventional RT-PCR and sequencing of the amplicon.

~~i) VHSV isolation in cell culture, followed by virus identification by conventional RT-PCR, and sequencing of the amplicon;~~

~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;~~

~~iii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;~~

~~iv) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and a positive result from immunohistopathology);~~

~~v) Detection of VHSV in tissue preparations by real-time RT-PCR and by conventional RT-PCR, followed by sequencing of the amplicon.~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

### 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with VHSV is provided in Table 6.3. This information can be used for the design of surveys for infection with VHSV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

***Table 6.3.*** *Diagnostic performance of tests recommended for surveillance or diagnosis*

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Cell culture | Surveillance | Experimentally infected fish | Kidney, heart and spleen | Rainbow trout | 86 (84) | – | Real-time RT-PCR | Jonstrup *et al.,* 2013 |
| Cell culture | Clinical diagnosis | Experimentally infected fish | Kidney | Atlantic salmon | 100 (100) | 94.4 (100) | Pseudo-gold standard* | Garver *et al.,* 2011 |
| Real-time RT-PCR | Surveillance | Experimentally infected fish | Kidney | Atlantic salmon | 93 (30) | 100 (70) | Cell culture | Garver *et al.,* 2011 |
| Real-time RT-PCR | Surveillance | Experimentally infected fish | Kidney, heart and spleen | Rainbow trout | 90 (84) | 100 (43) | Cell culture | Jonstrup *et al.,* 2013 |

* a compilation of 8 test results to evaluate both the real-time RT-PCR and virus isolation assay (Garver *et al,* 2011);
DSe = diagnostic sensitivity, DSp = diagnostic specificity; *n* = number of samples used in the study .

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**NB:** There are OIE Reference Laboratories for Infection with viral haemorrhagic septicaemia virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/ ).
Please contact the OIE Reference Laboratories for any further information on infection with viral haemorrhagic septicaemia virus

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