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

Paris, 26 August to 2 September 2020

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Due to the COVID-19 pandemic, the OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met electronically, instead of physically, between 26 August and 2 September 2020. The list of participants is presented in Annex 1.

The Commission reminded Members that as a consequence of the postponement of the OIE 88th General Session until 2021, all chapters in the OIE *Aquatic Animal Health Code* (hereinafter referred to as the *Aquatic Code*) or the *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter referred to as the *Aquatic Manual*) that were to be proposed for adoption in May 2020, will be proposed for adoption in May 2021.

The Aquatic Animals Commission thanked the following Members for providing written comments on draft texts for the *Aquatic Code* and the *Aquatic Manual* circulated in the Commission's February 2020 meeting report: Australia, Canada, Chile, Chinese Taipei, China (People's Rep. of), Cuba, Ecuador, Japan, Korea (Rep. of), New Caledonia, New Zealand, Peru, Singapore, Switzerland, Thailand, the United Kingdom (the UK), the United States of America (the USA), the Member States of European Union (the EU) and the African Union Interafrican Bureau for Animal Resources (AU-IBAR) on behalf of African Member Countries of the OIE.

The Commission considered 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 to distinguish them from those made previously. The Commission did not consider comments where a rationale had not been provided or were difficult to interpret.

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.

Comments on **Annexes 3** to **17** of this report must reach OIE Headquarters by the **6 January 2021** to be considered at the February 2021 meeting of the Aquatic Animals Commission.

All comments should be sent to the OIE Standards Department at: AAC.Secretariat@oie.int (a new address to use exclusively for the submission of Member comments on the Aquatic Animals Commission's reports).

Comments should be submitted as Word files rather than pdf files because pdf files are difficult to incorporate into the Commission's working documents.

Comments should be presented in the relevant Annex, and include new proposed text, supported by a structured rationale or by published scientific references. Proposed deletions should be indicated in '~~strikethrough~~' and proposed additions with 'double underline'. Members should not use the automatic 'track-changes' function provided by Word processing software, as such changes may be lost in the process of collating Members' submissions into the Aquatic Animals Commission's working documents. Members are also requested not to reproduce the full text of a chapter as this makes it easy to miss comments while preparing the working documents.

The Aquatic Animals Commission strongly encourages Members to participate in the development of the OIE's international standards by submitting comments on this report and participate in the process of adoption at the General Session.

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1. WELCOME FROM THE DEPUTY DIRECTOR GENERAL

Dr Matthew Stone, Deputy Director General (International Standards and Science), welcomed the Aquatic Animals Commission and thanked the members for taking time from their busy schedules to support the work of the OIE, and their employers and national governments.

Dr Stone thanked the Commission for its support during the Organisation's COVID-19 response, including the reports prepared to ensure OIE Members remain well briefed on the activities of the Specialist Commissions following the cancellation of the General Session for 2020. He noted the OIE's ongoing adaptation of its work programmes to the restrictions imposed as a result of the COVID-19 pandemic, with many successful virtual expert meetings now having been held, ensuring that the OIE's productive output has continued thanks to the hard work of staff and the understanding and dedication of the OIE's community of experts. Although the impacts of the global pandemic continue, and the scientific understanding of its root causes, mitigating and exacerbating factors remains incomplete, the OIE continues its internal reflection on its role to support its Members in the face of new priorities around emerging disease risk mitigation, resilience and preparedness. Concrete proposals in this respect will soon emerge, and the OIE will look to the expert networks of its Members and partners for implementation support, and funding support from resource partners. These activities will also engage the Specialist Commissions, and therefore need to be considered in work programme prioritisation. Dr Stone noted the currently open call for nominations for the elections in 2021 for Specialist Commissions.

He also provided the Commission with a summary of the performance evaluation process that all experts of Specialist Commissions would be participating in, as the concluding phase of the new Specialist Commission performance management system. This would result in a confidential report to OIE Council in February 2021.

2. COOPERATION WITH OTHER SPECIALIST COMMISSIONS

Dr Ingo Ernst, President of the Aquatic Animals Commission, and Dr Etienne Bonbon, President of the Terrestrial Animal Health Standards Commission (hereinafter referred to as the Code Commission) held a virtual meeting in July 2020 to ensure that any proposed amendments are aligned as far as possible in both the *Terrestrial* and *Aquatic Codes*.

3. WORK PLAN FOR THE AQUATIC ANIMAL HEALTH COMMISSION

The Aquatic Animals Commission reviewed and updated its work plan considering that the Commission's February 2021 meeting will be the last meeting of its term and that many texts will be proposed for adoption in May 2021.

The revised work plan is presented as Annex 2 for Member information.

4. TEXTS FOR MEMBER COMMENTS: OIE *AQUATIC ANIMAL HEALTH CODE*

The Aquatic Animals Commission noted that where amendments were of an editorial nature, no explanatory text has been provided in this report.

4.1. New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X)

Comments were received from Canada, Chile, China (People's Rep. of), Peru 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. The draft chapter has been circulated four times for comments between September 2018 and September 2020.

At its February 2020 meeting, the Aquatic Animals Commission revised the chapter only to respond to substantive comments that had not been submitted before.

Previous Commission 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 meeting

The Commission was grateful that Members submitted new substantial comments on this chapter. The Commission reviewed and considered all comments received and made changes as deemed appropriate.

The word 'staff' was replaced by 'personnel' throughout the chapter for consistency.

Article 4.X.3 Introduction

The Commission agreed with a comment to add to the 'Introduction' that the implementation of good biosecurity measures may reduce the rate of emergence of antimicrobial resistance (AMR).

Article 4.X.4 General principles

The Commission agreed to add 'environmental conditions' to the introductory text on General principles because the required biosecurity measures may depend on several factors including environmental conditions. The Commission did not agree to replace the terms 'infection' and 'disease' with 'the introduction, spread and release of pathogenic agents' when describing risks in this article because the wording proposed would be a repetition of the Glossary definition of 'biosecurity'.

In response to a comment, the Commission agreed to move point 5, concerning the display of clear signage, to Article 4.X.6 as a new point 7 c) because the content is not considered a principle and is more appropriate under the point on 'personnel and visitors'.

Article 4.X.5 Categories of aquaculture production systems

The Commission did not agree with the suggestion to add 'pathogenic agents' to the 'Open systems' and 'Semi-open systems' sections because it was deemed already implicit in the current wording.

The Commission also did not find it necessary to add 'raft cages' as an example of semi-open aquaculture production systems because it is already covered by net pens.

The Commission agreed to change 'may' for 'can' in the text of the 'Semi-closed systems' to emphasise that it is easier to prevent aquatic animals and vectors from entering and exiting a semi-closed system compared to a semi-open system.

Article 4.X.5 bis Area management

The Commission did not agree with a proposal to add 'Aquatic Animal Health Services' as one of the entities that should implement the biosecurity measures because it is for each Member to determine how the interventions should be applied.

Article 4.X.6 Transmission pathways and mitigation measures

1. Aquatic animals

The Commission agreed with a proposal to include the concept of 'gametes' as a more comprehensive example of aquatic animals that can be intentionally introduced into, or moved within an aquaculture establishment, but opted to use the term 'and milt' instead of 'and gametes'.

The Commission did not agree to add explanatory text under point b) to the meaning of 'quarantine' because it is covered by the Glossary definition of 'quarantine'.

In response to a comment, the Commission agreed to add the words 'and release of' to point d) because biosecure transport must also include measures to prevent pathogenic agents from being released during transport.

The Commission agreed to replace 'sick' with 'moribund' to improve the clarity of point g).

The Commission further agreed to add:

– 'by aquatic animal health professionals or veterinarians' to point h) to clarify that investigation and diagnosis of the cause of mortality should always be undertaken by both professionals;

– 'drying' to the text in point i) as allowing production installations to dry is a common mitigation measure when aquaculture establishments are being fallowed.

The Commission did not agree to add a new point k) 'establishing of a surveillance and monitoring plan aimed at the early detection of diseases' because the issue is addressed in the 'biosecurity plan' section.

3. Water

The Commission did not agree to include 'poorly managed toilets' under point e) because it was deemed an environmental and human health issue, and thus outside the scope of the chapter.

4. Feed

The Commission did not agree to add the term 'live feed' because it is included within the Glossary definition of 'feed'.

5. Fomites

The Commission did not agree to replace the term 'transferring' with 'transmitting' because it considered the former to be a more accurate term in the context of spread of pathogenic agents by fomites. However, the Commission did decide to reorder the sentence describing the sharing of equipment to improve readability and avoid misunderstanding.

6. Vectors

The Commission replaced the term 'transport' with 'transfer' in the first sentence to be consistent with the revised definition of 'vector', and the use of 'transfer' elsewhere in the chapter.

The Commission considered the proposal to replace 'wild' by 'any non-susceptible' and decided that neither of the terms were needed given the context of the sentence and therefore 'wild' was removed. The Commission did add the missing 'and' in the list of the first paragraph (second sentence).

The Commission considered replacing 'outdoor' with 'semi-open and semi-close' but decided that 'unenclosed' was a more appropriate term to use in this context.

7. Personnel and visitors

The Commission considered the proposals for additional points in this section and further changes were made.

A proposal to specify the type of production system was not supported by the Commission because mitigation measures should be considered in any type of production system.

The Commission added a point i) under a) to include the registration of visitors; it agreed to add the term 'gloves' as an additional example under point a) ii) and added the text moved from point 5 (Article 4.X.4, General principles) as a new point c).

The Commission agreed to add text regarding the training of personnel because it considered that it was a valuable concept to include but decided that it was more appropriate under Article 4.X.8.

Article 4.X.8 Biosecurity plan development

The Commission agreed to add 'aquatic animal health professionals or veterinarians' to point 1 g) to improve clarity. It also agreed to add 'laboratory test results' to point 2 d) as an additional health monitoring record.

In response to a comment on Article 4.X.6, the Commission agreed with the proposal to add training programme for personnel as relevant part of a biosecurity plan. This was added as a new point f).

The adoption of the chapter has been postponed until May 2021. As the chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

The revised new Chapter 4.X, Biosecurity for Aquaculture Establishments, is presented in Annex 3 for Member comments.

4.2. Listing of infection with decapod iridescent virus 1 (DIV1) – Revised Article 1.3.3 of Chapter 1.3

Comments were received from Australia.

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 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. At this meeting, the name was changed to 'Infection with decapod iridescent virus 1 (DIV1)' in accordance with the classification of the pathogenic agent in the database of the International Committee of Taxonomy of Viruses (ICTV).

The proposal to list infection with DIV1 and the assessment have been reviewed, updated and circulated three times for comments between February 2019 and September 2020.

Previous Commission 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 and Item 7.3.4, page 17).

September 2020 meeting

The Commission updated the assessment according to new available information, including the detection of DIV1 in giant tiger prawns, *Penaeus monodon*, and the associated reference.

The Commission is aware that new scientific papers on DIV1 will be published soon and asked Members to provide any relevant information, as it becomes available, for the Commission to consider at its February 2021 meeting.

Noting that the adoption of this revised article has been postponed until May 2021, and that the article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

The revised Article 1.3.3 of Chapter 1.3, Diseases listed by the OIE, and the updated assessment are presented in Annex 4 for Member comments.

4.3. Model Article 10.X.13 for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4)

Comments were received from Canada.

Background

Revision of 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, for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for 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. The model article has been circulated three times for comments.

At its February 2020 meeting, the Commission agreed to include in the first paragraph a cross reference to Chapter 4.4, Recommendation for surface disinfection of salmonid eggs, to provide guidance to Members on disinfection protocols. The Commission emphasised that Chapter 4.4 is restricted to salmonid species.

Previous Commission reports where this item was discussed:

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

September 2020 meeting

Further to the discussion at its February 2020 meeting, the Commission emphasised that the scope of Chapter 4.4 could be extended to address additional species in the future. In the meantime, methods for the disinfection of eggs from non-salmonid species can be found in some disease-specific chapters of the *Aquatic Manual*.

The Commission wished to thank a Member for sharing a scientific evaluation of Atlantic halibut (*Hippoglossus hippoglossus*) egg disinfection protocols for inactivation of infectious pancreatic necrosis virus and viral haemorrhagic septicaemia virus. The Commission confirmed that it would review this evaluation at its February 2021 meeting, and wished to encourage Members to submit supplementary information on disinfection protocols for other non-salmonid species.

Noting that the adoption of this revised article has been postponed until May 2021, and that the article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

The revised Model Article 10.X.13 for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4) is presented in Annex 5 for Member comments.

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

Comments were received from Canada and New Caledonia.

Background

The *ad hoc* Group on Susceptibility of fish species to OIE listed diseases applied the criteria for listing species as susceptible to infection with viral haemorrhagic septicaemia virus (VHSV) in accordance with Chapter 1.5, Criteria for listing species as susceptible to infection with a specific pathogen (report available at https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/). The assessments were reviewed by the Aquatic Animals Commission at its September 2019 meeting, and the amended list of susceptible species in Article 10.10.2 was circulated for comment in the Commission's September 2019 report and again in February 2020.

Previous Commission 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 meeting

In response to a request to re-instate genotypes in the table of susceptible species in Article 10.10.2, the Commission confirmed its decision that genotypes should not be included, noting that an approach to strain differentiation has been developed by the OIE and previously applied to infection with infectious salmon anaemia (ISAV). Unlike ISAV, the OIE has not assessed whether VHSV genotypes can be differentiated for the purpose of distinguishing risk management measures for traded commodities. It

is, therefore, not currently appropriate for genotype information to appear in the *Aquatic Code* chapter. However, 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.

Noting that the adoption of this revised article has been postponed until May 2021, and that the article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

The revised Article 10.10.2 of Chapter 10.10, Infection with viral haemorrhagic septicaemia virus, is presented as Annex 6 for Member comments.

4.5. Glossary Definitions

4.5.1. Glossary definitions for 'Aquatic animal waste' and 'Aquatic animal products'

Comments were received from Australia, China (People's Rep. of), New Caledonia, New Zealand, the USA and AU-IBAR.

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 on Biosecurity for aquaculture establishments (Chapter 4.X) as well as in Chapter 4.7, Handling, disposal and treatment of aquatic animal waste. The new Glossary definition was circulated for comment in the Commission's September 2019 and February 2020 reports.

Previous Commission reports where this item was discussed:

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

September 2020 meeting

'Aquatic animal waste'

The Commission agreed with a comment to replace 'its parts' with 'parts of aquatic animals' for clarity and consistency.

The Commission received several comments about consequential changes throughout the *Aquatic Code* resulting from the application of the new defined term 'aquatic animal waste'. In response to these comments, the Commission agreed to delete 'tissue' in the last sentence of point 7 of Section C of the User's guide, and to add 'or contaminated' to the third paragraph in Article 4.7.5.

'Aquatic animal products'

No comments were received on the proposed amendments.

Noting that the adoption of these revised Glossary definitions has been postponed until May 2021, and that the definitions have already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

The revised Glossary definitions for 'aquatic animal waste' and 'aquatic animal products' are presented in Annex 7 for Member comments.

4.5.2. Amending the Glossary definition of 'Vector'

Comments were received from Canada, China (People's Rep. of) and AU-IBAR.

Background

At its February 2020 meeting, the Aquatic Animals Commission amended the Glossary definition of 'vector' to make it clear that vectors for a specified infectious agent cannot be listed as a susceptible

species for the same pathogenic agent. This was in response to a request from the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases.

Previous Commission reports where this item was discussed:

February 2020 report (Item 7.2.1, page 15).

September 2020 meeting

The Commission considered the submitted comments and proposed a revised version of the definition for ‘vector’ to address potential misinterpretations.

The Commission added the text ‘that has been demonstrated to’ to clarify that for an organism to be classified as a vector, there must be evidence that it can transfer the specific pathogenic agent to susceptible populations. The Commission also agreed to replace the term ‘transport’ with ‘transfer’ to align the terminology with the new draft Chapter 4.X, Biosecurity for aquaculture establishments. It also reworded the definition to clearly separate ‘vectors’ from ‘susceptible species’.

The revised Glossary definition for ‘vector’ is presented in Annex 7 for Member comments.

4.5.3. Revision of the definitions of ‘Competent Authority’, ‘Veterinary Authority’ and ‘Aquatic Animal Health Services

Background

Following feedback from the *ad hoc* Group on Evaluation of Veterinary Services who met in May 2018 to develop a revised edition of the PVS Tool, the Code Commission agreed to revise the definitions for ‘Competent Authority’, ‘Veterinary Authority’ and ‘Veterinary Services’ in the *Terrestrial Code* Glossary. Proposed amendments were circulated for Member comments in the Code Commission’s September 2018 and September 2019 reports. An *ad hoc* Group on Veterinary Services, who met in July 2019, addressed the comments received, and produced draft definitions and an internal report for the OIE Headquarters.

Dr Ingo Ernst, President of the Aquatic Animals Commission, and Dr Etienne Bonbon, President of the Code Commission, held a virtual meeting in July 2020 to discuss the changes being proposed by the Code Commission to the *Terrestrial Code*.

The Presidents agreed that amendments to the three glossary definitions should be presented for consideration of both Commissions at their September 2020 meetings and that the revised definitions would then be circulated in both the Aquatic Animals Commission and Code Commission September 2020 reports for Member comments. For the purpose of the *Aquatic Code,* ‘Aquatic Animal Health Services’ will be used instead of ‘Veterinary Services’ because the latter is not used in this *Code*.

September 2020 meeting

The Aquatic Animals Commission noted that the Code Commission circulated to Members the revised amendments to these terms, for use in the *Terrestrial Code*, and encouraged Members to review both Commission reports to ensure alignment of comments, as appropriate.

The Commission reviewed the definitions and supported the amended versions provided by the Code Commission and added a reference to ‘Competent Authority’ in the Glossary definition of ‘Veterinary Authority’ for the purpose of the *Aquatic Code*. The Commission decided that this reference would add clarity, given the way ‘Competent Authority’ is used in the *Aquatic Code*.

The Commission encouraged Members to consider these amendments together with those being proposed in the Code Commission’s September 2020 report, to ensure alignment of the terms in both *Codes*.

The revised Glossary definitions of ‘Competent Authority’, ‘Veterinary Authority’ and ‘Aquatic Animal Health Services’ are presented as Annex 8 for Member comments.

4.6. De-listing of infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)

Comments were received from Australia, Ecuador and the UK.

Background

At its February 2020 meeting, the Aquatic Animals Commission considered a request from a Member to remove infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) from the list of diseases in Article 1.3.3 of Chapter 1.3, Diseases listed by the OIE. The Commission agreed to consider the issue further at its September 2020 meeting. It requested that Members provide any available information relevant to the Criteria for Listing Aquatic Animal Diseases; specifically listing criteria 4b) and 4c) of Article 1.2.2 (consequences for cultured or wild aquatic animals respectively).

Previous Commission reports where this item was discussed:

February 2020 (Item 7.3.1, page 16).

September 2020 meeting

The Commission reviewed information provided by Members and other peer-reviewed publications, and consulted with the OIE Reference Laboratory expert, to assess infection with infectious hypodermal and hematopoietic necrosis virus (IHHNV) against the criteria for listing aquatic animal diseases in Article 1.2.2 of the *Aquatic Code*. The Commission concluded that infection with IHHNV meets the listing criteria 1, 2, 3, and 4b. It should therefore remain listed in Article 1.3.3.

The Commission considered that whilst IHHNV has a wide global distribution, there are countries with a self-declared free status or that are likely to be able to declare freedom from infection with IHHNV. The Commission also agreed that a precise case definition is available, and that current assays provide a reliable means for detection.

The Commission acknowledged that the consequences of the disease have become less significant in many parts of the world due to improved biosecurity measures and the development of tolerant strains of shrimp. However, there is evidence that IHHNV may continue to cause significant economic losses in *P. vannamei*. In addition, significant levels of mortality and production loss due to infection with IHHNV have been reported in other species of shrimp.

The Commission noted that the inappropriate application of OIE standards, resulting in unjustified restrictions on trade, cannot be used as a rationale for delisting a disease.

The Commission's assessment of IHHNV against the criteria for listing aquatic animal diseases is presented as Annex 9 for Members' information.

4.7. Safe commodities (Article X.X.3 of disease-specific chapters)

Background

As reported in the Aquatic Animals Commission February 2020 report, the Commission had commenced its review of the structure of Article X.X.3 of all disease-specific chapters of the *Aquatic Code*. The review aims to address Members' requests for more clarity on the recommendations for safe commodities provided in these articles.

September 2020 meeting

The Commission noted that some Members had commented that the recommended time and temperature treatments in Article X.X.3 represented different levels of thermal treatment and that some were not commercially feasible as they would diminish product quality.

The Commission noted that the original approach to this article had been to list product types (e.g., hermetically sealed, pasteurised, cooked) and the standard commercial temperature treatments for those product types. This approach had resulted in the apparent lack of equivalence in time/temperature treatments (for example, between pasteurisation and hermetically sealed products) and had also reduced

flexibility for different product types to be considered safe even though they might exceed the heat treatment required to deactivate the relevant pathogenic agent.

The Commission proposed to amend Article X.X.3 of all disease-specific chapters to state more clearly the heat treatment required (i.e., core temperature and time period). These requirements would be based on those determined when assessments were originally undertaken against the criteria for safety of aquatic animal products in accordance with Article 5.4.1.

The Commission noted that it was not possible to propose a model Article X.X.3 because of differences in time/temperature treatments as well as products in this article between disease-specific chapters. Therefore, the Commission proposed to present an example article to Members to demonstrate the suggested approach. The Commission proposed to present Article 9.8.3 of Chapter 9.8, Infection with white spot syndrome virus, as the example article for Member comments. After considering Member comments, the Commission will review this example at its February 2021 meeting. It will then circulate, for each disease-specific chapter, the amended Articles X.X.3 for Member comments.

The Commission proposed the following amendments to point 1 of Article 3:

- the introductory (chapeau) text was edited to remove repetitions and improve readability;
- the reference to Article X.X.2 was deleted as this article has no relevance to the safety of an aquatic animal product but rather sets the scope of the chapter;
- reference to Article 5.4.1 was added to make clearer that the aquatic animal products listed have been assessed as meeting criteria for safety in accordance with Article 5.4.1;
- the approach to presenting heat-treated safe commodities was changed by describing the minimum core temperature/time treatment required to inactivate the pathogenic agent, as well as the type of products that would comply. This change accounts for product-related variables such as pre-treatment temperature and product size;
- 'hermetically sealed' was replaced by 'canned or retorted' to specify more clearly the type of product that has been hermetically sealed. The Commission explained that a common form of packaging of food products is retorted, and it means 'heated in an unopened hermetically sealed container for a time, and to a temperature, by superheated steam under pressure. Retorted goods may be in cans, jars or pouches'.

The Commission considered that points 2 and 3 of Article X.X.3 do not relate directly to the safety of aquatic animal products, but rather provide cross references to other guidance on risk management, either within or outside the disease-specific chapters. The Commission agreed to delete these two points, as they considered this text as general guidance rather than specific recommendations.

Additionally, the Commission noted that the assessments against the criteria for safety had been completed in 2009, and that scientific evidence for commodity safety published since that time should be reviewed and considered in a revised assessment. This work would be included in the Commission's work plan for completion later. Until that time, existing assessments will continue to be used as the basis for the temperature treatments provided in Article X.X.3 of disease-specific chapters.

Revisions to Article 9.8.3 of Chapter 9.8, Infection with white spot syndrome virus, are presented as Annex 10 as an example article for Member comments.

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

The Aquatic Animals Commission reviewed the report of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases, which 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 *Bonamia ostreae* (see also Annex 11).

The Commission agreed to amend the list of susceptible species in Article 11.3.2 in line with recommendations made by the *ad hoc* Group. It noted 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 would 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 Code* were amended in line with the recommendations of the *ad hoc* Group as described in Item 5.7.

The report of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases is presented as Annex 11 for Member's information.

The revised Articles 11.3.1 and 11.3.2 of Chapter 11.3, Infection with *Bonamia ostreae*, are presented as Annex 11 for Member comments.

5. TEXT FOR MEMBER COMMENTS: OIE *AQUATIC ANIMAL HEALTH 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, at its meeting in September 2019, 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 would be indicated in the usual style (i.e., strikethrough for deletions and double underline for additions).

A software-generated document that compares the adopted version of a chapter and the proposed new text will be created. This comparison document will not be included in the Commission's report, but will be available upon request from the OIE Standards Department (standards.dept@oie.int).

5.1. Status of *Aquatic Manual* revisions

The Aquatic Animals Commission examined the status of chapters that had previously been identified for substantial revision. The adoption of the three fish disease and a new chapter on Infection with *Batrachochytrium salamandrivorans* had been postponed to 2021. Two updated fish disease chapters had been circulated with the February 2020 report, and first drafts of another two fish disease chapters had been submitted for review at this meeting. All the updated chapters have been revised using the new template.

The remaining three chapters on listed diseases of fish are expected to be available for the Commission's review at its meeting in February 2021. The Commission wished to acknowledge the substantial contributions of Reference Laboratory experts in assisting with the comprehensive revision of *Aquatic Manual* chapters.

The Commission noted, with appreciation, the OIE Director General's decision to appoint a technical editor to assist the Commission with the substantial task of revising all disease-specific chapters of the *Aquatic Manual* into the new template. The Commission recognised that the successful candidate, Dr Mark Crane, brought substantial experience to this role and would be able to assist the Commission in its endeavour to apply a high level of scientific rigour and consistency to the revision of *Aquatic Manual* chapters.

The Commission wished to assure Members that all comments that had been received on the chapters listed below will be considered prior to their finalisation and proposal for adoption at the May 2021 General Session:

- Infection with *Batrachochytrium salamandrivorans* (Chapter 2.1.3) – Comments were received from New Zealand, Switzerland, Thailand, the UK, and the EU.
- Infection with spring viraemia of carp virus (Chapter 2.3.9) – Comments were received from Australia, Canada, New Zealand, Singapore, Switzerland, Thailand, the UK and the EU.
- Infection with infectious haematopoietic necrosis virus (Chapter 2.3.4) – Comments were received from Canada, China (People's Rep. of), Japan, New Zealand and the EU.
- Infection with viral haemorrhagic septicaemia virus (Chapter 2.3.10) – Comments were received from Canada, Japan, Korea (Rep. of), New Zealand and the EU.

5.2. Chapter 2.3.3 Infection with *Gyrodactylus salaris*

Comments were received from Australia, Canada, China (People's Rep. of), Chinese Taipei, the EU and the UK.

Previous Commission reports where this item was discussed:

February 2020 (Item 8.3.1, page 21).

September 2020 meeting

The Aquatic Animals Commission amended the wording of the scope of the chapter so that it aligns with the scope given in the *Aquatic Code* chapter on Infection with *Gyrodactylus salaris*.

The order of Sections 2.1.2 and 2.1.3 was reversed, and the title of Section 2.1.2 was left unchanged to make the chapter consistent with the template.

The Commission did not agree to a request to change 'none known' to 'under study' in Section 2.2.2, Species with incomplete evidence for susceptibility. The criterion for susceptibility is whether the parasite replicated on the host at a level that resulted in persistence at a population level, a binary outcome. Therefore 'none known' is accurate. For the same reason, the Commission also modified the text in Section 2.2.3, Non-susceptible species, from 'under study' to 'None known'.

In response to a request to delete the statement 'there is no evidence that they [any fish species] are important in the epidemiology of *G. salaris*' from Section 2.2.7, Vectors, the Commission stressed that the statement is based on the absence of published literature and amended the sentence accordingly.

The text in Section 2.3.2, Clinical signs, including behavioural changes, was amended to improve the clarity of the description and to include mention of susceptible species other than Atlantic salmon.

The Commission decided to remove the words 'and management' from the title of Section 2.3.5, so that it is now 'Environmental factors'. Information on disease management was moved to Section 2.4.7, General husbandry. This amendment will be made horizontally to all *Aquatic Manual* chapters and to the template. The Commission also agreed to add a sentence and reference to Section 2.3.5 on the relation between water temperature and reproduction of the parasite.

Section 2.3.6, Geographical distribution, was shortened, and mention of countries where *G. salaris* has never been detected was removed to achieve consistency with other chapters and the template.

The Commission reviewed the three sections 2.4.4, Breeding resistant strains, 2.4.5, Inactivation methods, and 2.4.7, General husbandry, and redistributed the text into the appropriate section.

The Commission decided to remove the word 'fixed' from the titles of Section 3.5.3, Fixed samples for histopathology, immunohistochemistry or in-situ hybridisation, and 3.5.4, Fixed samples for electron microscopy. This amendment will be made horizontally to all *Aquatic Manual* chapters and to the template.

The Commission moved the last paragraph from Section 3.2, Selection of organs or tissues, to a new Section 4.1, Parasite detection, where it fitted better.

The Commission did not agree with a proposal to delete the text in Section 3.6, Pooling of samples, as it considered the information to be useful and best placed in this section.

In response to a Member comment on clades/haplotypes, the Commission agreed to the proposal to include a list of haplotypes of the species *G. thymalli* and *G. salaris* and accession numbers to support species identification. The Reference Laboratory expert has tabulated accessions by species (*G. salaris* or *G. thymalli*), which are included in the chapter. The Reference Laboratory will maintain an up to date database of accessions and should be invited to confirm significant detections *G. salaris* and *G. thymalli.*

Finally, the Commission did not agree with a proposed change in Section 6.2.1, Definition of suspect case in clinically affected animals, preferring to keep each criterion listed in this section as a single independent criterion rather than grouping two or more together.

The revised Chapter 2.3.3, Infection with *Gyrodactylus salaris*, is presented as Annex 12 for Member comments.

5.3. Chapter 2.3.6 Infection with salmonid alphavirus

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

Previous Commission reports where this item was discussed:

February 2020 (Item 8.3.2, page 21).

September 2020 meeting

In the Section 2.1.1, Aetiological agent, the Aquatic Animals Commission removed a sentence that mentions the pathological characteristics of the disease as the information is provided elsewhere. The Commission agreed to delete Table 2.1. SAV genotypes by susceptible host species and environment, and to include the information as text.

In response to a request from a Member, the Commission added data on virus survival and water temperatures in Section 2.1.3, Survival and stability outside the host.

The Commission agreed to tabulate the list of susceptible host species in Section 2.2.1, Susceptible host species, and to include a column on genotype. The Commission also agreed to tabulate the list of species with incomplete evidence for susceptibility in Section 2.2.2.

In Section 2.2.5, Distribution of the pathogen in the host, the Commission agreed to include brain, but did not agree to include skeletal muscle as according to the reference provided, positive cells in muscle are seen only at one timepoint/day during clinical experiments.

In the second paragraph of Section 2.3.4, Modes of transmission and life cycle, the Commission found that the correct year of publication for the article written by Stene *et al.* was 2016 and amended the reference list accordingly.

As for Chapter 2.3.3, Infection with *Gyrodactylus salaris*, the Commission decided to remove the words 'and management' from the title of Section 2.3.5, so that it is now 'Environmental factors'. Information on disease management was moved to Section 2.4.7, General husbandry. This amendment will be made horizontally to all *Aquatic Manual* chapters and to the template.

The Commission did not agree to a proposal to list countries that have reported the infection in Section 2.3.6, Geographical distribution, as the text would need to be changed every time the disease is reported in a new country. The Commission reiterated its preference to include a link to the OIE WAHIS platform for recent information on distribution of infection with SAV at the country level.

The Commission added new details to Section 2.4.5, Inactivation methods, for clarity.

As for Chapter 2.3.3, Infection with *Gyrodactylus salaris*, the Commission decided to remove the word 'fixed' from the titles of Section 3.5.3, Fixed samples for histopathology, immunohistochemistry or in-situ hybridisation, and 3.5.4, Fixed samples for electron microscopy. This amendment will be made horizontally to all *Aquatic Manual* chapters and to the template.

In reply to a Member question on the low ratings of virus isolation and neutralisation in section A and B of 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 stressed that the neutralisation test has low sensitivity in the early stages of the disease, and that there are practical considerations when sampling from small fish. The Commission did not agree to the request to leave amplicon sequencing as the only confirmation tool. Although the requirement for sequencing

for confirmation of the first detection in a country is sound, sequencing is not necessary for confirmation in later detections, and confirmation should not be limited to sequencing alone.

In Section 6.1.2, Definition of confirmed case in apparently healthy animals, the Commission did not agree to include the immunofluorescence ELISA in point i) as this test is not given in the Table 4.1.

The revised Chapter 2.3.6, Infection with salmonid alphavirus, is presented as Annex 13 for Member comments.

5.4. Chapter 2.3.5 Infection with HPR-deleted or HPR0 infectious salmon anaemia virus

The Aquatic Animals Commission reviewed Chapter 2.3.5, Infection with HPR-deleted or HPR0 infectious salmon anaemia virus, which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include: updated lists of susceptible host species and species with incomplete evidence for susceptibility, and the inclusion of non-susceptible species, in accordance with the findings of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases (https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/); updated information on the distribution of the pathogen in the host; updated sections on disease pattern, biosecurity and disease control strategies, and specimen selection, sample collection, transportation and handling; and revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals.

The revised Chapter 2.3.5, Infection with HPR-deleted or HPR0 infectious salmon anaemia virus, is presented as Annex 14 for Member comments.

5.5. Chapter 2.3.7 Infection with koi herpesvirus

The Aquatic Animals Commission reviewed Chapter 2.3.7, Infection with koi herpesvirus (KHV), which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The chapter now recognises that infection with KHV includes all genotypes of cyprinid herpesvirus-3 (CyHV-3). The Commission agreed that the disease name 'infection with koi herpesvirus' should be retained and used in the *Aquatic Code* and *Aquatic Manual* for reasons of continuity and familiarity. CyHV-3, the virus name recognised by the ICTV is, however, referred to in Section 1 of the chapter. This is a similar approach used for other listed diseases where the official pathogen name may be relatively unfamiliar.

The main amendments to the chapter include: a new list of non-susceptible species in accordance with the findings of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases; updated sections on disease pattern, and biosecurity and disease control strategies; removal of text from Section 4 Diagnostic methods that is included in Chapter 2.3.0, General information [diseases of fish]; a revised section on conventional and real-time PCR to reduce the number of protocols described; and revised definitions of suspect and confirmed cases in apparently healthy and clinically affected animals.

The revised Chapter 2.3.7, Infection with koi herpesvirus, is presented as Annex 15 for Member comments.

5.6. Chapter 2.3.0 General information (diseases of fish)

At its February 2020 meeting, the Aquatic Animals Commission identified the need to update the introductory chapters on general information for each host species group (i.e., amphibians, crustaceans, fish and molluscs). Chapter 2.3.0, General information [for fish diseases], is the first of these chapters to be updated. All the OIE Reference Laboratories for diseases of fish were invited to contribute to the revision.

The revised Chapter 2.3.0, General information [for fish diseases], is presented as Annex 16 for Member comments.

5.7. Sections 2.2.1, 2.2.2 and a new 2.2.3bis of Chapter 2.4.3 Infection with *Bonamia ostreae*

The Aquatic Animals Commission amended Sections 2.2.1 and 2.2.2 and developed a new Section 2.2.3bis of Chapter 2.4.3, Infection with *Bonamia ostreae,* in line with the recommendations of *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases as described in Item 4.8.

The report of the *ad hoc* Group is presented as Annex 11 for Member's information.

The amended Sections 2.2.1, 2.2.2 and the new Section 2.2.3bis of Chapter 2.4.3, Infection with *Bonamia ostreae*, are presented as Annex 17 for Member comments.

6. TEXTS FOR MEMBER INFORMATION

6.1. New draft chapters on emergency disease preparedness and disease outbreak management

Background

The Aquatic Animals Commission commenced work on two new chapters for the revised Section 4, Chapter 4.X, Emergency disease preparedness, and Chapter 4.Y, Disease outbreak management, at its February 2020 meeting. The Commission agreed to define the article structure for both chapters, given how closely the two chapters are linked, and to review this work at its September 2020 meeting.

The Commission wished to remind Members that these two new chapters are part of the ongoing redevelopment of Section 4 which commenced with the development and adoption of Chapter 4.3, Disinfection of Aquaculture Establishments and Equipment. The second phase of this work has been the development of the new Chapter 4.X, Biosecurity of Aquaculture Establishments, which is presented at Item 4.1 of this report.

Previous Commission reports where this item was discussed:

February 2020 (Item 7.3.2, page 16).

September 2020 meeting

The Commission continued its work to develop the article structure for the two new chapters, Chapter 4.X, Emergency disease preparedness, and Chapter 4.Y, Disease outbreak management, and agreed that further work was required to ensure a logical and complementary structure of the two chapters before undertaking detailed drafting. The Commission decided that members of the Commission would continue this work and provide an update at the Commission's February 2021 meeting.

The previously agreed approach to the redevelopment of Section 4 of the *Aquatic Code* is presented as Annex 18 for Member's information.

6.2. Approaches for demonstrating disease freedom

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

Background

A discussion paper on approaches for determining periods required to demonstrate disease freedom, developed by the Aquatic Animals Commission, was first circulated for comments in the Commission's September 2018 report. The Commission considered comments received and circulated a revised discussion paper in its September 2019 report, and presented model Articles X.X.4, X.X.5 and X.X.6 for the disease-specific chapters of the *Aquatic Code* for Member comments in its February 2020 report.

Previous Commission reports where this item was discussed:

September 2018 report (Item 2.10, page 11); September 2019 report (Item 6.6, page 9); February 2020 report (Item 7.2.2, page 15).

September 2020 meeting

The Commission considered all comments received and agreed that response to these comments, including the revised Chapter 1.4., Aquatic animal health surveillance, and the model Articles X.X.4, X.X.5 and X.X.6, will be shared with Members in the February 2021 report.

6.3. Consideration of emerging diseases - Infection with carp edema virus (CEV)

Comments were received from Japan and the UK.

Background

The Aquatic Animals Commission reviewed scientific information on infection with carp edema virus (CEV) at its February 2020 meeting, given that the disease had been reported recently in several countries in the Asia-Pacific region and appears to be extending its geographic range. The Commission noted that the disease was listed as a notifiable disease by the Network of Aquaculture Centres in Asia-Pacific (NACA) in 2017 and that it has been reported to significantly impact carp production, which is the largest fish production in the world.

Based on available scientific information, the Commission agreed in February 2020 that infection with CEV meets the OIE definition of an 'emerging disease' and, as such, Members should report it in accordance with Article 1.1.4 of the *Aquatic Code*. The Commission also encouraged Members to investigate mortality and morbidity events linked to this disease, emphasising that a better understanding of the virus is essential for efforts to control its possible spread. Members were also encouraged to submit information on their experiences with infection with CEV and its impacts to the Commission.

Previous Commission reports where this item was discussed:

February 2020 report (Item 7.3.3, page 16).

September 2020 meeting

The Commission considered the comments received, including advice from some countries that infection with CEV had already emerged within their countries and that it has been detected for some years. The Commission agreed that it would continue to monitor the situation with CEV and requested that countries report infection with CEV as an emerging disease in accordance with Article 1.1.4 of the *Aquatic Code*.

6.4. The use of environmental DNA methods for aquatic animal disease surveillance

The Aquatic Animals Commission prioritised other agenda items at this meeting and decided to work on the discussion paper on guidance for the use of environmental DNA methods for aquatic animal disease surveillance at its February 2021 meeting.

7. OIE REFERENCE CENTRES OR CHANGE OF EXPERT

7.1. Evaluation of applications for OIE Reference Centres for Aquatic Animal Health issues or change of experts

The Delegate of the Member concerned had submitted to the OIE the following nomination for changes of experts at OIE Reference Laboratories. The Aquatic Animals Commission recommended their acceptance:

Infection with infectious salmon anaemia virus
Dr Ole Bendik Dale to replace Dr Knut Falk at the Norwegian Veterinary Institute, Oslo, NORWAY

Infection with hypodermal and haematopoietic necrosis virus
Dr Bing Yang to replace Dr Jie Huang at the Maricultural Organism Disease Control and Molecular Pathology Laboratory, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong, CHINA (PEOPLE'S REP. OF)

Infection with white spot syndrome virus
Dr Qingli Zhang to replace Dr Jie Huang at the Maricultural Organism Disease Control and Molecular Pathology Laboratory, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong, CHINA (PEOPLE'S REP. OF).

The Commission wished to convey its appreciation for the contributions of Dr Jie Huang and Dr Knut Falk in their roles as OIE Reference Laboratory experts.

7.2. Follow-up February meeting: feedback from the Laboratories that are not complying with the key ToR according to their 2019 annual report

The Aquatic Animals Commission reviewed additional information provided by an OIE Reference Laboratory following queries the Commission raised on the laboratory's 2019 annual report. The Commission appreciated and accepted the laboratory's response which included an action plan to address key Terms of Reference.

7.3. Review of the 5-year work plans received from Collaborating Centres

The Aquatic Animals Commission reviewed the 5-year work plans from two of the Collaborating Centres. The Commission was impressed by the range of activities and their relevance to the OIE Aquatic Animal Health Strategy. For one of the Centres, the Commission requested more information on international collaboration, and specific details in the work plan including time frames for activities. The Commission encouraged the OIE Headquarters to consider ways to make the activities of the Collaborating Centres more widely known to Members, for example through articles in the OIE *Bulletin*, the OIE social media tools, or webinars.

8. DATE OF NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 17–24 February 2021.

MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 26 August to 2 September 2020

List of participants

MEMBERS OF THE COMMISSION

Dr Ingo Ernst
(President)
Director Aquatic Pest and Health Policy
Animal Division
Department of Agriculture, Water and the Environment
GPO Box 858 Canberra ACT 2601
AUSTRALIA
Tel.: +61 2 6272 5615
ingo.ernst@awe.gov.au

Dr Kevin William Christison
Department of Agriculture Forestry and Fisheries
Directorate: Aquaculture Research and Development
Private Bag X 2V
Vlaeberg, 8018
SOUTH AFRICA
kevinCH@daff.gov.za

Dr Alicia Gallardo Lagno
(Vice-President)
Subdirectora nacional de acuicultura
Servicio Nacional de Pesca y Acuicultura
Calle Victoria 2832
CHILE
Tel.: +56 32 281 9282
agallardol@sernapesca.cl

Dr Atle Lillehaug
Head of Section
Section for Fish Health and Biosecurity
Norwegian Veterinary Institute
Ullevålsveien 68, 0454 Oslo
Pb 750 Sentrum, N-0106 Oslo
NORWAY
atle.lillehaug@vetinst.no

Dr Prof. Hong Liu
Deputy Director
Animal and Plant Inspection and Quarantine Technical Center
Shenzhen Customs District
General Administration of Customs,
1011 building of Fuqiang Road
Futianqu, Shenzhen City, Guangdong province
CHINA (People's Rep of)
szc_liuhong@customs.gov.cn
709274714@qq.com

Dr Edmund Peeler
(Vice-President)
Principal epidemiologist
CEFAS
Barrack Road, Weymouth
Dorset, DT4 8UB
UNITED KINGDOM
Tel.: +44 (0)1305 206746
ed.peeler@cefas.co.uk

OTHER PARTICIPANTS

Dr Mark Crane
Senior Principal Research Scientist
AAHL Fish Diseases Laboratory
CSIRO Australian Animal Health Laboratory
5 Portarlington Road Geelong
VIC 3220
AUSTRALIA
Tel.: +61 3 5227 5118
mark.crane@csiro.au

Dr Chang Siow Foong
Group Director
Professional & Scientific Services
Animal and Veterinary Service
Ministry of National Development
SINGAPORE
chang_siow_foong@nparks.gov.sg

OIE HEADQUARTERS

Dr Gillian Mylrea
Head
Standards Department
g.mylrea@oie.int

Dr Gounalan Pavade
Chargé de mission
Science Department
g.pavade@oie.int

Dr Stian Johnsen
Chargé de mission
Standards Department
s.johnsen@oie.int

Ms Sara Linnane
Scientific editor
Science Department
s.linnane@oie.int

Ms Elizabeth Marier
Chargée de mission
Standards Department
e.marier@oie.int

WORK PLAN FOR THE AQUATIC ANIMALS COMMISSION

AQUATIC CODE		
Chapter/Subject	**Activity**	**Status**
Items for review at the February 2021 meeting – horizontal chapters		
Glossary definitions of ‘Aquatic animal waste’ and ‘vector’	Propose a new definition of ‘Aquatic animal waste’. Revise the definition of ‘Vector’.	February 2021 – review Member comments. The item is expected to be proposed for adoption in May 2021.
Glossary definitions of ‘Competent Authority’, ‘Veterinary Authority’ and ‘Aquatic Animal Health Services’	Revise the definitions in the *Aquatic Code* to harmonise with the *Terrestrial Code.*	February 2021 – review Member comments.
Listing of infection with decapod iridescent virus 1 (DIV1) – Revised Article 1.3.3 of Chapter 1.3	Assess infection with DIV1 against the criteria for listing aquatic animal diseases in Article 1.2.2.	The disease has been found to meet the criteria for listing. February 2021 – review Member comments. The item is expected to be proposed for adoption in May 2021.
De-listing of infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)	Assess infection with IHHNV against the criteria for listing aquatic animal diseases in Article 1.2.2.	September 2020 – The Commission concluded that the disease continues to meet the criteria for listing. February 2021 – review Member comments.
Listing of infection with tilapia lake virus	Assess the disease against the criteria for listing aquatic animal diseases in Article 1.2.2.	February 2021 – review the final report of the *ad hoc* Group on tilapia lake virus (if circumstances allow completion of its work) and revise the assessment against the criteria for listing.
New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X)	Develop a new draft chapter on Biosecurity for aquaculture establishments.	February 2021 – review Member comments. The item is expected to be proposed for adoption in May 2021.
New draft chapters on emergency disease preparedness and disease outbreak management	Develop two new chapters for the revised Section 4, Chapter 4.X, Emergency disease preparedness, and Chapter 4.Y, Disease outbreak management.	September 2020 - work to develop the article structure for the two new chapters. February 2021 – finalise the structure to ensure a logical and complementary structure of the two chapters before undertaking detailed drafting.
Approaches for demonstrating disease freedom	Make improvements to the standards of the *Aquatic Code* for demonstration of freedom from OIE listed diseases, including: 1) Articles X.X.4. (free country) and X.X.5. (free zone or compartment) of each disease-specific chapter (except Infection with ISAV, for which numbering differs); 2) Chapter 1.4 on Aquatic animal health surveillance.	February 2021 – respond to Member comments received on the Commission’s February 2020 meeting report and present the revised Chapter 1.4, Aquatic animal health surveillance, and the model Articles X.X.4, X.X.5 and X.X.6 for Member comments.
Consideration of emerging diseases - Infection with carp edema virus (CEV)	Review scientific information on the disease and assess it against the criteria for listing aquatic animal diseases in Article 1.2.2.	The disease has been defined as an emerging disease by the Commission and, as such, Members should report it in accordance with Article 1.1.4 of the *Aquatic Code*. February 2021 – review new scientific information and information received by Members.

Items for review at the February 2021 meeting – disease-specific chapters		
Safe commodities (Article X.X.3 of disease-specific chapters)	Review the structure of this article to provide more clarity on the recommendations on safe commodities provided in these articles.	February 2021 – review Member comments.
Model Article 10.X.13 for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4)	Revision of the article to clarify the intended purpose.	February 2021 – review Member comments. The item is expected to be proposed for adoption in May 2021.
Article 10.10.2 of Chapter 10.10 Infection with viral haemorrhagic septicaemia virus	Update list of susceptible species in Article 10.10.2.	February 2021 – review Member comments. The item is expected to be proposed for adoption in May 2021.
Articles 11.3.1 and 11.3.2 of Chapter 11.3 Infection with *Bonamia ostreae*	Update Article 11.3.1 and the list of susceptible species in Article 11.3.2.	February 2021 – review Member comments.
AQUATIC MANUAL		
Chapter/Subject	**Activity**	**Status**
Items for review at the February 2021 meeting		
General provisions	Review and update the introductory chapters for fish diseases.	September 2020 – review revised chapters. February 2021 – review Member comments.
	The use of environmental DNA methods for aquatic animal disease surveillance.	February 2021 – Develop a discussion paper on guidance for the use of environmental DNA methods for aquatic animal disease.
Update and reformat disease chapters using the new template	Infection with *Batrachochytrium salamandrivorans* (Chapter 2.1.3).	February 2021 – expected to be proposed for adoption in May 2021.
	Infection with spring viraemia of carp virus (Chapter 2.3.9).	February 2021 – expected to be proposed for adoption in May 2021.
	Infection with infectious haematopoietic necrosis virus (Chapter 2.3.4).	February 2021 – expected to be proposed for adoption in May 2021.
	Infection with viral haemorrhagic septicaemia virus (Chapter 2.3.10).	February 2021 – expected to be proposed for adoption in May 2021.
	Infection with *Gyrodactylus salaris* (Chapter 2.3.3).	February 2021 – review Member comments. Expected to be proposed for adoption in May 2021.
	Infection with salmonid alphavirus (Chapter 2.3.6).	February 2021 – review Member comments. Expected to be proposed for adoption in May 2021.
	Infection with HPR-deleted or HPR0 infectious salmon anaemia virus (Chapter 2.3.5).	February 2021 – review Member comments.
	Infection with koi herpesvirus (Chapter 2.3.7)	February 2021 – review Member comments.
	Infection with epizootic haematopoietic necrosis virus (Chapter 2.3.1)	February 2021 – draft updated and reformatted chapter for Member comments.
	Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome) (Chapter 2.3.2)	February 2021 – draft updated and reformatted chapter for Member comments.
	Red sea bream iridoviral disease (Chapter 2.3.8)	February 2021 – draft updated and reformatted chapter for Member comments.
Sections 2.2.1, 2.2.2 and a new 2.2.3bis of Chapter 2.4.3 Infection with *Bonamia ostreae*	Amend Sections 2.2.1 and 2.2.2 and developed a new Section 2.2.3bis of Chapter 2.4.3, Infection with *Bonamia ostreae* in line with the recommendations of the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases	February 2021 – review Member comments.

Return to Agenda

New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X)

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* ~~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,~~ 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 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 physical and management measures to mitigate the identified risks according to the circumstances of the *aquaculture establishment*. *Aquaculture establishment* personnel ~~sStaff~~, ~~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*), 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 *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 *aquatic animal* farmedspecies, 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.

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

32) 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.

43) *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.

54) 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.

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

676) A routine review schedule for routine reviews and audits of the *biosecurity plan* should be described. and identified tTriggers 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 in Article 4.X.8.

Article 4.X.5.

Categories of aquaculture production systems

Aquatic animals can be produced in fFour 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 systems, 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* from *aquaculture establishments* to 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 for finfish and suspended baskets 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 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 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 *pathogenic agent* 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 animal*s intentionally ~~brought~~ introduced into, or moved within, an *aquaculture establishment*~~, or moved within it,~~ may include broodstock, 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 *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* 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, 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 following the guidance 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 provides 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* 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.*

cd) 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* may be initially infected with *pathogenic agents* or 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 feeds*. 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).

The *risk* of transmitting *pathogenic agents* via *aquatic animal feed* can should be assessed, and managed by mitigation measures as described provided 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 eEquipment 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* 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 *pathogenic agents* to susceptible *aquatic animals* in *aquaculture establishments*. These They 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 *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 *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;

   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 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.

cb) 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.

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* (see Chapter 2.1.). This article elaborates the principles in Chapter 2.1. and applies them for 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 Iidentification

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 Aassessment

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 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 impact.
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

		Consequence rating				
		insignificant	minor	moderate	major	catastrophic
Likelihood estimate	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, 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 *Rrisk* 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~~ pathways (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 separationmethods), 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;

hg) 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*;

ih) monitoring and audit schedule;

ji) 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) 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) 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) Health monitoring

Health monitoring as part of the *biosecurity plan* involves monitoring of the health status of *aquatic animals* in *aquaculture establishments*. 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) 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*.

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CHAPTER 1.3.

DISEASES LISTED BY THE OIE

[…]

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

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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.,* 2017), giant freshwater prawn (*Macrobrachium rosenbergii*) (Qiu *et al*., 2019a), red swamp crayfish (*Procambarus clarkii*) (Qiu *et al*., 2019a), oriental river prawn (*Macrobrachium nipponense*) (Qiu *et al*., 2019a), ridgetail white prawn (*Exopalaemon carinicauda*) and giant tiger prawn *Penaeus monodon* (OIE, 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). 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, gills, hepatopancreas, periopods and muscle (Qiu *et al*., 2017a). 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) and Chinese Taipei but the geographic distribution of the virus may be wider if mortality events have not 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 nested PCR method (Qiu *et al.,* 2017a), a TaqMan probe based real-time PCR (TaqMan qPCR) method (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) have been published and are available for DIV1 detection. The PCR primers and TaqMan probe have been shown to be specific for DIV1 (no cross-reaction with other shrimp pathogens), with a low detection limit (4 copies per reaction) and high diagnostic sensitivity and diagnostic specificity (95.3% and 99.2%, respectively). Validation of the nested PCR method and 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 2 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). Targeted surveillance in China in 2017 and 2018 detected DIV1 in 11 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). 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. 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 THE FISH DISEASE-SPECIFIC CHAPTERS 10.5, 10.6 AND 10.10 (AND ARTICLE 10.4.17 FOR CHAPTER 10.4)

[…]

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.

[…]

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CHAPTER 10.10.

INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

[…]

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~~
Cyprinidae	*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~~
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~~

Family	Scientific name	Common name	~~Genotype~~
	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~~
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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GLOSSARY

AQUATIC ANIMAL WASTE

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 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 *pathogenic agent.*

VECTOR (CLEAN VERSION)

means any living organism that has been demonstrated to transfer a pathogenic agent to a population of susceptible species. Susceptible species of a pathogenic agent are not considered as vectors for that pathogenic agent.

Proposed amendments of the term 'waste' to '*aquatic animal waste*' in the *Aquatic Code*

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 waste*~~s~~ 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: ~~*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.~~ High risk waste means *aquatic animal* ~~waste~~ *waste* that constitutes, or is suspected of constituting, a serious health risk to *aquatic animals* or humans. 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*. […]: 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*; 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. […] 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. *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*. […]. Containers used for transport of *aquatic animal* ~~waste~~ *waste* should be leak-proof and labelled regarding content. […]
4.7.6.	72	1. Requirement for approval All disposal plants dealing with *aquatic animal* ~~waste~~ *waste* should be approved by the *Competent Authority*. […]

		2. Conditions for approval For a disposal plant to be approved to deal with *aquatic animal* ~~waste~~ *waste*, it should: […] d) fulfil requirements for handling the *aquatic animal waste* and products specified by the *Competent Authority*. […] 3. Operating requirements […] c) handling and treatment of *aquatic animal* ~~waste~~ *waste* should take place as soon as possible after being received; […]
4.7.7.	73	1. Rendering […] 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. […] 2. Incineration […] Mobile air curtain incinerators enable the process to be carried out on site thus removing the need to transport the *aquatic animal waste*. Incinerators may only be capable of handling limited volumes of *aquatic animal* ~~waste~~ *waste*.
4.7.7.	74	6. Ensiling […] 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 […] Whenever possible, the *aquatic animal* ~~waste~~ *waste* should be subjected to a treatment that ensures inactivation of the *pathogenic agents* prior to burial. In selecting an acceptable burial site, consideration should be given to the following: […] b) Access – easy access for equipment and delivery of *aquatic animal* ~~waste~~ *waste*. Fencing and restricted admittance may be necessary. c) Pit construction – […]Pit dimensions depend on the volume of the *aquatic animal* ~~waste~~ *waste* to be buried and should be easy to fill. 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. 8. Pyre-burning Pyre-burning may not be suitable for large amounts of *aquatic animal* ~~waste~~ *waste*. […] b) Access – for equipment to construct the pyre and maintain the fire, for the delivery of fuel and *aquatic animal waste*. […] If the pyre-burning is carried out correctly, *aquatic animal* ~~wastes~~ *waste* will be destroyed within 48 hours.
4.7.8.	75	1. Ensiling 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*. […] 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** […] EITHER 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*; OR 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 […] - data on trends and occurrence of resistant microorganisms obtained through *surveillance* of *aquatic animals* and *aquatic animal products* and ~~waste~~ *aquatic animal waste* ~~products~~. 4. Exposure assessment […] - 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~~; […]

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GLOSSARY CONTINUED

AQUATIC ANIMAL HEALTH SERVICES

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

AQUATIC ANIMAL HEALTH SERVICES (CLEAN VERSION)

means the combination of governmental and non-governmental individuals and organisations that perform activities to implement the standards of the *Aquatic Code*.

COMPETENT AUTHORITY

means the a *Veterinary Authority* or other Governmental Authority of a Member Country having the responsibility and competence for ensuring or supervising the implementation of *aquatic animal* health and welfare measures, international health certification and other in the whole or part of the territory for the implementation of certain standards and recommendations in of the *Aquatic Code* in the whole territory.

COMPETENT AUTHORITY (CLEAN VERSION)

means a Governmental Authority of a Member Country having the responsibility in the whole or part of the territory for the implementation of certain standards of the *Aquatic Code*.

VETERINARY AUTHORITY

means the Governmental Authority of a Member Country, comprising *veterinarians*, other professionals and paraprofessionals, having the primary responsibility and competence for ensuring or supervising in the whole territory for coordinating the implementation of *aquatic animal* health and welfare measures, international *aquatic animal* health certification and other the standards and recommendations in of the *Aquatic Code* by *Competent Authorities* in the whole *territory*. The *Veterinary Authority* is a *Competent Authority*.

VETERINARY AUTHORITY (CLEAN VERSION)

means the Governmental Authority of a Member Country having the primary responsibility in the whole territory for coordinating the implementation of the standards of the *Aquatic Code* by *Competent Authorities*. The *Veterinary Authority* is a *Competent Authority*.

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ASSESSMENT OF THE INFECTION WITH INFECTIOUS HYPODERMAL AND HEMATOPOIETIC NECROSIS VIRUS (IHHNV) FOR DELISTING IN THE *AQUATIC CODE*

Overall Assessment

The Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) assessed infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code*, and agreed that infection with IHHNV meets the listing criteria 1, 2, 3, and 4b (see Table 1 below), and should, therefore, remain listed in Article 1.3.3.

Table 1. Summary of assessment of infection with IHHNV

	Listing criteria						Conclusion
	1	2	3	4a	4b	4c	
IHHN	+	+	+	NA	+	-	The disease meets the criteria for listing

NA = not applicable.

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

1. International spread of the pathogenic agent (via aquatic animals, aquatic animal products, vectors or fomites) is likely.

AND

2. At least one country may demonstrate country or zone freedom from the disease in susceptible aquatic animals, based on provisions of Chapter 1.4.

AND

3. A precise case definition is available and a reliable means of detection and diagnosis exists.

AND

4a. Natural transmission to humans has been proven, and human infection is associated with severe consequences.

OR

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.

OR

4c. The disease has been shown to, or scientific evidence indicates that it would affect the health of wild resulting in significant consequences e.g. morbidity or mortality at a population level, reduced productivity or ecological impacts.

Note

In this assessment the term ‘shrimp’ is used for both marine and freshwater species, however, where the term prawn is used in common names of species, e.g. giant tiger prawn, it has been retained.

Background

The first case of hypodermal and haematopoietic necrosis was reported in Hawaii in 1981, where it had caused mass mortalities in blue shrimp (*Penaeus stylirostris*) farmed in super-intensive raceways (Lightner *et al.*, 1983). Later it was discovered in *P. stylirostris* and white leg shrimp (*Penaeus vannamei)* in America and the Gulf of

California (Morales-Covarrubias *et al.*, 1999; Pantoja *et al.*, 1999). Some reports suggested that it might have contributed to the collapse of the *P. stylirostris* fishery in the Gulf of California. IHHNV has also been identified as the cause of 'runt deformity syndrome' (RDS) in *P. vannamei*.

IHHNV is classified with the subfamily Densovirinae of the virus family Parvoviridae. It was listed by the OIE in 1995. IHHNV is the smallest of the known penaeid shrimp viruses (the virion is a 20–22 nm, non-enveloped icosahedron). At least two distinct genotypes of IHHNV have been identified: type 1 from the Americas and East Asia (principally the Philippines) and type 2 from South-East Asia. Two sequences homologous to part of the IHHNV genome are found embedded in the genome of penaeids. The virus is widespread in shrimp production in Asia and Latin America.

Susceptible species listed by the OIE are: yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*P. stylirostris*), and white leg shrimp (*P. vannamei*). Northern brown shrimp (*Penaeus aztecus*) has incomplete evidence for susceptibility. Several other species have tested PCR positive, but an active infection has not been demonstrated.

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

Assessment

Marine and freshwater shrimp farming is currently carried out around the globe in at least 60 countries with production about 4,496,775 metric tons (MT) in 2018. The production is mostly concentrated in 15 nations in Asia and Latin America, including China (People's Rep. of), Indonesia, Vietnam, India, Ecuador, Thailand, Mexico, Bangladesh, Philippines, Brazil, Saudi Arabia, Iran (Islamic Republic of), Malaysia, Honduras and Peru (FAO, 2020; GAA, www.aquaculturealliance.org). In 2018, shrimp exports accounted for approximately 15 percent of the total global trade in aquatic animal products by value. Shrimps have historically been one of the most heavily traded aquatic animal products, with major markets located in the United States of America, the European Union and Japan. The China (People's Rep. of) is becoming a new rapid growing market (FAO, 2020).

Transmission of IHHNV can be horizontal or vertical. Horizontal transmission via ingestion of infected tissues or by contaminated water has been demonstrated, as has vertical transmission via contaminated eggs (OIE, 2019).

International trade in species susceptible to IHHNV includes live animals such as shrimp larvae and broodstock, and frozen shrimp products. Trade in these products provides pathways for international spread of IHHNV. Some examples demonstrating international spread, or presence of IHHNV in traded commodities are summarised below.

In 2019, the UK found IHHNV positive cases in imported *P. vannamei* broodstock at two indoor shrimp farms. At one site, no clinical signs or mortality were observed, but at the other site variable growth rates and stunting were observed. The detections were reported to the OIE. The affected animals were imported as free from IHHNV and other pathogens, i.e. they were sold as specific pathogen-free (SPF) post larval shrimp.

In 2019, Canada detected IHHNV in four premises in imported *P. vannamei* without clinical signs and mortality. The detections were reported to the OIE.

In 2015, 329 samples of *P. monodon* imported to China were tested, and 36.8% samples tested positive for IHHNV (Yu *et al.*, 2016). In 2019, samples of frozen *P. vannamei* imported to South Korea were tested and 40% of batches tested positive for IHHNV (Park *et al.*, 2020).

Conclusion

The criterion is met.

Criterion No. 2 At least one country may demonstrate country or *zone* freedom from the *disease* in susceptible *aquatic animals*, based on provisions of Chapter 1.4.

Assessment

New Caledonia self-declared freedom from IHHNV in 2016. The UK has two shrimp farms both of which became infected with IHHNV in 2019 but which have re-established with IHHNV free stock, and the UK is in a position to demonstrate freedom.

OIE WAHIS data demonstrates that IHHNV occurs in most shrimp producing countries, as shown in the following table. However, countries in the Middle East that are currently producing shrimp (e.g. Saudi Arabia and Iran), or commencing shrimp production (e.g. Oman) may be in a position to claim freedom from IHHNV. Other important shrimp producers, such as Madagascar and Bangladesh have not reported the occurrence of IHHNV.

Table 1. Reporting of IHHNV by country and year (taken from WAHIS)

Region or Country	2016	2017	2018	2019	2020
Africa					
Europe					
UK				**2**	
America					
Brazil	+..	+..	+..	+..	
Canada				**1**	
Costa Rica	**3**	**5**			
Ecuador	**38**	**96**	**111**	**31**	
Guatemala	**2**	**2**			
Honduras	**34**	**72**			
Mexico	**346**	**176**	**237**	**516**	
Nicaragua	**37**	**21**	**31**	**37**	
Peru	**5**	**15**			
El Salvador				**6**	
USA				**4**	
Asia					
China (People's Rep. of)		**64**	**69**	**40**	
Chinese Taipei	**26**	**7**	**1**		
India		**12**	**3**	**3**	
Indonesia	**14**	**7**	**4**		
Thailand	**4**	**8**	**2**	**6**	
Philippines	+..	+..	+..	+..	
Oceania					
Australia		**2**	**3**	**6**	

Note: the top 15 shrimp producing countries are China (People's Rep. of), Indonesia, Vietnam, India, Ecuador, Thailand, Mexico, Bangladesh, Philippines, Brazil, Saudi Arabia, Iran (Islamic Republic of), Malaysia, Honduras and Peru.

Conclusion

The criterion is met.

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

Assessment

Case definitions for suspicion and confirmation of infection with IHHNV have been developed by the OIE. Reliable conventional PCR (Tang *et al.*, 2007) and real-time PCR assays have been developed for the detection of IHHNV (Dhar *et al.*, 2001).

In recent years, some rapid tests have been developed, such as loop-mediated isothermal amplification (LAMP), modified PCR, recombinase polymerase amplification (RPA) and real-time PCR with higher sensitivity (Cowley *et al.*, 2018; Qian *et al.*, 2018; Xia *et al.*, 2015; Arunrut *et al.*, 2011). These tests have demonstrated utility and could be recommended in the OIE *Aquatic Manual* pending further validation in accordance with the OIE standards.

Conclusion

Criterion is met.

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

Assessment

There is no evidence of transmission to humans.

Conclusion

Criterion not applicable.

Criterion No. 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.

Assessment

Infection with IHHNV is known to have most severe impact in penaeids native to the Americas, *P. stylirostris* and *P. vannamei*. The disease has been reported to be most severe in *P. stylirostris* resulting in high mortality. In *P. vannamei*, infection with IHHNV is known to cause runting and deformities, resulting in significantly reduced crop value (Lightner *et al.*, 1996; Lightner *et al.*, 2011). Of the major commercial species, the disease has been considered to have least impact on *P. monodon* (Withyachumnarknkul *et al.*, 2006).

IHHNV was first described by Lightner *et al.* (1983) who reported mortalities of up to 90% in *P. stylirostris* post-larvae and juveniles. Subsequently, other studies have shown that in populations of *P. stylirostris*, infection with IHHNV results in an acute disease with high mortalities approaching 100% (Lightner *et al.* 1996). IHHNV outbreaks in farmed *P. stylirostris* caused such severe levels of mortality that some farms in Mexico closed permanently while others shifted to cultivating *P. vannamei* (Pantoja *et al.*, 1999). Although the impacts of IHHNV on *P. stylirostris* production are known to have been historically severe, domesticated populations of *P. stylirostris* have been developed which are considered to be tolerant to infection (Tang *et al.*, 2000).

Infection with IHHNV in populations of *P. vannamei* have resulted in a more subtle, chronic disease in which mortalities may not be significant, but where animals show cuticular deformities and reduced, highly disparate growth – a condition known as runt deformity syndrome (RDS) (Kalagayan *et al.*, 1991). Growth retardation has been reported to be greater than 30% (Wyban *et al.*, 1992, cited by Hsieh *et al.*, 2006) and runted animals have lower economic value resulting in significant economic loss (Kalagayan *et al.*, 1991). Infection with IHHNV also interferes with normal egg, larval, and post-larval development (Motte *et al.*, 2003).

The impacts of IHHNV appear to have declined due to the use of specific (i.e. IHHNV) pathogen free shrimp, changing to cultivation of less susceptible species and the breeding of more IHHNV-tolerant shrimp. However, several recent examples demonstrate that IHHNV continues to affect the health of cultured aquatic animals and results in significant production losses. Some of these examples are highlighted below.

In 2019, IHHNV positive cases were detected in imported *P. vannamei* broodstock at two indoor shrimp farms in the UK. At one of these sites, variable growth rates and stunting were observed. The farms were depopulated and decontaminated.

In surveillance of Indian *P. vannamei* farms from 2013 to 2018, 30 farms were found to be positive for IHHNV (Jagadeesan *et al.,* 2019). Animals at these farms exhibited classical IHHNV cuticular deformities and a wide size variation in growth in the affected farms.

Considerable differences in susceptibility to IHHNV infection were found in three batches of *P. vannamei* from different hatcheries in Northern Mexico. The results indicate varying levels of IHHNV resistance in farmed populations, although possible impacts on productivity were not explored (Escobedo-Bonilla *et al.*, 2014).

A recent study in Australia found an association between sustained presence of high level IHHNV infection with reduced growth performance and survival of *P. monodon* reared under simulated commercial conditions (Sellars *et al.*, 2019).

Conclusion

Criterion is met.

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

Assessment

IHHNV was detected in farmed *P. stylirostris* and *P. vannamei* in Mexico in the late 1980s and was later detected in wild *P. stylirostris* populations in the Gulf of California (Morales Covarrubias *et al.*, 1999). The detection of IHHNV in wild *P. stylirostris* coincided with declines in fishery landings of up to 50% and it has been suggested that IHHNV contributed to the collapse of the fishery (Morales Covarrubias *et al.*, 1999; Pantoja *et al.*, 1999). Further sampling in 1996 demonstrated high IHHNV prevalence; however, wild populations were recovering (Morales Covarrubias *et al.*, 1999).

IHHNV has been detected in wild populations of other crustacean species. High prevalence of IHHNV was found in wild *P. vannamei* from the Pacific coast of Panama, Ecuador, Colombia and Panama (Nunan *et al.*, 2001; Motte *et al.*, 2003). In the Pacific coast of Mexico, IHHNV was detected in wild shrimp and crabs with 19.5% prevalence rate (Macías-rodríguez *et al.*, 2014). In the East China Sea, IHHNV was detected in wild *P. penicillatus* and at a prevalence of 19.2% in wild *P. vannamei* (Hu, 2015).

Although IHHNV is thought to have impacted wild populations of *P. stylirostris*, definitive evidence of a causative role is not available. However, it is well known that demonstrating the impact of diseases on wild populations of aquatic animals is difficult, except in the most extreme examples where observable mortality occurs (Miller *et al.*, 2014).

Conclusion

Criterion is not met.

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EXAMPLE ARTICLE X.X.3 FOR ALL DISEASE-SPECIFIC CHAPTERS OF THE *AQUATIC CODE* (TRACK CHANGES VERSION)

CHAPTER 9.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

[…]

Article 9.8.3.

Measures for the ~~I~~importation or transit of aquatic animal products for any purpose regardless of the infection with WSSV status of the exporting country, zone or compartment

1) The following *aquatic animal products* have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of the *aquatic animal products* listed below, *Competent Authorities* should not require any sanitary measures ~~conditions~~ related to WSSV, regardless of the infection with WSSV status of the *exporting country*, *zone* or *compartment*.~~: when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 9.8.2. that are intended for any purpose and comply with Article 5.4.1.:~~

   a) cooked, canned, pasteurised or retorted *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least one minute (or a time/temperature equivalent that has been demonstrated to inactivate WSSV).

   ~~a) heat sterilised hermetically sealed crustacean products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate WSSV);~~

   ~~b) cooked crustacean products that have been subjected to heat treatment at 60°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate WSSV);~~

   ~~c) pasteurised crustacean products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate WSSV);~~

   ~~d)~~b) crustacean oil;

   ~~e)~~c) crustacean *meal*;

   ~~f)~~d) chemically extracted chitin.

~~2. When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 9.8.2., other than those referred to in point 1 of Article 9.8.3., *Competent Authorities* should require the~~ conditions ~~prescribed in Articles 9.8.7. to 9.8.12. relevant to the infection with WSSV status of the *exporting country*, *zone* or *compartment*.~~

~~3. When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 9.8.2. but which could reasonably be expected to pose a risk of transmission of WSSV, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis.~~

[…]

EXAMPLE ARTICLE X.X.3 FOR THE ALL DISEASE-SPECIFIC CHAPTERS OF THE *AQUATIC CODE* (CLEAN VERSION)

CHAPTER 9.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

[…]

Article 9.8.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with WSSV status of the exporting country, zone or compartment

1) The following *aquatic animal products* have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of the *aquatic animal products* listed below, *Competent Authorities* should not require any sanitary measures related to WSSV, regardless of the infection with WSSV status of the *exporting country*, *zone* or *compartment*:

   a) cooked, canned, pasteurised or retorted *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least one minute (or a time/temperature equivalent that has been demonstrated to inactivate WSSV);

   b) crustacean oil;

   c) crustacean *meal*;

   d) chemically extracted chitin.

[…]

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CHAPTER 11.3

INFECTION WITH BONAMIA OSTREAE

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.

Original: English
Jannuary–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) OR 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) OR PCR-RFLP (as described in Cochennec *et al*., 2003) OR 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. ISH is currently not sufficiently specific to resolve species level identifications. For early studies without molecular information, corroborating evidence from later studies was considered. 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:** Histopathology OR Cytology (usually gill or heart imprint or haemolymph smears) OR *In-situ* hybridization (ISH) OR TEM OR 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 OR 3) Demonstration of viability of cells isolated from tissues **by:** Flow cytometry OR Vital stains OR Successful infection of uninfected animals by inoculation	Mortality OR <u>Macroscopic lesions</u> such as - Discolouration of tissue - Gill ulceration OR Rapid loss of condition OR <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	Inconclu sive[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 *Ostrea edulis* 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

List of participants

MEMBERS OF THE *AD HOC* GROUP

Dr Isabelle Arzul (Chair)
IFREMER
Laboratoire de Génétique Aquaculture et Pathologie de Mollusques Marins
17390 La Tremblade
FRANCE
Tel: +33 5 46 76 2610
iarzul@ifremer.fr
isabelle.arzul@ifremer.fr

Dr Robert Adlard
Marine Biodiversity at Queensland Museum Network
PO Box 3300, South Brisbane BC
Queensland 4101
AUSTRALIA
robert.adlard@qm.qld.gov.au

Dr Changming Bai
Yellow Sea Fisheries Research Institute, CAFS
Division of Maricultural Organism Disease control and Molecular Pathology
No. 106 Nanjing Road
Qingdao, 266071
CHINA
baicm@ysfri.ac.cn

Dr Lori Gustafson
Surveillance Design and Analysis
USDA/APHIS/VS/CEAH
2150 Centre Ave, Bldg B
Mail Stop 2E6
Fort Collins, CO 80526-8117
UNITED STATES
lori.l.gustafson@aphis.usda.gov

Dr Karin B. Lohrmann
Departamento de Biología Marina
Facultad de Ciencias del Mar,
Universidad Católica del Norte,
Larrondo 1281, Coquimbo
CHILE
klohrman@ucn.cl

REPRESENTATIVE OF THE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Dr Kevin William Christison
Department of Agriculture Forestry and Fisheries
Directorate: Aquaculture Research and Development
Private Bag X 2V
Vlaeberg, 8018
SOUTH AFRICA
KevinCH@daff.gov.za

OIE HEADQUARTERS

Jeannine Fischer
Chargée de mission
Standards Department
j.fischer@oie.int

Dr Stian Johnsen
Chargé de mission
Standards Department
s.johnsen@oie.int

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

January–June 2020

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.

Return to the Agenda

CHAPTER 2.3.3.

INFECTION WITH *GYRODACTYLUS SALARIS*

1. Scope

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

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 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. 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 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.

2.2.5. 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. 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. Vectors

Gyrodactylus salaris parasites may attach themselves to species not considered susceptible species, for short periods of time. Thus, any fish species could act as a vector, however, there is no evidence from the published literature that they are important in the epidemiology of *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). 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 infections 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). *Gyrodactylus salaris* can survive longer in higher salinities at lower temperatures (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.*, 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 and by a commonly used oxidising disinfectant (Koski *et al.,* 2016) 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 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). 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 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. 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 fish host. Material from parasites should not be pooled for molecular diagnostics.

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]												
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 opisthaptor (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 opisthaptoral hard parts in *Gyrodactylus* varies extensively with, for example, temperature, whereas shape is more stable (see e.g. Mo, 1991a). 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 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 other species than *G. salaris/G. thymalli*, and the method is therefore mentioned briefly here. Conventional PCR and sequencing of the mitochondrial cytochrome oxidase I 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.

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 were reported as ranging from 93.1% to 101.1% and the limit of detection (dilution) 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 for species confirmation (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. 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) 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
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 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 COI 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

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 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* are 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.

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[9] For example, transboundary commodities.

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	–	–

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study;

7. References

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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*.

NB: FIRST ADOPTED IN 1997 AS GYRODACTYLOSIS OF ATLANTIC SALMON (*GYRODACTYLUS* F);
MOST RECENT UPDATES ADOPTED IN 2018.

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CHAPTER 2.3.6.

INFECTION WITH SALMONID ALPHAVIRUS

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. 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 2 and SAV 3 have been detected in rainbow trout.

2.2.5. 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. 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. 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 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 content~~s~~ is a usual post-mortem finding, typically seen in inappettant 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 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, 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,~~ The heart should be sampled from vaccinated fish without opening the abdominal cavity. Sampling of mid-kidney, spleen or other internal organs is not recommended, to avoid contamination of 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. However, no validated methods are currently available.

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

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.

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. 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 been evaluated (Hall *et al.*,2014). The results suggest that the use of individual samples rather than pools is 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~~
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 are commonly used for primary SAV isolation, but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used. Variation in cell line susceptibility among different SAV field isolates has been reported (Graham *et al.,* 2008; Herath *et al.,* 2009), and it is therefore recommended that several cell lines are tested for initial cell culture isolation of SAV in a new laboratory or for a new virus strain. 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 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 for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]) 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 qPCR 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. *Primer~~s~~ 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’ QnsP1R: 5’-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3’ QnsP1probe: 5’FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3’ (Taqman®probe)	Real-time RT- PCR	QnsP1	107 nt	Hodneland *et al.*, 2006
E2F: 5’-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3’ E2R: 5’-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3’	RT-PCR	E2	516 nt	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 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 of 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.

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.*

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 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 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 immunoglobulin for 1 hour (or if the primary Ab is polyclonal from rabbits, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity of the test, FITC-conjugated anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with rinsing as in step d) in between the steps. The nuclei can be stained with propidium iodide (100 µg ml–1 in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in the dark until examination. For long periods of storage (more than 2–3 weeks) a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.10. 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 Cell lines. 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)~~,~~ 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. in this chapter.

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) 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~~

[11] For example, transboundary commodities.

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.

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) 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 are 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*

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

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 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

NB: FIRST ADOPTED IN 2014. MOST RECENT UPDATES ADOPTED IN 2019.

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CHAPTER 2.3.5.

INFECTION WITH HPR-DELETED OR HPR0 INFECTIOUS SALMON ANAEMIA VIRUS

1. Scope

Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR)-deleted ISAV, or the non-pathogenic HPR0 (non-deleted HPR) ISAV of the Genus *Isavirus* and Family *Orthomyxoviridae*.

HPR-deleted ISAV may cause disease in Atlantic salmon (*Salmo salar*), which is a generalised and lethal condition characterised by severe anaemia, and variable haemorrhages and necrosis in several organs.

Detection of HPR0 ISAV has never been associated with clinical signs of disease in Atlantic salmon (Christiansen *et al.,* 2011). A link between non-pathogenic HPR0 ISAV and pathogenic HPR-deleted ISAV has been suggested, with some disease outbreaks potentially occurring as a result of the emergence of HPR-deleted ISAV from HPR0 ISAV (Cardenas *et al.,* 2014; Christiansen *et al.,* 2017; Cunningham *et al*., 2002; Gagne & Leblanc, 2017; Mjaaland, *et al*., 2002).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

ISAV is an enveloped virus, 100–130 nm in diameter, however, there are studies that indicate greater size heterogeneity in cells of epithelial origin (Ramirez & Marshall, 2018). The virus genome consists of eight single-stranded RNA segments with negative polarity (Dannevig *et al.,* 1995). The virus has haemagglutinating, receptor-destroying and fusion activity (Falk *et al*., 1997; Mjaaland *et al*., 1997; Rimstad *et al*., 2011).

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

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas. According to sequence differences in a partial sequence of segment 6, two groups have been defined: one designated as a European clade and one designated as a North American clade (Gagne & LeBlanc, 2017). In the HE gene, a small HPR near the transmembrane domain has been identified. This region is characterised by the presence of gaps rather than single-nucleotide substitutions (Cunningham *et al*., 2002; Mjaaland *et al*., 2002). A full-length gene (HPR0) has been suggested to represent a precursor from which all ISAV HPR-deleted (pathogenic) variants of ISAV originate. The presence of non-pathogenic HPR0 ISAV genome has been reported in both apparently healthy wild and farmed Atlantic salmon, but has not been detected in fish with clinical disease and pathological signs consistent with infection with HPR-deleted ISAV (Christiansen *et al*., 2011; Cunningham *et al*., 2002; Markussen *et al*., 2008; McBeath *et al*., 2009). A mixed infection with HPR-deleted and HPR0 ISAV variants has been reported in the same fish (Cardenas *et al.,* 2014; Kibenge *et al.,* 2009). Recent studies show that HPR0 ISAV variants occur frequently in sea-reared Atlantic salmon.

HPR0 ISAV is seasonal and transient in nature and displays a tissue tropism with high prevalence in gills (Christiansen *et al.*, 2011; Lyngstad *et al.*, 2011). To date there has been no direct evidence linking the presence of HPR0 ISAV to a clinical disease outbreak. The risk of emergence of pathogenic HPR-deleted ISAV variants from a reservoir of HPR0 ISAV is considered to be low but not negligible (Cardenas *et al.,* 2014; Christiansen *et al.,* 2011; 2017; EFSA, 2012).

In addition to the variations seen in the HPR of the HE gene, other gene segments may also be of importance for development of clinical disease. A putative virulence marker has been identified in the fusion (F) protein. Here, a single amino acid substitution, or different sequence insertion, near the protein's putative cleavage site has been found to be a prerequisite for virulence (Kibenge *et al.,* 2007; Markussen *et al.*, 2008). Aside from insertion/recombination, ISAV also uses gene segment reassortment in its evolution, with potential links to virulence (Cardenas *et al.,* 2014; Devold *et al.*, 2006; Gagne & Leblanc, 2017; Markussen *et al.*, 2008; Mjaaland *et al.*, 2005).

2.1.2. Survival and stability in processed or stored samples

A scientific study concluded that ISAV retains infectivity for at least 6 months at –80°C in tissue homogenates (Smail & Grant, 2012). Isolation in cell culture has been successful even from fish kept frozen whole at –20°C for several years. The experience of diagnostic laboratories has indicated the suitability of general procedures for sample handling (see Chapter 2.3.0) for ISAV.

2.1.3. Survival and stability outside the host

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

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 ISAV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*).

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 ISAV according to Chapter 1.5 of the *Aquatic Code* are: Atlantic herring (*Clupea harengus*) and amago trout (*Oncorhynchus masou*).

In addition, pathogen-specific positive PCR results have been reported in the following species, but an active infection has not been demonstrated *in vivo*: Coho salmon (*Oncorhynchus kisutch*).

2.2.3. Non-susceptible species

Species that have been found to be non-susceptible to infection with ISAV according to Chapter 1.5. of the *Aquatic Code* are:

Family	Scientific name	Common name	Reference
Caligidae	*Caligus rogercresseyi*	sea lice	Ito *et al.*, 2015
Cyclopteridae	*Cyclopterus lumpus*	lumpfish	Ito *et al.*, 2015
Cyprinidae	*Cyprinus carpio*	common carp	Ito *et al.*, 2015
Gadidae	*Gadus morhua*	Atlantic cod	MacLean *et al.*, 2003; Snow & Raynard, 2005
	Pollachius virens	saithe	Snow *et al.*, 2002
	Pollachius virens	pollack	Ito *et al.*, 2015
Mytilidae	*Mytilus edulis*	blue mussel	Molloy *et al.*, 2014; Skar & Mortensen, 2007
Pleuronectidae	*Hippoglossus hippoglossus*	Atlantic halibut	Ito *et al.*, 2015
Salmonidae	*Onchorhynchus tshawytscha*	Chinook salmon	Rolland & Winton, 2003
	Carassius auratus	goldfish	Ito *et al.*, 2015

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

In Atlantic salmon, life stages from yolk sac fry to adults are known to be susceptible. Disease outbreaks are mainly reported in seawater cages, and only a few cases have been reported in the freshwater stage, including one case in yolk sac fry (Rimstad *et al*., 2011). Infection with HPR-deleted ISAV has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater.

2.2.5. Distribution of the pathogen in the host

There is evidence of the presence of the virus in practically all organs of the fish, as well as in ovarian fluids and ova (Marshall *et al*., 2014), however, the HPR0 variant has a predilection for gills.

HPR-deleted ISAV: Endothelial cells lining blood vessels seem to be the primary target cells for ISAV replication as demonstrated by electron microscopy, immunohistochemistry and *in-situ* hybridisation. Virus replication has also been demonstrated in leukocytes, and sinusoidal macrophages in kidney tissue stain positive for ISAV using immunohistochemistry (IHC). Furthermore, red blood cells may have virus aggregates on the outer cell membrane as indicated by IFAT with a monoclonal antibody (MAb) against the HE protein. As endothelial cells support replication and virus may be carried on red blood cells, virus may occur in any organ. Repeated sampling over the course of a chronic infection point to kidney and heart as the organs most likely to become test-positive. Clinical disease and macroscopic organ lesions appear foremost in severely anaemic Atlantic salmon (Aamelfot *et al.,* 2012; Rimstad *et al*., 2011).

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

HPR0 ISAV: As HPR0 ISAV has not been isolated in cell culture, controlled, experimental studies on virus distribution within the host are generally lacking. Observed tissue tropism was foremost in the gills when PCR testing was carried out on various organs of Atlantic salmon (Christiansen *et al*., 2011). *In-situ* immunostaining of HPR0 ISAV PCR-positive gills show staining limited to the epithelium indicating replication and shedding to water, rather than invasive infection. Immunostaining was unable to demonstrate HPR0 ISAV infection of internal organs.

2.2.6. Aquatic animal reservoirs of infection

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

2.2.7. Vectors

Transmission of ISAV by salmon lice and sea lice (*Lepeophtheirus salmonis and Caligus rogercresseyi* (Oelckers *et al.,* 2014) has been demonstrated under experimental conditions.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

During outbreaks of infection with HPR-deleted ISAV, morbidity and mortality may vary greatly between net pens in a seawater fish farm, and between farms. Morbidity and mortality within a net pen may start at very low levels, with typical daily mortality between 0.5 to 1% in affected cages. Without intervention, mortality increases and often peaks in early summer and winter. The range of cumulative mortality during an outbreak is generally insignificant to moderate, but in severe cases, lasting several months, cumulative mortality may exceed 90%. Initially, a clinical disease outbreak may be limited to one or two net pens. In such cases, if affected fish are slaughtered immediately, further development of clinical infection with HPR-deleted ISAV at the site may be prevented. In outbreaks where smolts have been infected in well boats, simultaneous outbreaks on several farms may occur.

HPR0 ISAV has not been associated with clinical disease in Atlantic salmon.

2.3.2. Clinical signs, including behavioural changes

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

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

Affected fish are generally in good condition, but diseased fish have no feed in the digestive tract.

2.3.3. Gross pathology

Fish infected with HPR-deleted ISAV may show a range of pathological changes, from none to severe, depending on factors such as infective dose, virus strain, temperature, age and immune status of the fish. No lesions are pathognomonic to infection with HPR-deleted ISAV, but anaemia and circulatory disturbances are always present. The following findings have been described to be consistent with infection with HPR-deleted ISAV, though all changes are seldom observed in a single fish: i) yellowish or blood-tinged fluid in peritoneal and pericardial cavities; ii) oedema of the swim bladder; iii) small haemorrhages of the visceral and parietal peritoneum; iv) focal or diffusely dark red liver (a thin fibrin layer may be present on the surface); v) swollen, dark red spleen with rounded margins; vi) dark redness of the intestinal wall mucosa in the blind sacs, mid- and hind-gut, without blood in the gut lumen of fresh specimens; vii) swollen, dark red kidney with blood and liquid effusing from cut surfaces; and viii) pinpoint haemorrhages of the skeletal muscle.

2.3.4. Modes of transmission and life cycle

The main route of infection is most likely through the gills for both HPR0 and HPR-deleted ISAV, but infection via the intestine or skin cannot be excluded.

ISAV may be shed in mucous, urine, faeces (Totland *et al.,* 1996), ovarian fluid and ova (Marshall *et al*., 2014), but shedding from localised gill infection may be most important.

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

2.3.5. Environmental factors

Generally, outbreaks of infection with HPR-deleted ISAV tend to be seasonal, occurring in early summer and winter; however, outbreaks can occur at any time of the year.

2.3.6. Geographical distribution

ISAV was initially reported in Norway in the mid-1980s (Thorud & Djupvik, 1988). It has since been reported in other countries in Europe, North America and South America. The presence of the HPR0 ISAV variant has been reported in all countries where infection with HPR-deleted ISAV has occurred. For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Vaccination against infection with ISAV has been carried out in North America since 1999 and the Faroe Islands since 2005. In Norway, vaccination is not normally done, but was carried out for the first time in 2009 in a region where outbreaks were associated with a high rate of infection with HPR-deleted ISAV. Chile started vaccinating against infection with ISAV in 2010. However, vaccine efficacy seems insufficient given all cases of both HPR0 and HPR-deleted ISAV that occurred in the Faroe Islands have occurred in vaccinated fish. The same lack of efficacy has been observed in Norway after vaccination around outbreak areas.

2.4.2. Chemotherapy including blocking agents

Chemotherapy is currently not available. However, the broad-spectrum antiviral drug Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is effective in inhibiting ISAV replication both *in vitro* and *in vivo* (Rivas-Aravena *et al.,* 2011). It should also be noted that interfering peptides have recently been shown to have a non-toxic antiviral effect against ISAV (Cardenas *et al.*, 2020).

2.4.3. Immunostimulation

Not applicable.

2.4.4. Breeding resistant strains

Differences in susceptibility among different family groups of Atlantic salmon in freshwater have been observed in challenge experiments and in field tests (Gjoen *et al*., 1997). Breeding companies are using infection trials, family selection and genomic selection to improve ISA resistance, but scientific information on the effect of this on disease incidence or prevalence of subclinical infection is lacking.

2.4.5. Inactivation methods

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

2.4.6. Disinfection of eggs and larvae

Disinfection of eggs according to standard procedures is suggested as an important control measure (see chapter 4.4 of the *Aquatic Code*).

2.4.7. General husbandry

The incidence of infection with ISAV may be greatly reduced by implementation of legislative measures or husbandry practices regarding the movement of fish, mandatory health control, transport and slaughterhouse regulations. Specific measures including restrictions on affected, suspected and neighbouring farms, enforced sanitary slaughtering, generation segregation ('all in/all out') as well as disinfection of offal and wastewater from fish slaughterhouses and fish processing plants may also contribute to reducing the incidence of the disease.

Handling of fish (e.g. sorting or treatment, splitting or moving of cages) may initiate disease outbreaks on infected farms, especially if long-term undiagnosed problems have been experienced (Lyngstad *et al*., 2008).

The experience from the Faroe Islands, where the prevalence of HPR0 ISAV is high, demostrates that the combination of good biosecurity and husbandry substantially reduces the risk of outbreaks of infection with HPR-deleted ISAV.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

For detection of HPR-deleted ISAV, fish displaying clinical signs, gross pathology and anaemia should be sampled.

For detection of HPR0 ISAV, randomly selected individuals should be sampled at different time points throughout the production cycle.

3.2. Selection of organs or tissues

3.2.1. Detection of HPR-deleted ISAV

Only internal organs that have not been exposed to the environment should be used for diagnostic testing.

The organs or tissue material to be sampled and examined must be: i) for histology: mid-kidney, liver, heart, pancreas, intestine, spleen and gill; ii) for immunohistochemistry: mid-kidney and heart including valves and bulbus arteriosus; iii) for conventional RT-PCR and real-time RT-PCR analysis: mid-kidney and heart; and iv) for virus culture: mid-kidney, heart, liver and spleen.

3.2.2. Detection of HPR0 ISAV

Gill tissue is recommended, however, HPR0 ISAV has also been detected in the mid-kidney and heart. It is, therefore, suggested to use pools of the three organs for detection purposes.

3.3. Samples or tissues not suitable for pathogen detection

Information on samples or tissues not suitable for pathogen detection is lacking; follow recommendations in Section 3.2 for virus detection.

3.4. Non-lethal sampling

Blood is preferred for non-lethal sampling based on a study by Giray *et al*. (2005) in which blood and mucus was compared with kidney samples derived from both clinical and non-clinical fish and tested by RT-PCR and virus isolation in cell culture.

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

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. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of 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 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen. Commercial RNA preservatives are available, such as RNAlater, which have better efficacy than ethanol at room temperature. Commercial fixatives validated to be at least as effective as the fixatives described above may be used.

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

Tissue samples for histopathology should be fixed immediately after collection. Gills need to be fixed immediately after euthanasia. Thickness of tissues for fixation must not exceed 4–5 mm. The recommended ratio of fixative to tissue is 10:1, and neutral, phosphate-buffered, 10% formalin is recommended as this fixative is compatible with the immunohistochemistry procedure for ISAV.

3.5.4. Samples for electron microscopy

ISAV has been characterised by transmission electron microscopy (TEM) using general procedures (Falk *et al*., 1997).

3.5.5. Samples for other tests

At present, other tests, for example serology tests, are not used for diagnostic purposes.

3.6. Pooling of samples

Data are available regarding the effect of pooling samples on the detection of ISAV that indicate the effects are related to the prevalence of the disease in the fish population (Hall *et al*., 2013; 2014). Small life stages such as fry or specimens up to 0.5 g can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

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
Gross signs					+	+	+	1				
Histopathology[3]					++	++	++	1				
Cell or artificial media culture					++	++	++	1	+++	+++	+++	NA
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	3				
Conventional RT-PCR	+	+	+	1	++	++	++	1	+	+	+	NA
Amplicon sequencing[4]									+++	+++	+++	NA
In-situ hybridisation												
Immunohistochemistry					++	++	++	1	++	++	++	NA
IFAT on kidney imprints or blood					++	++	++	1	+++	+++	+++	NA
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods[5]												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not applicable; RT-PCR = reverse-transcription polymerase chain reaction; 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 2.2.4.
[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. Histopathology and cytopathology

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

i) Numerous erythrocytes in the central venous sinus and lamellar capillaries where erythrocyte thrombi also form in the gills.

ii) Multifocal to confluent haemorrhages and/or hepatocyte necrosis at some distance from larger vessels in the liver. Focal accumulations of erythrocytes in dilated hepatic sinusoids.

iii) Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria.

iv) Spleen stroma distended by erythrocyte accumulation.

v) Slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney.

vi) Erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

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

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

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

4.3. Cell or artificial media culture for isolation

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

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

For virus isolation, cells grown in 25 cm^2 tissue culture flasks or multi-well cell culture plates, which may be sealed with parafilm or a plate sealer to stabilise the pH of the medium, may be used. Cells grown in 24-well plates may not grow very well into monolayers, but this trait may vary between laboratories and according to the type of cell culture plates used. Serially diluted HPR-deleted ISAV-positive controls should be inoculated in parallel with the tissue samples as a test for cell susceptibility to HPR-deleted ISAV (this should be performed in a separate location from that of the test samples). See Chapter 2.3.0 for the methods used for inoculation of cell monolayers, monitoring the cultures and sub-cultivation.

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

Cell lines should be monitored to ensure that their susceptibility to targeted pathogens has not changed.

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

The primers and probes shown in Table 4.4.1 for real-time RT-PCR will detect both European and North-American HPR-deleted ISAV and HPR0 ISAV. Real-time RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.2) and is recommended over RT-PCR (see Section 4.4.2.) as it has increased specificity and, probably, also sensitivity. The primer sets derived from genomic segment 8 and segment 7 have been used by several laboratories and have been found suitable for detection of ISAV during disease outbreaks and in apparently healthy carrier fish.

With the widespread occurrence of HPR0 ISAV variants, it is essential to follow up any positive PCR results based on segment 7 or 8 primer sets by sequencing the HPR of segment 6 in order to determine if the isolate is either HPR-deleted or HPR0 ISAV or both. Primers, designed and validated by the OIE Reference Laboratory, are given in Table 4.4.2. Validation of the HPR primer set for the North American HPR0 isolates is restricted by the limited sequence data available in the Genbank for the 3' end of ISAV segment 6.

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

Table 4.4.1. *Primer and probes sequences and cycling conditions for ISAV real-time RT-PCR*

Primer and probe sequences (5'–>3') (concentration)	**Cycling conditions**	**Genomic segment**	**Amplicon size (bp)**	**Reference**
For: CAG-GGT-TGT-ATC-CAT-GGT-TGA-AAT-G (900nM) Rev: GTC-CAG-CCC-TAA-GCT-CAA-CTC- (900nM) Probe: *6FAM*-CTC-TCT-CAT-TGT-GAT-CCC-*MGBNFQ* (250nM)	1 × 2 minutes @ 50°C 1 × 10 minutes @ 95°C 45 × 15 seconds @ 95°C and 1 minute @ 60°C	7	155	Snow *et al.*, 2006
For: CTA-CAC-AGC-AGG-ATG-CAG-ATG-T (900 nM) Rev: CAG-GAT-GCC-GGA-AGT-CGA-T (900 nM) Probe: *6FAM*-CAT-CGT-CGC-TGC-AGT-TC-*MGBNFQ* (250 nM)		8	104	Snow *et al.*, 2006

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control. The positive control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results.

4.4.2. Conventional PCR

The primers described in Table 4.4.2 for RT-PCR will detect both European and North-American HPR-deleted ISAV and HPR0 ISAV. RT-PCR may be used for detection of ISAV from total RNA (or total nucleic acid) extracted from recommended organs/tissues (see Section 3.2). However, the real-time RT-PCR (see Section 4.4.1.) for the detection of ISAV is recommended as it has increased specificity and, probably, also sensitivity.

Table 4.4.2. *Primer sequences and cycling conditions for ISAV Segment 6 RT-PCR*

Primer sequences (5'–>3') (concentration)	**Cycling conditions**	**Amplicon size (bp)**	**Reference**
For: GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA (200 nM) Rev: GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG-TA (200 nM)	1 × 30 minutes @ 50°C 1 × 2 minutes @ 94°C 40 × 1 minute @ 94°C, 1 minute @ 50°C, 1 minute @ 68°C 1 × 7 minutes @ 68°C	304 if HPR0	Designed by OIE Ref. Lab.

With the widespread occurrence of HPR0 ISAV variants, it is essential to follow up any positive PCR results based on segment 7 or 8 primer sets by sequencing the HPR of segment 6 in order to determine if the isolate is either HPR-deleted or HPR0 ISAV or both. Primers, designed and validated by the OIE Reference Laboratory, are given in Table 4.4.2. Validation of the HPR primer set for the North-American

HPR0 isolates is restricted by the limited sequence data available in the Genbank for the 3' end of ISAV segment 6.

The primers for segment 7 and 8 may also be used for conventional RT-PCR if necessary.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control. The positive control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results.

4.5. Amplicon sequencing

There is evidence of the generation of complete amplicons for the eight segments of the viral genome that include the 5' and 3' ends of each one (Toro-Ascuy *et al*., 2015).

The segment 6 assay primers given in Section 4.4.2 are used for PCR and amplicon sequencing.

4.6. *In-situ* hybridisation

Published methods are available but not recommended due to lack of validation.

4.7. Immunohistochemistry

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

i) Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene or isopropanol and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for IHC sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for at least 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for pathomorphology and IHC as described below.

ii) Staining procedure for IHC

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

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

b) Sections are then incubated overnight at 4°C with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein) diluted in TBS with 1% non-fat dry milk, followed by three washes in TBS, the last wash with 0.1% Tween 20.

c) For detection of bound antibodies, sections are incubated with biotinylated goat anti rabbit IgG (diluted 1/200 in 2.5% BSA in Tris) for 60 minutes, followed by ABC-AP (diluted 1/100 in Tris) for 45 minutes. Following a final wash, Fast Red (1 mg ml–1) and Naphthol AS-MX phosphate (0.2 mg ml^{-1}) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) are added to develop for 20 minutes. Sections are then washed in tap water before counterstaining with Harris haematoxylin and mounted in aqueous mounting medium. ISAV positive and ISAV negative tissue sections are included as controls in every setup.

iii) Interpretation

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

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

4.7.1. Indirect fluorescent antibody test on tissue smears

An indirect fluorescent antibody test (IFAT) using validated MAbs against ISAV haemagglutinin-esterase (HE) on kidney smears (imprints), on blood or on frozen tissue sections of kidney, heart and liver has given positive reactions in both experimentally and naturally infected Atlantic salmon. Suspect cases (see Section 6.1) may be confirmed with a positive IFAT.

i) Preparations of tissue smears (imprints)

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

ii) Staining procedure

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

iii) Preparation of blood smear (imprint)

Blood fraction is obtain using a discontinuous Percoll gradient. A small fraction is smeared on poly-L-lysine-coated microscope slide. The imprint is air-dried, fixed in chilled 100% acetone for 10 minutes and stored either at 4°C for a few days or at –80°C until use.

iv) Staining procedure

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

4.8. Bioassay

Not available.

4.9. Antibody- or antigen-based detection methods

Virus identification by IFAT

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

i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well or 24-well plates), in slide flasks or on cover-slips dependent on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). SHK-1 cells grow rather poorly on glass cover-slips. The necessary monolayers for negative and positive controls must be included.

ii) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 7 days or, if CPE appears, for a shorter time.

iii) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. The fixed cell cultures may be stored dry for less than 1 week at 4°C or at –20°C for longer storage.

iv) Incubate the cell monolayers with anti-HPR-deleted ISAV MAb in an appropriate dilution in PBS for 1 hour, and rinse twice with PBS/0.05% Tween 20. If non-specific binding is observed, incubate with PBS containing 0.5% dry skimmed milk.

v) Incubate with FITC-conjugated goat anti-mouse immunoglobulin for 1 hour (or if antibody raised in rabbits is used as the primary antibody, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity, FITC-conjugated goat anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with the described rinsing in between the additional step. Rinse once with PBS/0.05% Tween 20, as described above. The nuclei can be stained with propidium iodide (100 µg ml^{-1} in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in the dark until examination. For long periods of storage (more than 2–3 weeks a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.10. Other methods

None published or validated.

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

Real-time RT-PCR is validated for surveillance to demonstrate freedom in apparently healthy populations.

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[12]

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 HPR0 or HPR-deleted ISAV shall be suspected if at least one of the following criteria is met:

i) ISAV-typical CPE in cell cultures (HPR-deleted only)

ii) Positive result by conventional RT-PCR

iii) Positive result by real-time RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

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.

Definition of confirmed case of infection with HPR-deleted ISAV

[12] For example: transboundary commodities.

The presence of infection with HPR-deleted ISAV is considered to be confirmed if, in addition to the criteria in Section 6.1.1, one or more of the following criteria are met:

i) ISAV-typical CPE in ASK cell culture and virus identification by by conventional RT-PCR and sequencing of the HE-gene to verify HPR-deletion

ii) Detection of ISAV in tissue preparations by conventional RT-PCR and detection of ISAV in histological sections by immunoassay using specific anti-ISAV antibodies (IFAT or immunohistochemistry)

iii) Detection of ISAV in tissue preparations by real time RT-PCR and detection of ISAV in tissue preparations by conventional PCR followed by sequencing of the HE-gene to verify HPR-deletion

iv) Detection of ISAV in tissue preparations by real time RT-PCR and detection of ISAV in histological sections by immunoassay using specific anti-ISAV antibodies (IFAT or immunohistochemistry)

v) Detection of ISAV in tissue preparations by real-time RT-PCR and ISAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon

vi) Detection of ISAV in tissue preparations by conventional PCR followed by sequencing of the amplicon

Definition of confirmed case of infection with HPR0 ISAV

The presence of infection with HPR0 ISAV is considered to be confirmed if the following criterion is met:

i) Detection of ISAV by conventional RT-PCR followed by amplification and sequencing of the HPR region of segment 6

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 HPR-deleted ISAV 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

ii) Histo- or cytopathological changes consistent with the presence of the pathogen or the disease

iii) ISAV-typical CPE in ASK cell culture

iv) Positive result by a real-time RT-PCR

v) Positive result of a conventional RT-PCR

vi) Positive result by immunohistochemistry

vii) Positive result by IFAT tissue imprints

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with HPR-deleted ISAV is considered to be confirmed if one or more of the following criteria is met:

i) ISAV-typical CPE in ASK cell culture and virus identification by conventional RT-PCR and sequencing of the HE-gene to verify HPR-deletion

ii) Detection of ISAV in tissue preparations by conventional RT-PCR and detection of ISAV in histological sections by immunoassay using specific anti-ISAV antibodies (IFAT or immunohistochemistry)

iii) Detection of ISAV in tissue preparations by real-time RT-PCR, followed by conventional RT-PCR and sequencing of the HE-gene to verify HPR-deletion

iv) Detection of ISAV in tissue preparations by real-time RT-PCR and detection of ISAV in tissue preparations by means of specific antibodies against ISAV (IFAT or immunohistochemistry)

v) Detection of ISAV in tissue preparations by real-time RT-PCR and ISAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon

vi) Detection of ISAV in tissue preparations by conventional 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: under study

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with ISAV are provided in Table 6.3. This information can be used for the design of surveys for infection with ISAV, 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.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction; NA = not available.

6.3.2. For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.

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

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS SALMON ANAEMIA; MOST RECENT UPDATES ADOPTED IN 2018.

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CHAPTER 2.3.7.

INFECTION WITH KOI HERPESVIRUS

1. Scope

Infection with koi herpesvirus means infection with all genotypes of the pathogenic agent cyprinid herpesvirus-3 (CyHV-3), of the Genus *Cyprinivirus* in the Family *Alloherpesviridae* (Haramoto *et al*., 2007; Waltzek *et al.,* 2009).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Koi herpesvirus (KHV), also known as carp interstitial nephritis and gill necrosis virus (CNGV) (Ilouze *et al.,* 2010), has been classified as cyprinid herpesvirus-3 (CyHV-3) following the nomenclature of other cyprinid herpesviruses: CyHV-1 (carp pox virus, fish papilloma virus) and CyHV-2 (goldfish haematopoietic necrosis virus). Analysis of the complete genome has shown that CyHV-3 is closely related to CyHV-1, CyHV-2, anguillid herpesvirus-1 (AngHV-1) and distantly related to channel catfish virus (Ictalurid herpesvirus: IcHV-1) and Ranid (frog) herpesvirus (RaHV-1) (Waltzek *et al.,* 2005). CyHV-3 was designated the type species of the new *Cyprinivirus* genus within the *Alloherpesviridae* family, that also contains CyHV-1 and CyHV-2. However, the designation KHV has been retained in the *Aquatic Code* and *Aquatic Manual* for reasons of continuity and is used here synonymously with CyHV-3.

Early estimates of the genome size of KHV varied from at least 150 kbp to 277 kbp; the size is now confirmed as 295 kbp. Virus nucleocapsids have been measured at 100–110 nm in diameter and are surrounded by an envelope (review: Ilouze *et al.,* 2010). Aoki *et al.* (2007) initially described the complete genome sequence of three isolates of CyHV-3 and 156 unique protein-coding genes. They suggested that the finding that 15 KHV genes are homologous with genes in IcHV-1 confirms the proposed place of KHV in the family Herpesviridae. Forty viral proteins and 18 cellular proteins are incorporated into mature virions.

2.1.2. Survival and stability in processed or stored samples

No information available.

2.1.3. Survival and stability outside the host

Studies in Israel have shown that KHV remains viable in water for at least 4 hours, but less than 21 hours, at water temperatures of 23–25°C (Perelberg *et al.,* 2003). Studies in Japan have shown a significant reduction in the infectious titre of KHV within 3 days in river or pond water or sediment samples at 15°C. However, KHV remained infective for >7 days when kept in environmental water samples that had been sterilised by autoclaving or filtration.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with KHV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: all varieties of common carp (*Cyprinus carpio*), and common carp/goldfish hybrids (e.g. *Cyprinus carpio* × *Carassius auratus*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is insufficient evidence to fulfil the criteria for listing as susceptible to infection with KHV according to Chapter 1.5 of the *Aquatic Code* are: Goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and Crucian carp (*Carassius carassius*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) and or *in-situ* hybridisation results have been reported in the following organisms, but an active infection has not been demonstrated:

Family	Scientific name	Common name
Acipenseridae	*Acipenser gueldenstaedtii*	Atlantic sturgeon
	Acipenser ruthenus × *Huso huso*	hybrid sterlet × beluga
	Acipencer oxyrinchus	Russian sturgeon
Cyprinidae	*Leuciscus idus*	blue back ide
	Rutilus rutilus	common roach
	Tinca tinca	tench
	Hypophthalmichthys molitrix	silver carp
Gammaridae	*Gammarus pulex*	scud (crustacean)
Nemacheilidae	*Barbatula barbatula*	stone loach
Percidae	*Gymnocephalus cernuus*	Euraseas ruffe
	Perca fluviatilis	European perch
Salmonidae	*Oncorhynchus mykiss*	rainbow trout
Unionidae	*Anodonta cygnea*	swan mussel

2.2.3. Non-susceptible species

Species that have been found non-susceptible to infection with KHV according to Chapter 1.5. of the *Aquatic Code* are:

Family	Scientific name	Common name
Agamidae	*Intellagama lesueurii*	Eastern water dragon
Ambassidae	*Ambassis agassizii*	olive perchlet
Anguillidae	*Anguilla australis*	short-finned eel
Ariidae	*Neoarius graeffei*	salmon catfish
Chelidae	*Emydura macquarii*	Macquarie short-necked turtle
Clupeidae	*Nematalosa erebi*	bony bream
Eleotridae	*Hypseleotris* sp.	carp gudgeon
Galaxiidae	*Galaxias maculatus*	common galaxias
Limnodynastidae	*Limnodynastes tasmaniensis*	spotted marsh frogs
Melanotaeniidae	*Melanotaenia duboulayi*	crimson-spotted rainbowfish
Mordaciidae	*Mordacia mordax*	short-headed lamprey ammocoetes
Mugilidae	*Mugil cephalus*	sea mullet
Parastacidae	*Cherax destructor*	common yabby
Pelodryadidae	*Litoria peronii*	Peron's tree frog
Percichthyidae	*Maccullochella peelii*	Murray cod
	Macquaria ambigua	golden perch
Plotosidae	*Tandanus tandanus*	eel-tailed catfish
Retropinna	*Retropinna semoni*	Australian smelt
Terapontidae	*Bidyanus bidyanus*	silver perch

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

All age groups of fish, from juveniles upwards, appear to be susceptible to infection with KHV but, under experimental conditions, 2.5–6 g fish were more susceptible than 230 g fish (Perelberg *et al.,* 2003). Carp larvae appear to be tolerant to infection with KHV.

Common carp or varieties, such as koi or ghost (koi × common) carp, are most susceptible and should be preferentially selected for virus detection, followed by any common carp hybrids, such as goldfish × common carp or crucian carp × common carp. Experimental challenges studies by Ito *et al.,* 2014a; 2014b, demonstrated that mortality due to infection with KHV was higher in indigenous Japanese carp (95–100%) compare with domesticated common carp and koi carp, where mortality varied from 30% to 95% and from 35% to 100%, respectively.

2.2.5. Distribution of the pathogen in the host

Gill, kidney, gut and spleen are the organs in which KHV is most abundant during the course of clinical disease (Gilad *et al.,* 2004). In fish surviving experiment challenge by immersion, KHV DNA was more likely to be detected from the caudal fin and brain compared with gill and kidney (Ito *et al*., 2014b).

2.2.6. Aquatic animal reservoirs of infection

There is evidence to indicate that survivors of infection with KHV may become persistently infected with virus and may retain the virus for long periods without expression of clinical signs of infection. The virus has been shown to persist in common carp experimentally infected at a permissive temperature and

subsequently maintained at a lower than permissive temperature (St-Hilaire *et al.,* 2005). Researchers in Japan conducted a PCR and serological survey of CyHV-3 in Lake Biwa in 2006, where episodic outbreaks of infection with KHV had been reported in the 2 years following a major outbreak in 2004. Further analysis of the surviving population showed that 54% of the older carp were seropositive and 31% PCR positive. The maintenance of high levels of antibody to the virus suggests that latent virus may be reactivating periodically in some animals, leading to excretion and a low level of virus circulation in the population, which boosts herd immunity.

2.2.7. Vectors

Studies in Japan have reported the detection of CyHV-3 DNA in plankton samples and, in particular, Rotifera species. Plankton samples were collected in 2008 from Iba-naiko, a shallow lagoon connected to Lake Biwa, a favoured carp spawning area (Minamoto *et al*., 2011). Statistical analysis revealed a significant positive correlation between CyHV-3 in plankton and the numbers of Rotifera and the authors suggested that CyHV-3 binds to or is concentrated by the filter feeding behaviour of Rotifera species. In an earlier report of a study in Poland, CyHV-3 was detected in swan mussels (*Anodonta cygnea*) and freshwater shrimp (*Gammarus pulex*) (Kielpinski *et al*., 2010). The invertebrates were collected from ponds in Southern Poland where outbreaks had occurred in common carp populations over 5 to 6 years. More work is needed to determine how long the infectious virus persists and remains viable in the invertebrates in the absence of the host species.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The clinical signs of infection may become apparent 3–21 days after naïve fish have been introduced to a pond containing infected fish (Bretzinger *et al.,* 1999; Hedrick *et al*., 2000). Morbidity of affected populations can be 100%, and mortality 70–100% (Bretzinger *et al.,* 1999; Haenen *et al.,* 2004). However, in several experiments, differential resistance to infection with KHV among common carp strains was reported (Dixon *et al*., 2009; Ito *et al*., 2014a; Shapira *et al*., 2005). In these reports, the cumulative mortalities of the most resistant strains were approximately 40%. Secondary and concomitant bacterial or parasitic infections are commonly seen in diseased carp and may affect both the mortality rate and clinical signs of infection (Haenen *et al.,* 2004).

2.3.2. Clinical signs, including behavioural changes

During an outbreak of infection with KHV there will be a noticeable increase in mortality in the population. All age groups of fish, except larvae, appear to be susceptible to infection with KHV, although, under experimental infection, younger fish (up to 1 year of age) are more susceptible to infection. Changes to the skin are also commonly observed and include: focal or total loss of epidermis, irregular patches of pale colouration or reddening, excessive or reduced mucous secretion (on skin or gills) and sandpaper-like skin texture. Other clinical signs include enophthalmia (sunken eyes) and haemorrhages on the skin and base of the fins, and fin erosion.

Fish become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and gasp at the surface of the water. Some fish may experience loss of equilibrium and disorientation, but others may show signs of hyperactivity.

2.3.3. Gross pathology

There are no pathognomic gross lesions. However, the most consistent gross pathology is seen in the gills, which can vary in extent from pale necrotic patches to extensive discolouration, severe necrosis and inflammation. Internal lesions are variable in occurrence and often absent in cases of sudden mortality. Other gross pathologies that have been reported include adhesions in the abdominal cavity, with or without abnormal colouration of internal organs (lighter or darker). The kidney or liver may be enlarged, and they may also exhibit petechial haemorrhages. Co-infections, for example with ectoparasites such as gill monogeneans, may alter the observed gross pathology.

2.3.4. Modes of transmission and life cycle

Virus is shed via faeces, urine, gills and skin and the main mode of transmission of KHV is horizontal. Early reports suggested that the gills are the major portal of virus entry in carp (Dishon *et al.,* 2005; Gilad *et al.,* 2004; Pikarsky *et al*., 2004). However, a more recent experimental study has demonstrated that the skin covering the fins and body of the carp is the major portal of entry for KHV (Costes *et al.,* 2009). Another study has shown that KHV DNA was detected in two of three fish from the caudal fin and gill, and caudal fin and spleen one day after exposure to sub-clinically infected fish (Ito *et al*., 2014a; 2014b). The virus spreads systemically from main points of entry to the internal organs; high levels of KHV DNA have been detected in kidney, spleen, liver and gut tissue (Dishon *et al.,* 2005; Pikarsky *et al*., 2004).

The assembly and morphogenesis of KHV in infected cells is the same as other herpesviruses (Miwa *et al.*, 2007). An ultrastructural examination of experimentally infected carp has provided evidence for immature capsids and mature nucleocapsid assembly in the nucleus and further maturation of the virion in the cytoplasm of infected cells. Hyper-secretion of mucous is very evident in the early stages of infection with KHV and KHV DNA has been detected at high levels in mucous sampled from experimentally infected carp (Gilad *et al.,* 2004). This is further evidence for active involvement of the skin in viral pathogenesis and an important site of virus shedding. Excretion of virus via urine and faeces may also be an important mechanism for virus shedding; infectious virus has been detected in faeces sampled from infected carp (Dishon *et al.,* 2005; Gilad *et al*., 2004).

2.3.5. Environmental factors

Disease patterns are influenced by water temperature, virulence of the virus, age, population genetics and condition of the fish, population density and stress factors (e.g. transportation, spawning, poor water quality). The disease is temperature dependent, occurring mainly between 16 and 29°C (Haenen *et al.,* 2004; Hedrick *et al*., 2000; Perelberg *et al.,* 2003; Sano *et al.,* 2004). Under experimental conditions, infectious virus was continually shed for a longer period from infected common carp at 16°C than those kept at 23°C or 28°C (Yuasa *et al.,* 2008). However, experimental challenge resulted in high mortality at 28°C but not at 29°C or 30°C, nor at 13°C (Gilad *et al.,* 2004; Ilouze *et al.,* 2010) (optimal temperature range for viral replication may vary with the virus strain).

2.3.6. Geographical distribution

Following the first reports of infection with KHV in Israel and Germany in 1998 and detection of KHV DNA in tissue samples taken during a mass mortality of carp in the UK in 1996, the geographical range of the disease has become extensive and includes most continents, including Europe, Asia, the Middle East, Southern Africa, and North America.

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

A safe and effective commercial vaccine is not currently widely available. However, live attenuated virus has been used to vaccinate carp. The vaccine preparation induced antibody against the virus and the duration of the protection was at least 8 months (Ilouze *et al.,* 2010). The vaccine was licensed for emergency use in Israel and has been widely used in carp farms across the country. Results of studies in Japan have shown that oral administration of a liposome-based vaccine containing inactivated KHV was also effective in protecting carp against clinical disease (reviewed by Ilouze *et al.,* 2010). The effectiveness of attenuated recombinant vaccines has been demonstrated in experimental challenge experiments (Boutier *et al*., 2015).

2.4.2. Chemotherapy including blocking agents

Chemotherapy is not currently available, however, the antiviral activity of exopolysaccharides against KHV *in vitro* has been reported (Reichert *et al*., 2017)

2.4.3. Immunostimulation

There is currently no published information on the use of immunostimulants to control infection with KHV in carp. However, it is known to be an area of research interest (Reichert *et al.,* 2017).

2.4.4. Breeding resistant strains

Differential resistance to infection with KHV, but not to virus entry, has been shown among different carp strains (Dixon *et al.,* 2009; Ito *et al*., 2014a; 2014b; Shapira *et al.,* 2005). The progeny of crosses of two strains of domesticated carp and one strain of wild carp were challenged by experimental or natural infection. The lowest survival rate was approximately 8% but the survival rate of the most resistant strain was 60.7% for experimental exposure and 63.5% for natural exposure in ponds (Shapira *et al.,* 2005). In a more recent resistance study, 96 families derived from di-allele crossing of four European/Asian strains of common carp were experimentally challenged with KHV. Survival rates of the five most resistant crosses in the final virus challenge trial ranged from 42.9 to 53.4% (Dixon *et al.,* 2009).

2.4.5. Inactivation methods

The virus is inactivated by UV radiation at a dose of 4.0×10^3 μ Ws/cm^2, temperatures above 50°C for 1 minute and by iodophor (200 mg litre^{-1}) treatment for 30 seconds at 15°C (Kasai *et al.*, 2005).

2.4.6. Disinfection of eggs and larvae

Disinfection of eggs can be achieved by iodophor treatment (Kasai *et al.,* 2005). There are no publications on the disinfection of larvae.

2.4.7. General husbandry

Biosecurity measures should include ensuring that new introductions of fish are from disease-free sources and installation of a quarantine system where new fish can be held with sentinel fish at permissive temperatures for infection with KHV. The fish should be quarantined for a minimum of 4 weeks to 2 months before transfer to the main site and mixing with naïve fish. Hygiene measures on site should include disinfection of eggs, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish.

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 above 16°C. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. If moribund fish or fish showing clinical signs are sampled, the probability of detecting KHV is higher than if randomly selected, apparently healthy fish are sampled.

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). Younger fish up to 1 year are more susceptible to clinical disease and are recommended for sampling.

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.

3.2. Selection of organs or tissues

When testing clinically affected fish by PCR methods, and particularly if virus isolation is to be attempted, it is recommended to sample gill, kidney, and spleen tissues. The virus is most abundant in these tissues during the course of overt infection and high levels of virus have also been detected in encephalon (brain) and intestine (gut) tissue (Dishon *et al.,* 2005; Gilad *et al.,* 2004). Moreover, KHV DNA was detected with high probability from the encephalon of the surviving fish at 120 days post-infection (Ito *et al.,* 2014a). When testing subclinical, apparently healthy, fish by PCR methods, it is recommended to also include intestine (gut) and encephalon in a separate sample. In addition, KHV DNA was detected in the caudal and pectoral fin of all sampled dead fish from the field. As fins can be easily collected using tweezers and scissors, the fins are a suitable organ for PCR detection of KHV in clinically affected fish (Ito *et al.*, 2014a; 2014b).

3.3. Samples or tissues not suitable for pathogen detection

Fish carcasses showing very advanced signs of tissue decomposition are not suitable for testing by any method.

3.4. Non-lethal sampling

While some research has been carried out on the use of non-lethal sampling during the first few days after experimental challenge (Monaghan *et al*., 2017), due to the lack of formal validation non-lethal sampling is currently not recommended for the detection of KHV.

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

The success of pathogen isolation depends strongly on the quality of samples (which is influenced by 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. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 80–100% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health and will ensure that the ethanol does not fall to below 70% The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

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

Tissue samples for histopathology should be fixed in neutral buffered formalin immediately after collection. To ensure adequate penetration of the fixative the recommended ratio of fixative to tissue is 10:1.

3.5.4. 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. Samples for other tests

Blood samples extracted from the caudal vessel into a vacuum blood collection tube 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 maintenance of virus infectivity.

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.

Small life stages such as fry or specimens up to 0.5 g, can be pooled to obtain the minimum amount of 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 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 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
Histopathology						++	++	1				
Cell or artificial media culture						++	++	1				
Real-time PCR	++	++	++	1	++	++	++	1				
Conventional PCR					++	+++	+++	1	++	++	++	1
Conventional nested PCR	++	++	++	1	+++	+++	+++	1	++	++	++	1
Amplicon sequencing[3]									+++	+++	+++	1
In-situ hybridisation												
Bioassay												
LAMP						+++	+++	1				
IFAT						+	+	1				
Serology (ELISA)												

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; ELISA = 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]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.

4.2. Histopathology and cytopathology

Examination of the gills by low-power light microscopy can reveal erosion of primary lamellae, fusion of secondary lamellae, and swelling at the tips of the primary and secondary lamella. The histopathology of the disease is variable and not pathognomonic, but inflammation and necrosis of gill tissues is a consistent feature. Gills also exhibit hyperplasia and hypertrophy of branchial epithelium, and fusion of secondary lamellae and adhesion of gill filaments can be seen. Gill necrosis, ranging from small areas of necrotic epithelial cells of secondary lamellae to complete loss of the lamellae is observed. Branchial epithelial cells and leucocytes may have prominent nuclear swelling, margination of chromatin to give a 'signet ring' appearance, and pale diffuse eosinophilic intranuclear inclusions can be observed. Inflammation, necrosis and nuclear inclusions have also been observed (individually or together) in other organs, particularly the kidney, but also in the spleen, pancreas, liver, brain, gut and oral epithelium.

4.3. Cell or artificial media culture for virus isolation

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

Diagnosis of infection with KHV in clinically affected fish can be achieved by virus isolation in cell culture. However, the virus is isolated in only a limited number of cell lines which can be difficult to handle. Also, cell culture isolation is not as sensitive as the published PCR-based methods to detect KHV DNA and is not considered to be a reliable diagnostic method for KHV (Haenen *et al.,* 2004).

Cell line to be used: KF-1, KFC or CCB

Use the procedure described in Chapter 2.3.0 *General information* (on diseases of fish), Section A.2.2.2.

Confirmatory identification

The most reliable method for confirmatory identification of a CPE is by PCR, followed by sequence analysis of the PCR product. The PCR methods recommended for identification of KHV are the same methods recommended for direct detection in fish tissues (Section 4.3.1.2.3 below). For final confirmation, PCR products of the correct size should be identified as KHV in origin by sequence analysis.

i) Using a suitable DNA extraction kit or reagent, extract DNA from a sample of the virus culture that includes both cellular and supernatant material.

ii) Extracted DNA is then amplified using the PCR protocols described below. Amplified PCR products may then be excised from the gel and sequenced as described in Section 4.3.1.2.3.

4.4. Nucleic acid amplification

4.4.1. Sample preparation and extraction of DNA

DNA from infected cells and/or tissues is extracted using a phase-separation method or by use of a commercially available DNA isolation kit used according to the manufacturer's instructions.

4.4.2. Real-time PCR

Real-time PCR assays, such as TaqMan real-time PCR, are favoured by many diagnostic laboratories over conventional PCR, and real-time Taqman PCR is now a common diagnostic procedure that has been shown to detect and quantitatively assess very low copy numbers of target nucleic acid sequences. The most commonly used quantitative assay for detection of KHV is the Gilad Taqman real-time PCR assay (Gilad *et al.,* 2004). However, it should be noted that real-time PCR positive results are presumptive only and should be confirmed by convention PCR and sequence analysis.

Further, it should be noted that there is evidence that the published conventional PCR and real-time PCR methods, developed for the detection of KHV DNA in fresh tissue samples from clinically diseased carp, fail to detect some KHV variants in clinically affected fish (Engelsma *et al.,* 2013). Until this is resolved, it is highly recommended that the assay described by Engelsma *et al*. (2013) is used in place

of the current assays; using the nested or one-tube semi-nested PCR assay or increasing the cycle number of the single-round assay to detect the virus in apparently healthy carriers.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control. Ideally, the positive control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results. The primer and probe sequences and cycling conditions for the Gilad *et al.* (2004) KHV and koi glucokinase (used as the internal PCR control) real-time PCRs are shown in Table 4.4.1.

Table 4.4.1. *Primer and probe sequences and cycling conditions for the KHV real-time PCR (Gilad* et al.*, 2004).*

Target	Primer/probe sequence (5'->3') (concentration)	Cycling conditions	Amplicon size (bp)	Reference
KHV	KHV-86f: GAC-GCC-GGA-GAC-CTT-GTG (400 nM)	1 × 2 minutes @ 50°C 1 × 10 minutes @ 95°C 40 × 15 seconds @ 95°C and 60 seconds @60°C	78	Gilad *et al.* (2004)
	KHV-163r: CGG-GTT-CTT-ATT-TTT-GTC-CTT-GTT (400 nM)			
	KHV-109p: 6FAM-CTT-CCT-CTG-CTC-GGC-GAG-CAC-G-TAMRA (80 nM)			
Glucokinase	CgGluc-162f: ACT-GCG-AGT-GGA-GAC-ACA-TGA-T (400 nM)		69	
	CgGluc-230r: TCA-GGT-GTG-GAG-CGG-ACA-T (400 nM)			
	CgGluc-185p: 6FAM-AAG-CCA-GTG-TCA-AAA-TGC-TGC-CCA-CT-TAMRA (80 nM)			

4.4.3. Conventional PCR

Engelsma *et al.* (2013) reported that the published single-round PCR methods traditionally thought to be the most sensitive for detection of KHV DNA in fresh tissue samples fail to detect some KHV genotypes in clinically affected fish. Therefore, the assay described by Engelsma *et al.* (2013) is highly recommended when detecting KHV variants. By extending the number of cycles to 50 or using the nested second round of amplification the assay may also be suitable to detect virus in sub-clinical carriers. This method and other commonly used PCR protocols are shown in Table 4.4.3.

Table 4.4.3. *Primer sequences and cycling conditions for KHV PCR methods*

Primer sequence (5'->3') (concentration)	Cycling conditions	Amplicon size (bp)	References
Primary step: CyHVpolfor: CCA-GCA-ACA-TGT-GCG-ACG-G (200 nM) CyHVpolrev: CCG-TAR-TGA-GAG-TTG-GCG-CA (200 nM) Nested PCR: CyHVpolforint: CGA-CGG-VGG-YAT-CAG-CCC (200 nM) CyHVpolrevint: GAG-TTG-GCG-CAY-ACY-TTC-ATC (200 nM)	1 × 2 minutes @ 95°C 40 × 30 seconds @ 95°C, 30 seconds @ 55°C and 45 seconds @ 72°C 1 × 10 minutes @ 72°C	361 339	Engelsma *et al.* (2013)
For: GGG-TTA-CCT-GTA-CGA-G (200 nM)	1 × 15 minutes @ 95°C 40 × 45 seconds @ 95°C, 45 seconds @ 55°C and 60 seconds @ 72°C	409	Bercouvier *et al.* (2005)

Rev: CAC-CCA-GTA-GAT-TAT-GC (200 nM)	1 × 7 minutes @ 72°C		
For: GAC-ACC-ACA-TCT-GCA-AGG-AG (1000 nM) Rev: GAC-ACA-TGT-TAC-AAT-GGT-CGC (1000 nM)	1 × 30 seconds @ 94°C 40 × 30 seconds @ 94°C, 30 seconds @ 63°C and 30 seconds @ 72°C 1 ×7 minutes @ 72°C.	292	Gray *et al.* (2002) Yuasa *et al.* (2005)
For: GAC-GAC-GCC-GGA-GAC-CTT-GTG (300 nM) Rev: CAC-AAG-TTC-AGT-CTG-TTC-CTC-AAC (300 nM)	1 × 5 minutes @95°C 39 ×1 minute @ 94°C, 1 minute @ 68°C and 30 seconds @ 72°C 1 × 7 minutes @ 72°C	484	Gilad *et al.,* (2004)

4.4.4. Other nucleic acid amplification methods

A Loop-mediated isothermal amplification (LAMP) targeting TK gene has been developed for detection of KHV and shown to be more or equally sensitive as the single-round conventional PCR assays. An assay incorporating DNA hybridisation technology and antigen–antibody reactions in combination with LAMP has also been developed and reported to have improved sensitivity and specificity (Soliman & El-Matbouli, 2010).

4.5. Amplicon sequencing

PCR products are excised from the gel and purified using a commercial kit for gel purification. Single, intense (bright) PCR products, after purification, are sequenced directly in both directions with the primers used in the initial amplification. Alternatively, less intense (faint) PCR products are cloned using a TA cloning vector and both DNA strands are sequenced. The amplification, cloning and sequencing are performed in duplicate to eliminate potential errors introduced by the Taq polymerase. Sequence reactions are then analysed on a Genetic Analyser and the alignments and consensus sequences generated using appropriate computer software. Testing laboratories that have no sequencing facilities are recommended to use commercial companies that offer a sequencing service. Testing laboratories should follow the instructions supplied by the chosen sequencing service for submission of samples.

4.6. *In-situ* hybridisation

In-situ hybridisation (ISH) and immunofluorescence (IF) methods performed on separated fish leucocytes, have been used in research applications for detection, confirmation, or identification of KHV. Although these methods have not been thoroughly compared with other techniques, they are non-destructive (non-lethal) techniques and some laboratories may find them useful in a diagnostic setting and for confirmation of PCR results. Details of the methods are not given here but detailed protocols for separation of leucocytes from blood and for IF and ISH can be found in published reports by Bergmann *et al.* (2009; 2010).

4.7. Indirect fluorescent antibody test (IFAT)

KHV can be detected in touch imprints of liver, kidney and brain of infected fish by immunofluorescence (IF). Highest levels of positive IF were seen in the kidney and the virus could be detected by IF on a kidney imprint 1 day post-infection (Pikarsky *et al.,* 2004; Shapira *et al.,* 2005). The detection of KHV by immunostaining must be interpreted with care, as positive-staining cells could result from cross-reaction with serologically related virus (e.g. CyHV-1) or a non-viral protein (Pikarsky *et al.,* 2004).

A method for direct detection of KHV from kidney imprints by indirect fluorescent antibody test (IFAT) is detailed below.

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) Allow the imprint to air-dry for 20 minutes.

iv) 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 glass slides or a mixture of 30% acetone/70% ethanol, also stored at –20°C, for plastic wells.

v) Let the fixative act for 15 minutes. A volume of 0.5 ml/2 cm^2 well is adequate for imprints in cell culture plates.

vi) Allow the fixed imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vii) Rehydrate the dried imprints by four rinses with 0.01 M PBS solution, pH 7.2, containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinse.

viii) Prepare a solution of purified antibody or antiserum to CyHV-3 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Block with a solution of 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

x) Rinse four times with PBST.

xi) Treat the imprints with the antibody solution (prepared at step viii) for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur. A volume of 0.25 ml/2 cm^2 well is adequate for imprints in cell culture plates.

xii) Rinse four times with PBST.

xiii) Treat the imprints for 1 hour at 37°C 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.

xiv) Rinse four times with PBST.

xv) Add PBS (0.5 ml/2 cm^2 well) to the treated imprints in cell culture plates and examine immediately or mount the glass slides with cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.

xvi) Examine under incident UV light using a microscope. Positive and negative controls must be found to give the expected results prior to any other observation.

Paraffin wax tissue sections fixed in 10% neutral buffered formalin (NBF) are also suitable for detection of KHV antigen by IFAT. However, the deparaffinised sections, rehydrated in PBS, may need to be further treated to reveal antigen that may be masked by over fixation of the tissue. A common treatment is incubation of the sections with 0.1% trypsin in PBS at 37°C for 30 minutes. The sections are then washed in cold PBS before proceeding with steps viii–xvi above. Tissues collected for direct detect by IFAT (or other immunohistochemical staining, e.g., immunoperoxidase) should be fixed for 24–48 hours in 10% NBF and then the fixative should be replaced with 70% ethanol for prolonged storage.

4.8. Bioassay

Bioassay is not recommended as a diagnostic procedure.

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

Enzyme-linked immunosorbent assay (ELISA)-based methods for direct detection of KHV antigen in infected tissues are under development in a number of laboratories and these methods may also be suitable for confirmatory identification of KHV. Currently, two published ELISA methods are available and was developed in Israel to detect KHV in fish faeces (Dishon *et al.,* 2005) but also after isolation in cell culture using different KHV isolates at different temperatures (Bergmann *et al.* 2017b). The ELISA methods developed will have low sensitivity that may be suitable for detection of the high levels of KHV found in clinically diseased fish tissue but not suitable for KHV surveillance in healthy populations

4.10. Other methods

Infected carp produce antibodies against the virus, and ELISA-based tests that reliably detect these antibodies at high serum dilution have been published (Adkison *et al.,* 2005; Bergman *et al.,* 2017a; Ilouze *et al.,* 2010; St-Hilaire *et al.,* 2005). Antibody has been detected in the serum at 3 weeks after experimental infection and in survivors after 1 year following a natural infection (Adkison *et al.,* 2005; Ilouze *et al.,* 2010; St-Hilaire *et al.,* 2005; Taylor *et al.,* 2010).

Serum from koi containing antibodies to KHV has been shown to cross-react, in low dilutions, with CyHV-1, a further indication that these viruses are closely related. Evidence of cross-reacting antibodies was

demonstrated in ELISA and western blot analyses of serum from koi infected with CyHV-1 or KHV (Adkison *et al.,* 2005). Diagnostic virologists should also be aware that fish recently vaccinated against KHV may test positive in antibody detection ELISAs.

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

There are no well validated methods that are currently recommended for testing healthy populations of susceptible fish for declaration of freedom from infection with KHV; there is increasing evidence that the published real-time PCR assays may fail to detect all genotypes of KHV. Therefore, conventional nested PCR assays described by Engelsma *et al.* (2013) which will detect all known KHV genotypes is currently recommended for surveillance to demonstrate freedom in apparently health populations.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the presence (6.1) or absence of clinical signs (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[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.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection shall be suspected if: a positive result has been obtained on at least one animal from at least one of the following diagnostic tests:

i) A positive result from a real-time PCR assay

ii) A positive result from a conventional PCR assay

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with KHV is considered to be confirmed if the following criterion is met:

i) Detection of KHV in tissue preparations by real-time PCR and conventional PCR followed by sequencing of the amplicon

6.2. Clinically affected animals

No clinical signs are pathognomonic for infection with KHV however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection shall be suspected if at least one of the following criteria are met:

i) Gross pathology or clinical signs associated with infection with KHV as described in this chapter, with or without elevated mortality

ii) Histopathological changes consistent with infection with KHV as described in this chapter

iii) KHV typical CPE in cell culture.

[13] For example: transboundary commodities.

iv) A positive result by a real-time PCR

v) A positive result by a conventional (single round or nested) PCR

vi) A positive result by LAMP assay

vii) A positive result by IFAT

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection shall be confirmed if one of the following criteria is met:

i) KHV isolation in cell culture followed by virus identification by conventional PCR and sequencing of the amplicon

ii) Detection of KHV in tissue preparations by real-time PCR and by conventional PCR followed by sequencing of the amplicon.

iii) A positive result by LAMP assay and by conventional PCR followed by sequencing of the amplicon

iv) A positive result by IFAT and by conventional PCR followed by sequencing of the amplicon

v) Detection of KHV in tissue preparations by conventional 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: under study

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with KHV are provided in Table 6.3. This information can be used for the design of surveys for infection with KHV, 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.

The diagnostic sensitivity (DSe) and specificity (DSp) of PCR assays, based on an analysis of field collections and experimentally infected carp (Amita *et al.*, 2002, Ito *et al.*, 2014a; 2014b) demonstrated 94-100% DSe and 100% DSp.

6.3.1. For presumptive diagnosis of clinically affected animals:

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

6.3.2. For surveillance of apparently healthy animals:

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

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*
* *

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

NB: FIRST ADOPTED IN 2006; MOST RECENT UPDATES ADOPTED IN 2019.

Return to the Agenda

SECTION 2.3.

DISEASES OF FISH

CHAPTER 2.3.0.

GENERAL INFORMATION

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 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.

1.3. Specifications according to clinical status

For diagnosis of clinical infection for most 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. Samples from ten clinically diseased fish should be sufficient for the pathogen test(s) for each epidemiological unit.

For detecting subclinical infections ~~carriers of virus~~ or for targeted surveillance, 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 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: 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

42 recommended in section 3.4. of the specific disease chapter. Take the ovarian fluid, milt or tissues
43 appropriate for the specific pathogen being tested for (see individual disease chapter in the *Aquatic*
44 *Manual* for details).

45 **1.4.2. For infection with Aphanomyces invadans (epizootic ulcerative syndrome [EUS])**

46 *Any size of fish:* kidney, liver, muscular tissue (See Chapter 2.3.2 Infection with *Aphanomyces invadans*
47 [epizootic ulcerative syndrome] for specific details.

48 **1.4.3. For infection with Gyrodactylus salaris**

49 *Any size of fish:* skin and fins (See Chapter 2.3.3 Infection with *Gyrodactylus salaris* for specific details.

50 **1.4.4. For Koi herpesvirus (KHV)**

51 *Fish 4 cm to adult:* Take the gill, kidney, spleen, encephalon and gut tissues depending on test to be
52 used (See Chapter 2.3.7 *Infection with* *koi herpesvirus disease* for specific details.

53 **1.4.5. For viral encephalopathy and retinopathy (VER)**

54 *Fish 2–4 cm*: take the whole head.

55 *Fish 4 cm to adults:* take the encephalon and possibly the eyes and spinal cord (see Chapter 2.3.12
56 Viral encephalopathy and retinopathy for specific details).

57 ## 2. General processing of samples

58 ### 2.1. Macroscopic examination

59 For the listed diseases, macroscopic examination is mostly used for detecting clinical signs of epizootic
60 ulcerative syndrome infection with *Aphanomyces invadans* or *Gyrodactylus salaris,* but this is followed by
61 microscopic examination of histological slides for the former or by identification of parasites isolated from of
62 wet mounts of skin/fin scrapings the skin, fins or gills of fish for the latter.

63 For viral diseases, clinical signs (including increased mortality rate, surface discolouration, distended
64 abdomen, excess mucous production, exophthalmia, pale gills/anaemia, skin/fin/gill lesions, surface
65 haemorrhages, lethargy, abnormal swimming behaviour and inappetence) are non-specific.

66 ### 2.2. Preservation of samples for subsequent virological examination

67 Samples to be submitted are (i) fresh and chilled on ice for virus isolation, (ii) fixed in a nucleic acid stabilisation
68 solution (e.g. RNAlater or 80–90% ethanol) for polymerase chain reaction (PCR) detection and (iii) preserved
69 in 4–10% neutral-buffered formalin fixative for histology. See individual sections below for further details.

70 ### 2.3. Virological examination

71 **2.3.1. Transportation and antibiotic treatment of samples**

72 Pools of organs or of ovarian fluids/milt are placed in sterile vials and stored at 4°C or on ice until virus
73 extraction isolation is performed in the laboratory. Virus extraction isolation should optimally be carried
74 out within 24 hours after fish sampling, but is still acceptable for up to 48 hours if the storage temperature
75 is maintained at 0–4°C, or for longer periods for clinical disease samples held frozen at –80°C. Freezing
76 at –20°C for storage should be avoided. For testing of apparently healthy fish, freezing of samples (at
77 any temperature) for testing for subclinical carriers should be avoided.

78 Organ samples may also be transported to the laboratory by placing them in vials containing cell culture
79 medium or Hanks' balanced salt solution (HBSS) with added antibiotics to suppress the growth of
80 bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable
81 antibiotic concentrations are: gentamycin (1000 µg ml^{-1}), or penicillin (800 International Units [IU] ml^{-1})
82 and streptomycin (800 µg ml^{-1}). Antifungal compounds, such as Mycostatin® or Fungizone®, may also
83 be incorporated into the transport medium at a final concentration of 400 IU ml^{-1}. Serum or albumen (5–
84 10%) may be added to stabilise the virus if the transport time will exceed 12 hours.

85 **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, for example, PCR.

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)

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)

178 **1.3.1. Virus production**

179 For the *in-vitro* production of stock cultures of most of these viruses, monolayer cultures of susceptible
180 cells (see relevant sections in the *Aquatic Manual*) in suitable tissue culture vessels (e.g. plastic flasks)
181 should be inoculated with fairly low multiplicities of infection (m.o.i.), i.e. 10^{-2} to 10^{-3} plaque-forming units
182 (PFU) per cell or equivalent.

183 The preferred temperatures for virus propagation are included in the table below.

184 ~~15°C for IHNV, ISAV, OMV, and VERV (genotype BFNNV) and VHSV~~

185 ~~20°C for KHV SVCV and VERV (genotypes BFNNV, SJNNV and TPNNV)~~

186 ~~22°C for EHNV~~

187 ~~25°C for RSIV and VERV (genotypes RGNNV and SJNNV)~~

188 ~~30°C for VERV (genotype RGNNV)~~

Temperature	Virus
15°C	infectious haematopoietic necrosis virus (IHNV) infectious salmon anaemia virus (ISAV) salmonid alphavirus (SAV)
20°C	koi herpesvirus (KHV) spring viraemia of carp virus (SVCV)
22°C	epizootic haematopoietic necrosis virus (EHNV)
25°C	red sea bream iridovirus (RSIV)

189 **1.3.2. Preservation and storage of virus stock cultures**

190 1. Centrifuge infected cell cultures at 2–5°C and 2000–4000 ***g*** for 15 minutes then dilute the virus-
191 containing supernatants in order to obtain virus titres averaging 10^6 PFU ml^{-1} or equivalent.

192 2. Dispense the resulting viral suspensions into sterile vials at volumes of 0.3–0.5 ml each.

193 3. Freeze and store each series of standard virus stocks at –80°C or in liquid nitrogen vapour phase,
194 and check the titre of each virus stock at regular intervals (6–12 months) if it has not been used
195 during that time period.

196 *Lyophilisation:* long-term storage (decades) of ~~the seeds of~~ standard virus seed strains is achievable by
197 lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% ~~fetal~~
198 ~~calf serum~~ FBS are mixed (v/v) with an equal volume of cryopreservative medium (such as 20%
199 lactalbumin hydrolysate in distilled water) before processing. Seal or plug under vacuum and store at
200 4°C, in the dark.

201 ## 2. Techniques

202 ### ~~2.1. Serology~~

203 **~~2.1.1. Production of rabbit antisera and polyclonal antibodies to fish viruses~~**

204 ~~There are various ways in which antibodies against fish viruses can be raised in rabbits. Titre and~~
205 ~~specificity are influenced, however, by the inoculation programme used. The following immunisation~~
206 ~~protocols may be used to produce antisera for use in the virus isolation and/or identification procedures~~
207 ~~described later.~~

208 **~~2.1.1.1. Antisera to infectious pancreatic necrosis virus~~**

209 ~~Intravenous injection with 50–100 µg of purified virus on day 0, followed by an identical booster on day~~
210 ~~21, and bleeding 5–7 days later. Rabbits may be reused if not bled completely.~~

211 **~~2.1.1.2. Antisera to other viruses~~**

212 ~~The immunisation protocols alternate an intramuscular or intradermal injection with further intravenous~~
213 ~~boosters:~~

214 Day 0: primary injection, 500–1000 µg of purified virus is mixed (v/v) with adjuvant (Freund's
215 incomplete or other[14] adjuvants that are considered more acceptable) giving a total volume of
216 1.2 ml. This antigen is delivered to the rabbit as multipoint intradermal injections (2 points on each
217 side) after the animal has been shaved.

218 Day 21: collect about 2 ml of blood and check for reactivity (neutralisation, fluorescence); boost
219 intravenously with the same amount of purified virus as in the primary injection, but without
220 adjuvant. Prior to the intravenous booster injection, the rabbit should be treated with promethazine
221 (12 mg intramuscularly) to prevent a possible anaphylactic response.

222 Day 28: sample the blood, check the serum reactivity and bleed or boost according to the results.

223 For rhabdoviruses, this immunisation procedure is well suited to production of antisera to be used in
224 immunofluorescence and in the enzyme-linked immunosorbent assay. However, a more efficient
225 method for production of neutralising antisera is regular intravenous injection without adjuvant (0.2 ml)
226 every 3–4 days (twice a week). As many as 15 injections may be necessary; 1 week after the last
227 injection, a serum sample should be collected and tested.

228 **2.1.3. Processing and storage of immune sera**

229 After blood clotting, collect and centrifuge the serum at 20°C and heat it for 30 minutes at 56°C. Filter
230 the resulting heat-inactivated serum through a membrane filter (450 nm pore size) and temporarily store
231 it at 4°C for the time necessary for the screening of its reactivity and specificity and for checking that
232 these properties are not affected by preservation conditions (e.g. freezing or lyophilisation). Sterile rabbit
233 sera can be kept for at least 2 months at 4°C without any change in their properties. Dispense (usually
234 as small volumes) and freeze at –20°C or lyophilise.

235 Immunoglobulins (Ig) may be extracted from antisera using conventional methods suitable for Ig
236 purification. Selective attachment to protein A constitutes a reliable and effective method. The
237 concentration of Ig solutions is adjusted to the values required for further conjugate preparation or
238 storage.

239 *Preservation of Ig:* Mix a solution of Ig of concentration 2 mg litre^{-1} with sterile pure glycerol (v/v) and
240 keep at –20°C. Solutions of Ig with a higher concentration may also be prepared in glycerol.

241 **2.1.4. Mouse monoclonal antibodies**

242 Monoclonal antibodies (MAbs) to most of the fish viruses have been raised over the past years. Some
243 of them, singly or as two or three associated MAbs, have given rise to biological reagents suitable for
244 the identification of virus groups (IPN, VHS, IHN). Other MAbs, taken individually or as components of
245 Ab panels, allow accurate typing of VHSV and IHNV. These MAbs can be obtained from the Reference
246 Laboratories listed at the end of this *Aquatic Manual*.

247 In theory, mouse monoclonal IgGs can be processed and stored as for polyclonal IgGs. However, the
248 reactivity of certain MAbs may be impaired by processes such as enzymatic- or radio-labelling or
249 lyophilisation. It is thus necessary to test various MAbs for the conditions under which they will be used.

250 **2.1. Direct microscopy**

251 Samples for direct microscopic examination of smears or tissue imprints should be examined as soon as
252 possible after collection. Live specimens should be used whenever possible, or fresh specimens chilled at
253 4°C, or 10% neutral-buffered formalin-fixed specimens when live specimens are not practical. If an adequate
254 field laboratory is available, it should be used to process and examine samples near the site of collection. For
255 *G. salaris,* fresh specimens are examined or fish can be stored in ethanol prior to microscopic examination
256 (see Chapter 2.3.3 Infection with *G. salaris*).

257 **2.2. Histological techniques**

258 **2.2.1 Preparation of slides for histological examination**

259 **2.3.1. Tissue fixation and embedding**

14 Use of Freund's complete adjuvants may be restrictedon animal welfare grounds. Alternative synthetic adjuvants include trehalose dimycolate and monophosphate lipid A.

Only live or moribund specimens of fish with clinical lesions should be sampled 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 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

351 Mix to dissolve, adjust pH to 6.0 with 1 N HCl before adding Tween 20. Store this solution at room
352 temperature for 3 months or at 4°C for longer storage.

353 ## 2.3. Electron microscopy

354 Electron microscopy (transmission or scanning) is a valuable research tool for the study of aquatic animal
355 diseases (e.g. Hyatt *et al.,* 1991) and for the detection of previously unknown viruses for which there are no
356 specific diagnostic tests. However, these methods are not normally used for the routine diagnosis of the fish
357 diseases listed by the OIE so are not described in the *Aquatic Manual*.

358 ## 2.4. Virus isolation

359 ### 2.4.1. Introduction

360 For most viruses, the standard surveillance method (to detect subclinical carriers) is virus isolation in cell
361 culture followed by identification using either antibody-based or, more commonly, nucleic acid-based
362 (PCR) methods can be employed in the diagnosis of clinically affected animals or in the surveillance of
363 apparently healthy animals. Isolation of finfish viruses in cultures of a number of established fish cell lines
364 is well-documented (Crane *et al.,* 2005; Devold *et al.,* 2000; Graham *et al.,* 2008; Herath *et al.,* 2009;
365 Lorenzen *et al.,* 1999; Olesen & Vestergård Jørgensen, 1992). However for some viruses, such as While
366 inoculation of fish cell lines with fish tissues processed for virus isolation is considered the reference
367 standard for surveillance programmes with respect to sensitivity, the precise sensitivity of the procedure
368 is unknown. Moreover KHV, cell culture isolation is not as sensitive as the published PCR-based methods
369 and is not considered to be a reliable diagnostic method for KHV (Haenen *et al.,* 2004). Indeed, real-time
370 or conventional PCR methods and sequencing are, in general, highly sensitive and highly specific and,
371 following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared
372 from fish tissue. The technique has the potential to be used in direct surveillance programmes for
373 obtaining approved free status (e.g. Garver *et al.,* 2011; Jonstrup *et al.,* 2013). Duplicates of unfixed
374 samples testing positive using real-time or conventional PCR methods and sequencing can be processed
375 for virus isolation to confirm presence of infectious virus. At least every 6 months or if decreased cell
376 susceptibility is suspected, titration of frozen viral stocks is performed to verify cell line susceptibility to
377 infection.

378 ### 2.4.2. Inoculation of cell monolayers

379 Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively
380 growing (not confluent) at inoculation.

381 Prepared tissue samples (see Section A. *Sampling* above) are inoculated onto cell cultures in at least
382 two dilutions, i.e. the primary dilution and a 1/10 dilution thereof, resulting in final dilutions of tissue
383 material in cell culture medium of 1/100 and 1/1000, respectively (to prevent homologous interference).
384 The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each
385 dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well
386 cell culture plate, has to be used. Use of 24-well cell culture plates is recommended, but other units of a
387 similar or larger growth area are also acceptable.

388 ### 2.4.3. Incubation of cell cultures

389 Inoculated cell cultures are incubated at the pathogen-specific temperature for 7–14 days. If the colour
390 of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment
391 with sterile bicarbonate solution, or equivalent substances, has to be performed to ensure cell
392 susceptibility to virus infection.

393 ### 2.4.4. Microscopy

394 Using ×40–150 magnification, inoculated cell cultures must be inspected regularly (at least three times
395 a week) for the occurrence of cytopathic effect (CPE). The use of a phase-contrast microscope is
396 recommended. If obvious CPE is observed, virus identification procedures must be initiated immediately.

397 ### 2.4.5. Sub-cultivation

398 If no CPE has developed after the primary incubation for 7–14 days, sub-cultivation is performed with
399 fresh cell cultures using a cell area similar to that of the primary culture.

400 Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled
401 according to the cell line 7–14 days after inoculation. The pools are then inoculated onto homologous
402 cell cultures undiluted and diluted 1/10 (resulting in final dilutions of 1/10 and 1/100, respectively, of the
403 supernatant) as described above (Section B.2.4.2. *Inoculation of cell monolayers*). For SAV, and other
404 non- or slow CPE-forming viruses that are cell-bound, it is recommended that a freeze–thaw cycle or
405 sonication step be included prior to passage.

406 Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into
407 a well with a fresh cell culture (well-to-well sub-cultivation). In the case of salmonid samples, inoculation
408 may be preceded by preincubation of the dilutions with an anti-IPNV antiserum at an appropriate dilution,
409 as described above (see Section A.2.3.3. *Treatment to neutralise enzootic viruses*). The inoculated
410 cultures are then incubated for 7–14 days at the appropriate temperature, with observation, as described
411 above (see Section B.2.4.4. *Microscopy*).

412 If nonspecific cytotoxicity occurs within the first 3 days of incubation, sub-cultivation may be performed
413 at that stage, but the cells must then be incubated for 7 days and sub-cultivated again with a further
414 7 days' incubation. When nonspecific cytotoxicity develops after 3 days, the cells may be passed once
415 and incubated to achieve a total of 14 days from the primary inoculation. There should be no evidence
416 of toxicity in the final 7 days of incubation.

417 If bacterial contamination occurs despite treatment with antibiotics, sub-cultivation must be preceded by
418 centrifugation at 2000–4000 ***g*** for 15–30 minutes at 2–5°C, or filtration of the supernatant through a
419 0.45 µm filter (low protein-binding membrane). In addition to this, sub-cultivation procedures are the
420 same as for nonspecific cytotoxicity.

421 If no CPE occurs, the test may be declared negative, however, increased confidence of a negative result
422 can be achieved by testing for the presence of virus using antibody-based or nucleic acid-based (PCR)
423 methods. For SAV2/SAV3 no apparent CPE is common from field isolates. An IFAT for the detection of
424 SAV antigen is routinely performed.

425 Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.) that make
426 it impossible to inoculate cells within 48 hours of collection of the tissue samples, it is acceptable to
427 freeze the supernatant at –80°C and carry out virological examination within 14 days. If the collected
428 supernatant is stored at –80°C within 48 hours of sampling it may be reused only once for virological
429 examination.

430 #### 2.4.6. Virus identification

431 Supernatant from cultures demonstrating CPE is used for virus identification by either antibody-based
432 and/or nucleic acid-based techniques. The preferred method for confirmatory identification is by
433 sequence analysis of PCR amplicons (see *Aquatic Manual* chapters on individual pathogens for details).

434 ### 2.5. Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis

435 Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional and the
436 polymerase chain reaction (PCR) and real-time PCR, have been developed for the identification of many
437 pathogens of aquatic animals. However, as is the case with several other diagnostic techniques, an advantage
438 in sensitivity is frequently offset by problems in interpretation or susceptibility to technical problems. Real-time
439 PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used
440 for direct detection of viral nucleic acids in samples prepared from fish tissue. The technique can be used in
441 direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well
442 as in the diagnosis of clinically affected animals (e.g. Garver *et al.*, 2011; Jonstrup *et al.*, 2013). Duplicates of
443 unfixed samples testing positive using real-time PCR can be processed for virus isolation to confirm presence
444 of infectious virus.

445 When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative
446 controls, as well as validation of the PCR method chosen are important. PCR can be quite dependent on the
447 conditions under which it is run and can be highly subject to laboratory contamination by previous PCR
448 products, yielding false-positive results. Nevertheless, Real-time PCR is a powerful technique particularly for
449 analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. Thus, while
450 Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening,
451 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.

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 ~~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 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 or 80–90% ethanol). Small organs such as kidney, liver and spleen can be stored whole in RNAlater 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, simply remove the tissue from ethanol or RNAlater and treat it as though it was just harvested. Most fresh and RNAlater/ethanol-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). 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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NB: First adopted in 1995. Most recent updates adopted in 2012.

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CHAPTER 2.4.3.

INFECTION WITH *BONAMIA OSTREAE*

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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

~~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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THE AQUATIC ANIMALS COMMISSION'S APPROACH TO THE REDEVELOPMENT OF SECTION 4, DISEASE PREVENTION AND CONTROL, OF THE *AQUATIC CODE*

SECTION 4–DISEASE PREVENTION AND CONTROL		
	Activity	**Status/Next Steps**
Current chapters		
4.1. Zoning and compartmentalisation	Requires revision to improve readability and clarity on the general principles for establishing zones and compartments.	Revision to follow work on new chapters described below.
4.2. Application of compartmentalisation	Requires revision to improve readability and clarity and to improve guidance for establishing compartments for trade purposes. Would integrate with other chapters.	Revision to follow work on new chapters described below.
4.3. Disinfection of aquaculture establishments and equipment	Update and improve the readability and clarity of the chapter.	New chapter adopted in 2017 as the first stage of redeveloping Section 4.
4.4. Recommendations for surface disinfection of salmonid eggs		Chapter adopted in 2015. Most recent amendments adopted in 2017.
4.5. Contingency planning	Requires substantial revision to provide adequate guidance on the principles of contingency planning and emergency response. Required to support articles in each disease-specific chapter on returning to freedom following an outbreak. Would integrate with other chapters.	To be replaced by a chapter on emergency disease preparedness (see new chapters below).
4.6. Fallowing in aquaculture		The chapter will be retained until the new chapters on Biosecurity in aquaculture establishments, Emergency disease preparedness and Disease outbreak management have been adopted.
4.7. Handling, disposal and treatment of aquatic animal waste	May require some revision to integrate with other new and revised chapters in this section and to ensure recommendations are sound.	Revision will follow the adoption of other new and revised chapters.
4.8. Control of pathogenic agents in aquatic animal feed	The recommendations to be integrated in other chapters.	Most recent revisions adopted in 2015. Revision may be required once other chapters of Section 4 have been revised and new chapters adopted.

SECTION 4–DISEASE PREVENTION AND CONTROL		
	Activity	**Status/Next Steps**
New chapters		
4.X. Biosecurity for aquaculture establishments	Develop a new chapter on principles of aquaculture biosecurity. Would cover key approaches to biosecurity planning such as risk analysis and identification of transmission pathways. Would integrate with other chapters e.g., disinfection, compartmentalisation.	Will be proposed for adoption in May 2021.
4.X. Emergency disease preparedness	Develop a new chapter that focuses on a comprehensive emergency management framework (consisting of prevention, preparedness, detection, response and recovery).	September 2020 – The Commission developed the article structure for the new draft chapter.
4.X. Disease outbreak management	Develop a new chapter that focuses mainly on details of the response phase of aquatic animal disease outbreaks.	September 2020 – The Commission developed the article structure for the new draft chapter.
4.X. Application of zoning	Draft new chapter on application of zoning.	Yet to be prioritised.

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