Organisation Mondiale de la Santé Animale
World Organisation for Animal Health
Organización Mundial de Sanidad Animal

Original: English
February 2019

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

Paris, 7–14 February 2019

The OIE Aquatic Animal Health Standards Commission (hereinafter referred to as the Aquatic Animals Commission) met at OIE Headquarters in Paris from 7 to 14 February 2019. The list of participants is attached as **Annex 1**.

The Aquatic Animals Commission thanked the following Member Countries for providing written comments on draft texts for the OIE *Aquatic Animal Health Code* (hereinafter referred to as the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter referred to as the *Aquatic Manual*) circulated after the Commission's September 2018 meeting: Australia, Canada, China (People's Rep. of), Chinese Taipei, Japan, Malaysia, Mexico, New Caledonia, New Zealand, Norway, Switzerland, Thailand, Vietnam, the United States of America (USA), the Member States of the European Union (EU) and the African Union Interafrican Bureau for Animal Resources (AU-IBAR) on behalf of African Member Countries of the OIE.

The Aquatic Animals Commission reviewed Member Country comments and amended relevant chapters of the *Aquatic Code* and the *Aquatic Manual* where appropriate. The amendments are shown in the usual manner by 'double underline' and '~~strikethrough~~' and are presented in the Annexes to this report. For Annexes that have been circulated previously, amendments proposed at this meeting are highlighted with a coloured background in order to distinguish them from those proposed previously.

The Aquatic Animals Commission considered all Member Country comments that were submitted on time and supported by a rationale. However, the Commission was not able to draft a detailed explanation of the reasons for accepting or not each of the proposals received and focused its explanations on the most significant issues.

The Aquatic Animals Commission encourages Member Countries to refer to previous reports when preparing comments on longstanding issues. The Commission also draws the attention of Member Countries to the reports of relevant *ad hoc* Groups, which include important information, and encourages Member Countries to review these reports together with the report of the Commission. These reports are available on the OIE website.

The table below lists the texts as presented in the Annexes. Member Countries should note that texts in **Annexes 3 to 16** are proposed for adoption at the 87th General Session in May 2019; **Annexes 17 to 22** are presented for Member Country comment; and **Annexes 23 and 24** are presented for information.

Comments on **Annexes 17 to 22** of this report must reach OIE Headquarters by the **7 August 2019** to be considered at the September 2019 meeting of the Aquatic Animals Commission. Comments received after the due date will not be submitted to the Commission for its consideration.

All comments should be sent to the OIE Standards Department at: standards.dept@oie.int.

The Aquatic Animals Commission again strongly encourages Member Countries to participate in the development of the OIE's international standards by submitting comments on this report, and prepare to participate in the process of adoption at the General Session. 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 submitted as specific proposed text changes, supported by a structured rationale or by published scientific references. Proposed deletions should be indicated in '~~strikethrough~~' and proposed additions with 'double underline'. Member Countries should not use the automatic 'track-changes' function provided by word processing software as such changes are lost in the process of collating Member Country submissions into the Aquatic Animals Commission's working documents.

Item	**Texts proposed for adoption**	**Annex number**	**Page number**
	AQUATIC CODE		
1.2.	Glossary	3	27
1.3.	Criteria for listing species as susceptible to infection with a specific pathogen (Chapter 1.5.); in track changes (A) and clean text (B)	4 (A&B)	29
1.4.1.	Article 10.5.2. – Infection with salmonid alphavirus (Chapter 10.5.)	5	37
1.4.2.	Article 10.7.2. – Infection with koi herpesvirus (Chapter 10.7.)	6	39
1.4.3.	Article 10.9.2. – Infection with spring viraemia of carp virus (Chapter 10.9.)	7	41
1.5.	Infection with Ranavirus (Chapter 8.3.)	8	43
1.6.	Acute hepatopancreatic necrosis disease (Chapter 9.1.)	9	51
1.7.	Articles 10.2.1. and 10.2.2. – Infection with *Aphanomyces invadans* (Chapter 10.2.)	10	57
1.8.	Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)	11	59
1.9.	Model Article X.X.8.	12	67
	AQUATIC MANUAL		
5.1.	Scope and Sections 2.2.1. and 2.2.2. – Infection with yellow head virus genotype 1 (Chapter 2.2.9.)	13	69
5.2.	Infectious haematopoietic necrosis (Chapter 2.3.4.)	14	71
5.3.	Infection with salmonid alphavirus (Chapter 2.3.6.)	15	87
5.4.	Scope and Sections 2.2.1. and 2.2.2. – Koi herpesvirus disease (Chapter 2.3.7.)	16	101
	Texts for Member Country comments	**Annex number**	**Page number**
	AQUATIC CODE		
2.1.	New draft chapter on Biosecurity for Aquaculture Establishments (Chapter 4.X.)	17	103
3.1.1.	Infection with shrimp haemocyte iridescent virus (SHIV); assessment for SIHV against the criteria for listing an aquatic animal disease in accordance with Chapter 1.2. – Criteria for listing aquatic animal diseases (A) and revised Article 1.3.3. of Chapter 1.3 – Diseases listed by the OIE (B)	18 (A&B)	113

3.2.	Model Article 10.X.13.	19	119
1.8.	Article 10.6.13. – Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)	20	121
AQUATIC MANUAL			
6.1.1.	Spring viraemia of carp (Chapter 2.3.9.); in track changes (A) and clean text (B)	21 (A&B)	123
6.1.2.	New draft Chapter 2.1.X. – Infection with *Batrachochytrium salamandrivorans*	22	169
Item	**Annexes for Member Country information:**	**Annex number**	**Page number**
4.2.	Report of the electronic *ad hoc* Group on Tilapia lake virus	23	183
J	Work plan	24	185

A. INTRODUCTIONS AND THE PERFORMANCE MANAGEMENT FRAMEWORK

Matthew Stone, Deputy Director General, International Standards and Science, presented the new Performance Management Framework to the Aquatic Animals Commission. He explained that the objective of this framework is continuous improvement of the work all of the Specialist Commissions and the OIE Secretariat in order to improve their work for the benefit of the OIE Members. He noted that this process includes regular meetings between Commission members and himself, and between all Commission Presidents and the Director General and a brief review at the end of each meeting.

Ingo Ernst, President of the Aquatic Animals Commission, encouraged all Commission members to approach the Performance Management Framework in a positive way. He noted that transparency is a key aspect of any performance management framework requiring assessments to be made in an open way and clearly communicated. He commented that the initiative could assist the Commission in undertaking its work in a productive manner where expectations of roles and responsibilities are clearly understood.

B. ADOPTION OF THE AGENDA

The draft agenda circulated prior to the meeting was discussed, updated and agreed. The adopted agenda of the meeting is attached in **Annex 2.**

C. COOPERATION WITH OTHER SPECIALIST COMMISSIONS

1. The Aquatic Animals Commission and the Biological Standards Commission

The Aquatic Animals Commission and the Biological Standards Commission held a joint meeting on 13 February 2019 to share information and explore areas of common interest and how to strengthen ways of working together. Topics addressed included: each Commission's approach to working on the *Aquatic Manual* and the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (hereinafter referred to as the *Terrestrial Manual*); Reference Centre activities, specifically how to collaborate on the development of procedures and decision making.

All agreed that this meeting assisted in strengthening the collaboration between the two Commissions.

Given it is unlikely that the two Commissions will meet at the same time for future meetings, they agreed to hold teleconference calls between meetings to progress relevant items, e.g. guidance for Reference Centres networking.

D. OIE *AQUATIC ANIMAL HEALTH CODE*

1. Texts to be proposed for adoption at the 2019 General Session (that were circulated for Member Country comments in September 2018)

1.1. General comments

In response to a comment to develop new chapters for the *Aquatic Code* to ensure safe trade in broodstock and genetic material, the Aquatic Animals Commission added this topic to its work plan.

1.2. Glossary

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

Basic biosecurity conditions

The Aquatic Animals Commission reviewed the comments and noted that most were either in support of the proposed changes or of an editorial nature. In response to a Member Country suggestion to include additional text providing the option of disease notification to the OIE or the Competent Authority, the Commission did not agree as the definition of basic biosecurity conditions addresses biosecurity at the national level. The Commission highlighted that notification obligations to the OIE are addressed in Chapter 1.1.

The Aquatic Animals Commission agreed not to make any amendments to the text as they considered it to be clear as currently proposed.

The revised definition for 'basic biosecurity conditions' is presented in Annex 3 and will be proposed for adoption at the 87th General Session in May 2019.

1.3. Criteria for listing species as susceptible to infection with a specific pathogen (Chapter 1.5.)

Comments were received from Australia, Canada, China (People's Rep. of), Chinese Taipei, Japan, Malaysia, Mexico, Thailand, USA, Vietnam, the EU and AU-IBAR.

The Aquatic Animals Commission reviewed the comments and amended the chapter, where relevant.

Article 1.5.2.

The Aquatic Animals Commission did not agree with a comment to delete the text after the word 'infection', specifying the definition of infection to include presence of multiplying, developing pathogenic agent, as they considered it useful to be explicit about the meaning of infection at the start of the chapter.

In response to a comment, the Aquatic Animals Commission clarified that the definition for infection in the Glossary should continue to include latency and therefore does not need to be reviewed. The Commission reiterated that in terms of demonstrating susceptibility the criteria do not need to include demonstration of true latency as this offers no advantage in the identification of susceptible species over demonstration of other forms of infection.

Article 1.5.4.

The Aquatic Animals Commission did not agree with a comment to delete point 3 in Article 1.5.4. The Commission noted that this article describes stage 1 which is only for classification of the evidence. It considered that to support transparency and defensibility all relevant and available evidence should be classified before determining which evidence is used to assess species susceptibility. The Commission also noted that some experimental evidence could be used to demonstrate that some species are refractory to infection, which is useful for Article 1.5.9.

The Aquatic Animals Commission agreed to change 'inoculation' to 'injection' to clarify that this is not vaccination. It also agreed, for clarity, to change 'infectivity' to 'infective' in the last paragraph of Article 1.5.4.

In response to a comment requesting a definition or guidance for 'high loads' the Aquatic Animals Commission agreed to reinsert the word 'unnaturally' to indicate that high loads are meant to be a level greater than would be experienced under natural conditions.

Article 1.5.6.

The Aquatic Animals Commission did not agree with a comment to change 'naïve' to 'apparently healthy' as naive was the appropriate term and apparently healthy animals could in fact be infected or immune to infection.

Article 1.5.8.

The Aquatic Animals Commission agreed with a comment to change the cross-reference of Articl 1.5.3. to Article 1.5.7. noting that this amendment clarified the intention of this text which is to identify species having incomplete evidence for susceptibility. A similar edit was made in point 1a) of Article 1.5.9. to ensure consistency.

The Aquatic Animals Commission agreed with a comment to change 'risk analysis' to 'risk assessment' because in this context risk management is not included.

Article 1.5.9.

A Member Country requested the scientific rationale for setting the threshold for low host species specificity, namely at least one susceptible species in each of three or more taxa at the ranking of Family. The Aquatic Animals Commission explained that the threshold has been established at a level considered appropriate to restrict application of this article to pathogenic agents that have a broad host range, and where gaps in the scientific knowledge mean that new species would be likely to be judged as susceptible, if they were exposed to the pathogenic agent. For example, for infection with *Aphanomyces astaci*, all species of fresh water crayfish in the Families of Cambaridae, Astacidae and Parastacidae tested to date have proven to be susceptible. However, the susceptibility of many species within these families has not been investigated.

In response to a comment to provide examples to facilitate the understanding of this article, the Aquatic Animals Commission referred Members to page 7 of its February 2018 report, where it provided a table of examples of how the threshold for application of Article 1.5.9. might apply. http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/A_AAC_Feb_2018.pdf

The Aquatic Animals Commission agreed with comments to edit the numbering of points in this article to improve readability.

The Aquatic Animals Commission did not agree with a comment to replace 'appropriately designed experimental procedures' with 'invasive experimental procedures'. The Commission reiterated that it had replaced the words 'controlled challenges' as this was considered to be too limited, and that all relevant procedures, invasive or not, should be considered.

The Aquatic Animals Commission did not agree with a comment to amend point 2(a) as it considered it clear as written.

The revised Chapter 1.5. *Criteria for listing species as susceptible to infection with a specific pathogen* is presented in track changes (A) and clean text (B) in **Annex 4** and will be proposed for adoption at the 87th General Session in May 2019.

1.4. Amendments to fish disease-specific chapters regarding susceptible species

1.4.1. Article 10.5.2. – Infection with salmonid alphavirus (Chapter 10.5.)

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

A Member proposed that brown trout *(Salmo trutta)* be removed from Article 10.5.2. as it did not consider that this species met the criteria for susceptibility. The Aquatic Animals Commission requested the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases review its assessment of brown trout *(Salmo trutta)* to infection with salmonid alphavirus.

The *ad hoc* Group reviewed its assessment and agreed that brown trout *(Salmo trutta)* did not meet the criteria and should therefore not be included in Article 10.5.3. of the *Aquatic Code.* But it did meet the criteria to be included in Section 2.2.2. *Species with incomplete evidence for susceptibility* of the *Aquatic Manual*. The rationale for this change was because the study of Boucher *et al*. (1995) only included pathology and no virus isolation/detection for an invasive experimental trial, which is not sufficient to meet the listing criteria.

The Aquatic Animals Commission became aware of a new publication reporting susceptibility of ballan wrasse (*Labrus bergylta)* to infection with salmonid alphavirus (Ruane *et al.,* 2018) and requested that the *ad hoc* Group undertake an assessment of this species against the criteria in Chapter 1.5. in light of this study.

The *ad hoc* Group reviewed its assessment and noted that the results of the new study were based on a single positive result from a single location/survey and that supplementary evidence is needed to prove susceptibility. The Commission agreed that based on this information, ballan wrasse (*Labrus bergylta*) should not be included in Article 10.5.2. of the *Aquatic Code* before there is corroborating evidence. It would, however, be proposed for inclusion in Section 2.2.2. *Species with incomplete evidence for susceptibility* of the *Aquatic Manual* (refer to Item 5.2.).

<u>References</u>

Boucher, P., Raynard, R. S., Houghton, G., & Laurencin, F. B. (1995). Comparative experimental transmission of pancreas disease in Atlantic salmon, rainbow trout and brown trout. Diseases of Aquatic Organisms, **22 (1)**, 19–24.

Ruane, N. M., Swords, D., Morrissey, T., Geary, M., Hickey, G., Collins, E. M., Geoghegan, F., Swords, F. (2018). Isolation of salmonid alphavirus subtype 6 from wild -caught ballan w *Labrus bergylta* (Ascanius). *Journal Fish Diseases* 41 (11), 1643-1651).

The revised Article 10.5.2. of Chapter 10.5. *Infection with salmonid alphavirus* is presented in **<u>Annex 5</u>** and will be proposed for adoption at the 87th General Session in May 2019.

1.4.2. Article 10.7.2. – Infection with koi herpesvirus (Chapter 10.7.)

Comments were received from Australia and the EU.

The Aquatic Animals Commission reviewed a comment and agreed not to make any additional amendments as they considered the text clear as written.

The revised Article 10.7.2. of Chapter 10.7. *Infection with koi herpesvirus* is presented in **<u>Annex 6</u>** and will be proposed for adoption at the 87th General Session in May 2019.

1.4.3. Article 10.9.2. – Infection with spring viraemia of carp virus (Chapter 10.9.)

Comments were received from Australia, Canada, New Zealand and the EU.

A Member requested the rationale for delisting species such as tench *(Tinca tinca)* that have been recognised and controlled in some Member Countries as susceptible to infection with spring viraemia of carp (SVCV). The Aquatic Animals Commission noted that the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases had undertaken assessments of the susceptibility of all relevant species to infection with SVCV against the criteria in Chapter 1.5. The Commission noted that tench and several of the other species currently considered as susceptible in some Member Countries were found not to meet the criteria for listing when applying the criteria in Chapter 1.5.

The Commission reminded Members that all reports of the *ad hoc* Group are available at the OIE website: http://www.oie.int/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/

In response to a comment that raised concerns about the inclusion of zebrafish *(Danio rerio)* in Article 10.9.2., the Aquatic Animals Commission noted that the species had been assessed by the *ad hoc* Group and was found to meet the criteria for susceptibility. The Aquatic Animals Commission noted that the assessment for zebrafish *(Danio rerio)* had been based on one available study. The Commission agreed that based on this information, zebrafish *(Danio rerio)* should not be included in Article 10.9.2. of the *Aquatic Code* until there is corroborating evidence to support the assessment of susceptibility, even though the study provided strong evidence of susceptibility. The species would, however, be proposed for inclusion in Section 2.2.2. *Species with incomplete evidence for susceptibility* of the *Aquatic Manual* (see Item 6.1.1.).

The revised Article 10.9.2. of Chapter 10.9. *Infection with spring viraemia of carp virus* is presented in **Annex 7** and will be proposed for adoption at the 87th General Session in May 2019.

1.5. Infection with Ranavirus (Chapter 8.3.)

Comments were received from Australia, Canada, and the EU.

The Aquatic Animals Commission reviewed comments and noted that they were either in support of the proposed changes or of an editorial nature.

The Aquatic Animals Commission did not agree with a comment to include the word ‘infection’ before ‘status’ in the following phrase, ’infection with *Ranavirus* species status of the exporting country, zone or compartment’ that is used throughout this and other disease-specific chapters. The Commission reiterated that it had agreed some time ago to move away from referring to the disease name but rather to ‘infection with pathogenic agent’. The Commission noted this approach has now been applied in all disease-specific chapters with the exception of acute hepatopancreatic necrosis disease because of the aetiology of the disease.

Article 8.3.1.

The Aquatic Animals Commission agreed with a comment to remove ‘member virus’ as it was unnecessary. The Commission highlighted that, unlike other listed diseases, the pathogenic agent in this chapter is listed at the Genus level not at the species level, and so the wording differs for this chapter.

Article 8.3.8.

The Aquatic Animals Commission amended text to ensure alignment with amendments made to the model Article X.X.8. (see Item 1.8.).

The revised Chapter 8.3. *Infection with Ranavirus* is presented in **Annex 8** and will be proposed for adoption at the 87th General Session in May 2019.

1.6. Acute hepatopancreatic necrosis disease (Chapter 9.1.)

Comments were received from Australia, Malaysia, Mexico, New Caledonia and the EU.

The Aquatic Animals Commission reviewed comments and noted that they were either in support of the proposed changes or of an editorial nature.

Article 9.1.8.

The Aquatic Animals Commission amended text to ensure alignment with amendments made in the model Article X.X.8. (see Item 1.8.).

The revised Chapter 9.1. *Acute hepatopancreatic necrosis disease* is presented in **Annex 9** and will be proposed for adoption at the 87th General Session in May 2019.

1.7. Articles 10.2.1. and 10.2.2. – Infection with *Aphanomyces invadans* (Chapter 10.2.)

The Aquatic Animals Commission reminded Members that it had amended Article 10.2.1. at its September 2018 meeting to ensure consistency with other amended fish disease-specific chapters and it had also amended some Family names in Article 10.2.2. to remove the use of italics as Family names for fish should not appear in italics. The Commission considered these amendments to be of an editorial nature. The Commission noted that during the review of these articles it corrected misspelling of the names of torpedo-shaped catfishes (*Clarias* spp.) and terapon (*Terapon* sp.).

The revised Articles 10.2.1. and 10.2.2. of Chapter 10.2. *Infection with Aphanomyces invadans* is presented in **Annex 10** will be proposed for adoption at the 87th General Session in May 2019.

1.8. Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)

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

Article 10.6.1.

In response to comments to revert from 'Salmonid novirhabdovirus' back to 'infectious haematopoietic necrosis virus' as some susceptible species are non-salmonid, the Aquatic Animals Commission noted that it would use the official ICTV designation but that the previously used virus name, 'infectious haematopoietic necrosis virus', should also be referred to in Article 10.6.1. The Commission made minor edits consistent with the approach in other *Aquatic Code* chapters.

Article 10.6.2.

The Aquatic Animals Commission agreed with a comment to correct a misspelling of masu salmon *(Oncorhynchus masou).*

A Member Country recommended pike *(Esox lucius)*, grayling *(Thymallus thymallus)* and eel *(Anguilla anguilla)* be included in Article 10.6.2. of the *Aquatic Code* as susceptible to infection with infectious haematopoietic necrosis virus (IHNV) based on information in two scientific studies: Reschova *et al.*, 2008 and Dorson *et al.*, 1987. The Aquatic Animals Commission requested that the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases review its previous assessments of these species against the criteria in Chapter 1.5.

The *ad hoc* Group reviewed its previous assessments and agreed that the Northern pike met the criteria and should be included in Article 10.6.2. The Aquatic Animals Commission therefore proposed the inclusion of pike in Article 10.6.2. of the *Aquatic Code*.

The Aquatic Animals Commission agreed that it was not possible to include grayling *(Thymallus thymallus)* and eel *(Anguilla anguilla)* in Article 10.6.2. because there was insufficient available scientific evidence to assess the species against the criteria in Chapter 1.5.

References:

Dorson, M., Chevassus, B., & Torhy, C. (1987). Susceptibility of pike (*Esox lucius*) to different salmonid viruses (IPN, VHS, IHN) and to the perch rhabdovirus. *Bulletin français de la pêche et de la protection des milieux aquatiques*, 307, 91-101.

Reschova, S., Pokorova, D., Hulova, J., Kulich, P., & Vesely, T. (2008). Surveillance of viral fish diseases in the Czech Republic over the period January 1999 - December 2006. *Veterinarni Medicina*, 53(2), 86–92.

Article 10.6.8.

The Aquatic Animals Commission amended text to ensure alignment with amendments made to the model Article X.X.8. (see Item 1.9.).

Article 10.6.13.

In response to a comment the Aquatic Animals Commission amended point 1 to improve clarity regarding assessment of the disease risks associated with imported disinfected eggs. The Commission proposed to circulate this amendment for comment.

The revised Chapter 10.6. *Infection with infectious haematopoietic necrosis virus* is presented in **Annex 11** and will be proposed for adoption at the 87th General Session in May 2019.

The revised Article 10.6.13 of Chapter 10.6. *Infection with infectious haematopoietic necrosis virus* is presented in **Annex 20** for Member Country comments.

1.9. Model Article X.X.8.

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

In response to a comment seeking clarification for the meaning of 'high health status' in point 2(a)(ii) of Article X.X.8. the Aquatic Animals Commission clarified that 'high health status' means the highest feasible disease status for a source population from a country not declared free from 'infection with pathogen X' and is based on information from testing and surveillance of the source population. The Commission emphasised that this article addresses the importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from 'infection with pathogenic agent X'.

The Aquatic Animals Commission agreed to edit point 1(b) to clarify that aquatic animals could be killed and processed either in the original quarantine facility or following biosecure transport to another quarantine facility. This edit will be applied to Article X.X.8. of all disease-specific chapters of the *Aquatic Code* when they are being amended.

The Aquatic Animals Commission amended text in point 2(b)(iv) to clarify the period required for quarantine in response to a comment.

The revised model Article X.X.8. is presented in **Annex 12** and will be proposed for adoption at the 87th General Session in May 2019.

2. Texts circulated for Member Country comments

2.1. New draft chapter on Biosecurity for Aquaculture Establishments (Chapter 4.X.)

Comments were received from Australia, Canada, China (People's Rep. of), Chile, Japan, New Caledonia, New Zealand, Norway, Thailand, Vietnam, the EU and AU-IBAR.

The Aquatic Animals Commission considered all comments and amended the text to improve readability and clarity, where relevant.

General comments

In response to some comments, the Aquatic Animals Commission reminded Members that the purpose of this chapter is primarily to mitigate the risk of the introduction of specific pathogenic agents into aquaculture establishments.

A Member Country requested that the Aquatic Animals Commission edit the chapter to provide more context about the biosecurity framework provided by governance and regulation, in which biosecurity at the level of the aquaculture establishment operates. The Commission highlighted that this chapter is intended to focus on biosecurity at the aquaculture establishment level but Article 4.X.3. recognises that biosecurity can also be applied at the level of country, zone or compartment. The Commission acknowledged the importance of broader biosecurity frameworks but agreed that the scope of the chapter could not be extended to include this content without making it less accessible and applicable for aquaculture establishments. The Commission plans to revise other relevant chapters in Section 4 of the *Aquatic Code* to include the application of biosecurity for zoning and compartmentalisation.

In response to a comment, the Aquatic Animals Commission highlighted that they would ensure the inclusion of cross references to this new chapter in other *Aquatic Code* chapters and vice versa, as relevant, when this chapter is adopted.

Article 4.X.5.

In response to a comment that all input and output risk factors should be used to define three systems (open, semi-closed and closed), the Aquatic Animals Commission commented that this approach does not provide a model that allows categorisation of individual establishments. A suggestion that categories of semi-open and semi-closed systems should be merged was not accepted as the Commission considered that there are important differences between these systems that need to be distinguished.

The Aquatic Animals Commission did not agree with a comment to include open systems in the scope of the chapter, as they are not 'aquaculture establishments', which is the subject of this chapter. The Commission noted that the production of aquatic animals for stocking into open systems takes place within aquaculture establishments and therefore associated biosecurity matters are addressed by articles in this chapter. However, the Commission accepted the comment that the health status of aquatic animals stocked into open systems should be subject to disease mitigation measures and amended the text accordingly.

The Aquatic Animals Commission agreed with a comment to include 'mollusc aquaculture' as an example of a semi-open aquaculture production system.

In response to a comment that not all recirculating production systems discharge water that is effectively treated to inactivate pathogens and therefore they cannot be considered a closed production system, the Aquatic Animals Commission explained that for these systems to be regarded as closed, all incoming and outgoing water should be subjected to an effective treatment.

Article 4.X.6.

In response to a comment the Aquatic Animals Commission agreed to amend the title of this article to ‘Transmission pathways, associated risks and mitigation measures’ to better reflect its content.

The Aquatic Animals Commission also agreed to add some text prior to listing the risk mitigation measures in each point in this article specifying that the list of mitigation measures is not exhaustive, but rather is aimed at addressing the most important measures. Other pathways may be identified in some systems that also need to be mitigated.

Comments to include detailed information that can already be found in other chapters in the *Aquatic Code*, e.g. Chapter 4.3. *Disinfection of aquaculture establishments and equipment* and 4.8. *Control of pathogenic agents in aquatic animal feed,* were not accepted in order to avoid duplication.

Point 1. Aquatic animals

In response to a comment the Aquatic Animals Commission amended point b) to emphasise the defined term for ‘quarantine’ is being used as the glossary definition describes in detail the purpose of maintaining aquatic animals in isolation.

The Aquatic Animals Commission did not agree to include vectors in point h), noting that vectors are addressed in point 6.

Point 2. Aquatic animal products and aquatic animal waste

The Aquatic Animals Commission agreed to amend point 2 (aquatic animal products and waste) to be consistent with the rest of the text regarding waste moved out of establishments.

Point 5. Fomites

The Aquatic Animals Commission agreed to include ‘footwear’ after ‘clothing’ as a potential fomite.

Point 6. Vectors

The Aquatic Animals Commission agreed with a comment to provide more guidance on mitigation measures associated with vectors. Accordingly, the text was revised to clarify that the mitigation measures described in point 1 for aquatic animals can also be applied to mitigate risks associated with vectors. Mitigation measures for other types of vectors were also added.

Article 4.X.7.

In response to a comment the Aquatic Animals Commission clarified that this article on risk analysis is consistent with Chapter 2.1. *Import Risk Analysis* of the *Aquatic Code* but it agreed to add a new sentence noting that this article elaborates the principles in Chapter 2.1. and applies them for the development of biosecurity for aquaculture establishments.

Step 1 – Hazard Identification

The Aquatic Animals Commission revised text to clarify that many hazards will share the same pathway and that information and pathways of introduction need to be combined to identify the most effective mitigation measures. The Commission emphasised that the current text clearly describes a hazard as a specific pathogenic agent or as a group of pathogenic agents.

Step 2 – Risk Assessment

In response to a suggestion to include a visual representation of the pathway of all physical and biological events required for a hazard to occur, the Aquatic Animals Commission noted that it would explore this option and report back at its September 2019 meeting.

Table 2. Qualitative descriptors of consequences

The Aquatic Animals Commission agreed with a comment to amend text in Table 2 to provide clearer explanations of the impacts.

Table 4. Interpretation of risk estimates

The Aquatic Animals Commission agreed with a comment to amend text in Table 4 to provide clearer explanations of the management responses.

Step 3 – Risk Management

The Aquatic Animals Commission did not agree with a comment to address environmental factors, as they considered this to be outside the scope of the chapter.

Article 4.X.8.

The Aquatic Animals Commission did not agree with a comment to include risk communication in the title as they saw no need to highlight risk communication over and above other elements of the biosecurity plan.

The Aquatic Animals Commission agreed to expand section 1(b) to include epidemiologic units and separation measures.

The Aquatic Animals Commission found it necessary to reorganise the text in this article because it considered it important to highlight the key components of the biosecurity plan.

1. Development of a biosecurity plan

The Aquatic Animals Commission agreed with a comment to include additional examples in points b) and d) to provide more guidance to Member Countries.

2. Key components of a biosecurity plan

In response to comments the Aquatic Animals Commission agreed to add more examples of documentation required in point b) *Documentation and record keeping,* to strengthen biosecurity requirements and to have more robust evidence of the effectiveness of the biosecurity protocols. The Commission also decided to provide examples of who, e.g. producer, aquatic animal health professional or veterinarian, might carry out routine monitoring of stock for important health and production parameters in point d) *Health monitoring*.

The revised new draft Chapter 4.X. *Biosecurity for aquaculture establishments* is presented in **Annex 17** for Member Country comments.

2.2. Discussion paper on Approaches for determining periods required to demonstrate disease freedom

Comments were received from Australia, Canada, China (People's Rep. of), Chinese Taipei, Japan, Malaysia, Mexico, New Caledonia, New Zealand, Norway, Thailand, Vietnam, the EU and AU-IBAR.

The Aquatic Animals Commission acknowledged the extensive number of comments submitted from Member Countries and appreciated the quality of comments and the high level of engagement on this topic. The Commission indicated that it will develop a revised paper at its next meeting in September 2019, taking into account Member Country comments, sound science and with a view to achieving consensus, in particular focusing on refining the recommended approaches. The revised paper will be sent for Member Country comments in the September 2019 report of the Commission.

3. Other *Aquatic Code* topics

3.1. Disease listed by the OIE (Chapter 1.3.)

3.1.1. Infection with shrimp haemocyte iridescent virus (SHIV)

The Aquatic Animals Commission noted the identification of a novel virus which had been named shrimp haemocyte iridescent virus (SHIV) and undertook an assessment of SHIV against the criteria for listing an aquatic animal disease in accordance with Chapter 1.2. The Commission concluded that infection with SHIV meets the criteria for listing in Article 1.2.2. and should be proposed for listing under Chapter 1.3. *Diseases listed by the OIE*.

The Aquatic Animals Commission recognised the potential significance of infection with SHIV to many countries given the worldwide importance of crustacean farming and trade. The Commission reminded Member Countries that infection with SHIV meets the definition of an 'emerging disease' and, as such, should be reported in accordance with Article 1.1.4. of the *Aquatic Code*.

The Aquatic Animals Commission also encouraged Member Countries to investigate mortality and morbidity events in crustaceans, emphasising that an understanding of the geographic distribution of SHIV is essential for efforts to control its possible spread.

The Aquatic Animals Commission suggested that Member Countries wishing for more information or advice on diagnostic testing for SHIV could contact the Reference Laboratories experts for Infection with white spot syndrome virus (see link below).

http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/

The Aquatic Animals Commission agreed to develop a Technical Disease Card for SHIV to provide information for Member Countries on available detection methods and transmission risks for this virus. The Technical Disease Card will be made available on the OIE website once completed.

The revised Article 1.3.3. of Chapter 1.3. *Diseases listed by the OIE* (B) and the *assessment for SHIV against the listing criteria* (A) are presented at **Annex 18** for Member Country comments.

3.2. Model Article 10.X.13.

In response to a comment, the Aquatic Animals Commission acknowledged that the current text in point 1 of Article 10.X.13. was not very clear as written. Therefore, the Commission proposed some amendments to clarify the intended purpose of this text. The Commission agreed to present this change in a model article that, once adopted, would be applied to relevant disease-specific chapters when they are being amended (see also Item 1.8.).

The model Article 10.X.13. is presented in **Annex 19** for Member Country comments.

3.3. Infection with *Gyrodactylus salaris* (Chapter 10.3.)

The Aquatic Animals Commission continued discussions on the taxonomy of *Gyrodactylus salaris* and reviewed advice provided by the OIE Reference Laboratory expert for *G. salaris*. The Commission agreed to retain the position set out in the report of the September 2018 meeting that it does not support synonymisation of *G. salaris* and *G. thymalli*, given the clear phenotypic differences between the two parasites, notably host predilection and pathogenicity in different host species (see also Item 6.2.).

3.4. Infection with *Marteilia refringens* (Chapter 11.4.)

The Aquatic Animals Commission received no comments on the question, raised in its September 2018 meeting report, of splitting *Marteilia refringens* into two species (*M. refringens* and *M. pararefringens*) based on evidence presented in a paper by Kerr *et al.* (2018). The Commission agreed it will revise Chapter 11.4. Infection with *Marteilia refringens* and in addition, consider whether *M. pararefringens* needs to be assessed against criteria for listing diseases (see also Item 6.3.).

E. OIE *AD HOC* GROUPS

4.1. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases

The *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases met from 13 to 15 November 2018. The *ad hoc* Group continued its work in undertaking assessments of susceptible species to infection with viral haemorrhagic septicaemia virus (VHSV) using the ‘Criteria for listing species as susceptible to infection with a specific pathogen’ (Chapter 1.5. of the *Aquatic Code*). Due to the large number of potential susceptible species the *ad hoc* Group did not complete all the assessments.

The Aquatic Animals Commission requested that the *ad hoc* Group continue its work on VHSV and also undertake assessments to review the list of susceptible species for Infection with *Aphanomyces invadans* (Epizootic ulcerative syndrome) and Infection with red sea bream iridovirus.

4.2. Electronic *ad hoc* Group on Tilapia lake virus

The Aquatic Animals Commission reviewed the report of the *ad hoc* Group on Tilapia lake virus (TiLV) which worked electronically from October 2018 to January 2019 on the assessment of TiLV diagnostics and their validation.

The Aquatic Animals Commission noted that the *ad hoc* Group will undertake test validation studies in April 2019. The Commission will review the results at its September 2019 meeting and will, depending on the progress made with test validation, review its assessment for TiLV against the listing criteria in Chapter 1.2.

The *ad hoc* Group was requested to continue its work and report back to the next meeting of the Aquatic Animals Commission in September 2019.

The report of the OIE *ad hoc* Group on Tilapia lake virus is presented at **Annex 23** for Member Country information.

F. OIE *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS*

5. Texts to be proposed for adoption at the 2019 General Session (that were circulated for Member Country comments in September 2018)

Comments were received from Australia, Canada, China (People’s Rep. of), Japan, Malaysia, Thailand, and the EU.

5.1. Scope and Sections 2.2.1. and 2.2.2. – Infection with yellow head virus genotype 1 (Chapter 2.2.9.)

One comment was received regarding consistency of grammar and content in the standard sentence in the scope: that the scope refers to Genus and Family and not Order of the pathogenic agent. The Aquatic Animals Commission agreed with the comment and would check and amend the scope of chapters in the *Aquatic Code* and *Aquatic Manual* accordingly.

The revised scope and Sections 2.2.1. and 2.2.2. of Chapter 2.2.9. *Infection with yellow head virus genotype 1* is attached as **Annex 13** and is proposed for adoption at the 87th General Session in May 2019.

5.2. Infectious haematopoietic necrosis (Chapter 2.3.4.)

Section 1. *Scope*

A Member Country disagreed with the proposed amendment to the name of the pathogenic agent: Salmonid novirhabdovirus as some susceptible species are non-Salmonids. The Aquatic Animals Commission reiterated that the amendment was in accordance with the classification in the database of the International Committee of Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20171739).

Section 2.1.1. *Aetiological agent, agent strains*

In response to a comment to include 'with a mean period of occurrence of a single haplotype with a maximum of one calendar year, the genetic diversity of European IHNV is very high (Cieslak *et al.*, 2017)' at the end of the last paragraph, the Aquatic Animals Commission determined that the issue would be addressed when this chapter is reformatted using the new chapter template.

Section 2.2.1. *Susceptible host species*

The list of susceptible species was amended following the recommendations of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases (see Item 1.7.).

A Member Country asked for the rationale for not including Atlantic cod *(Gadus morhua)* and Japanese charr *(Salvelinus leucomaenis)* in the report of the *ad hoc* Group. The Aquatic Animals Commission clarified that the two species were not included in the report as they had not been scored in the assessments because of insufficient scientific evidence.

Section 2.2.6. *Persistent infection with lifelong carriers*

The Aquatic Animals Commission agreed to delete the words 'at normal temperatures' from the last sentence in this paragraph as they are inaccurate.

Section 2.4.3. *Immunostimulation*, Section 4. *Diagnostic methods* and Section 6. *Test(s) recommended for targeted surveillance to declare freedom from infectious haematopoietic necrosis*

In response to comments on immunostimulation, 'gold standard' diagnostic methods, and technical issues relating to the PCR protocol and recommendations for targeted surveillance, the Aquatic Animals Commission determined that these issues would be provided to the OIE Reference Laboratory expert who is currently updating and reformatting the chapter using the new chapter template.

The revised Chapter 2.3.4. *Infectious haematopoietic necrosis* is attached as **Annex 14** and is proposed for adoption at the 87th General Session in May 2019.

5.3. Infection with salmonid alphavirus (Chapter 2.3.6.)

The Aquatic Animals Commission reviewed the comments and amended text, where relevant.

Section 2.1.1. *Aetiological agent, agent strains*

The Aquatic Animals Commission agreed to delete the column on geographical distribution from Table 2.1. *SAV genotypes by host, environment and geographic distribution* as it would rapidly be out-dated. As the remaining information is important, the Commission agreed to retain it as Table 2.1. *SAV genotypes by susceptible species and environment*.

Section 2.1.2. *Survival outside the host*

A Member Country proposed deletion of text referring to long-distance spread of fat droplets from dead fish from which SAV can be detected. The Aquatic Animals Commission disagreed with the request as this is important information that is supported by a scientific publication.

Section 2.2.1. *Susceptible host species*

A Member Country has proposed adding ballan wrasse *(Labrus bergylta)* to Section 2.2.1. *Susceptible host species for this disease*. The *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases advised the Aquatic Animals Commission that to date there has only been one published study on this species and that until a corroborative study is published, the species should be added to Section 2.2.2. *Species with incomplete evidence for susceptibility*.

Section 7.1. *Definition of suspect case* and Section 7.2. *Definition of confirmed case*

The Aquatic Animals Commission agreed that the definitions of suspect and confirmed cases needed to be thoroughly reviewed. Rather than proposing amendments in a piecemeal manner, the Commission agreed that this revision would be undertaken systematically when the chapters are reformatted using the new chapter template.

4.3.1.1.2. *Reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and genotyping by sequencing*

The Aquatic Animals Commission agreed with a comment that for genotyping of different SAV-isolates, sequencing of the E2 gene is sufficient. Sequencing of the nsP3 gene may add information regarding deletions between different isolates but is not necessary and the Commission proposed deleting the reference to nsP3 of the third paragraph of the Section. Consequently, it also proposed deleting the nsP3-primerset in Table 3.1. *Characteristic of primers and probe sequences*.

The revised Chapter 2.3.6. *Infection with salmonid alphavirus* is attached as **Annex 15** and is proposed for adoption at the 87th General Session in May 2019.

5.4. Scope and Sections 2.2.1. and 2.2.2. – Koi herpesvirus disease (Chapter 2.3.7.)

Title

The Aquatic Animals Commission applied the naming convention “infection with [pathogenic agent]” to the title of the chapter and throughout.

Section 2.2.1. *Susceptible host species*

A Member Country reiterated a comment regarding the common name of one of the susceptible fish species given in this Section. The Aquatic Animals Commission stated that it uses the common names included in the FAOTERM database. If there is any confusion, Member Countries should rely on the Latin names, which are always given.

The revised Scope and Sections 2.2.1. and 2.2.2. of Chapter 2.3.7. *Koi herpesvirus disease* are attached as **Annex 16** and are proposed for adoption at the 87th General Session in May 2019.

6. Other *Aquatic Manual* chapters

6.1. Revision of disease-specific chapters using the new chapter template

6.1.1. Spring viraemia of carp (Chapter 2.3.9.)

The Aquatic Animals Commission reviewed Chapter 2.3.9. *Infection with spring viraemia of carp virus*, which had been updated and reformatted using the new disease chapter template. In accordance with OIE protocol, all new text is double underlined and deleted text is struck through; the existing text to be retained has been left unmarked. Amendments to the structure of the chapter has resulted in some changes to the order of the sub-heading titles.

The revised Chapter 2.3.9. *Spring viraemia of carp* is presented in track changes (A) and clean text (B) at **Annex 21** for Member Country comments.

6.1.2. Infection with *Batrachochytrium salamandrivorans* (Chapter 2.1.X.)

The Aquatic Animals Commission reviewed a new draft Chapter 2.1.X. *Infection with Batrachochytrium salamandrivorans*, which had been developed by experts using the new disease chapter template.

The new Chapter 2.1.X. *Infection with Batrachochytrium salamandrivorans* is presented at **Annex 22** for Member Country comments.

6.2. Infection with *Gyrodactylus salaris* (Chapter 2.3.3.)

The decision by NCBI GenBank to reclassify gene sequences submitted as *G. thymalli* to *G. salaris* necessitates a revision to the guidance in the *Aquatic Manual*. The Aquatic Animals Commission recommended that the current approach to distinguishing *G. salaris* from *G. thymalli*, by comparison of the sequenced amplified CO1 fragments to reference sequences, continues. Given that the GenBank/EMBL resource is no longer suitable for this purpose, the information would need to be retained by the OIE Reference Laboratory expert for *G. salaris*. In addition, it was recommended to Member Countries that they seek guidance from the Reference Laboratory expert if they need to distinguish *G. salaris* from *G. thymalli*. In the longer term it is hoped that further molecular analysis of *G. salaris* and *G. thymalli* isolates will identify differences that can be used for the development of improved diagnostic assays (see Item 3.3.).

6.3. Infection with *Marteilia refringens* (Chapter 2.4.4.)

The Aquatic Animals Commission requested that the OIE Reference Laboratory expert for *M. refringens* revises the *Aquatic Manual* chapter to take into account the scientific information presented by Kerr *et al*. (2018) and to highlight to the Commission any diagnostic challenges that may arise (see Item 3.4.).

6.4. Chapters that are being updated and reformatted using the new chapter template

The Aquatic Animals Commission selected a number of chapters for reformating by applying the new *Aquatic Manual* chapter template and revisions, where relevant. The Commission will review these revised chapters in a progressive manner at future meetings. The first chapters to be reformatted are:

Chapter 2.3.2. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)

Chapter 2.3.3. Infection with *Gyrodactylus salaris*

Chapter 2.3.4. Infection with infectious haematopoietic necrosis

Chapter 2.3.6. Infection with salmonid alphavirus

Chapter 2.3.7. Infection with koi herpesvirus disease

Chapter 2.3.8. Infection with red sea bream iridoviral disease

Chapter 2.3.10. Infection with viral haemorrhagic septicaemia.

G. OIE REFERENCE CENTRES

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

No applications were received.

7.2. Review of annual reports of Reference Centre activities in 2018

Annual reports had been received from all but one OIE Reference Laboratory for diseases of aquatic animals and both Collaborating Centres for aquatic animal issues. In accordance with the adopted *Procedures for designation of OIE Reference Laboratories* (the SOPs) (http://www.oie.int/en/scientific-expertise/reference-laboratories/sops/) and the *Procedures for designation of OIE Collaborating Centres* http://www.oie.int/en/scientific-expertise/collaborating-centres/sops/, the Aquatic Animals Commission reviewed all the reports, noting in particular the performance of each Reference Centre with regard to fulfilling the Terms of Reference (ToR) to the benefit of OIE Member Countries. The Commission expressed its on-going appreciation for the enthusiastic support and expert advice given to the OIE by the Reference Centres. A small number of Reference Laboratories did not provide evidence of communication with other Reference Laboratories. They will be reminded that one of the ToRs is to 'establish and maintain a network with other OIE Reference Laboratories designated for the same pathogen or disease'.

7.4. Twinning

As of February 2019, six aquatic animal health twinning projects have been completed: Canada and Chile for infection with infectious salmon anaemia virus; Denmark and Republic of Korea for infection with viral haemorrhagic septicaemia virus; Japan and Indonesia for infection with koi herpesvirus; Norway and Brazil for infection with infectious salmon anaemia virus; USA and China (People's Rep. of) for infection with infectious haematopoietic necrosis virus; USA and Indonesia for crustacean diseases. Two other twinning projects are underway: Italy and Tunisia for viral encephalopathy and retinopathy; USA and Saudi Arabia for shrimp diseases.

The Aquatic Animals Commission reviewed one twinning project proposal between China (People's Rep. of) and Indonesia on infection with white spot syndrome virus and infection with infectious haematopoietic necrosis virus. The Commission provide comments concerning the objectives and workplan of the project.

H. OTHER ISSUES

8.1. Technical disease cards

8.1.1. Tilapia lake virus

The Aquatic Animals Commission reviewed the technical disease card for tilapia lake virus and amended the sections on geographical distribution and confirmatory test methods in line with recent publications.

The technical disease card is available on the OIE website at: http://www.oie.int/en/international-standard-setting/specialists-commissions-groups/aquatic-animal-commission-reports/disease-information-cards/

8.1.2. Infection with *Batrachochytrium salamandrivorans*

The Aquatic Animals Commission reviewed the technical disease card for Infection with *Batrachochytrium salamandrivorans* and concluded that no amendments were necessary.

The technical disease card is available on the OIE website at: http://www.oie.int/en/international-standard-setting/specialists-commissions-groups/aquatic-animal-commission-reports/disease-information-cards/

I. OIE GLOBAL CONFERENCE ON AQUATIC ANIMAL HEALTH

The Aquatic Animals Commission finalised the programme for the OIE Global Conference on Aquatic Animal Health: Collaboration, sustainability: our future to be held from 2 to 4 April 2019 in Santiago (Chile), ensuring that the Global Conference would be engaging and relevant to all Member Countries.

J. WORK PLAN OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION FOR 2018/2019

The Aquatic Animals Commission reviewed and updated its work programme. The programme will be comprehensively reviewed at the next Commission meeting.

The revised 2018/2019 work programme is presented at **Annex 24** for Member Country information.

K. NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 25 September to 2 October 2019.

…/Annexes

Annex 1

MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 7–14 February 2019

List of participants

MEMBERS OF THE COMMISSION

Dr Ingo Ernst
(President)
Director Aquatic Pest and Health Policy
Animal Division
Department of Agriculture and Water Resources
GPO Box 858 Canberra ACT 2601
AUSTRALIA
Tel.: +61 2 6272 5615
ingo.ernst@agriculture.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
Director
OIE SVC reference laboratory
NACA regional resource centre
State Key laboratory of aquatic animal health
Shenzhen Custom
General administrations of China Customs
Room 907 of 1011 building, Fuqiang Road, Futian Qu
Shenzhen, Guangdong province, 518045
P. R. CHINA
szc_liuhong@customs.gov.cn
709274714@qq.com

Dr Edmund Peeler
(Vice-President)
Group Manager Aquatic Pest & Pathogens
CEFAS
Barrack Road, Weymouth
Dorset, DT4 8UB UK
UNITED KINGDOM
Tel.: +44 (0)1305 206746
ed.peeler@cefas.co.uk

OIE HEADQUARTERS

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

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

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

Annex 2

MEETING OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Paris, 7–14 February 2019

____

Agenda

A. INTRODUCTIONS AND THE PERFORMANCE MANAGEMENT FRAMEWORK

B. ADOPTION OF THE AGENDA

C. COOPERATION WITH OTHER SPECIALIST COMMISSIONS

a) Joint meeting of the Biological Standards Commission and the Aquatic Animals Commission

D. OIE *AQUATIC ANIMAL HEALTH CODE*

1. Texts proposed for adoption at the General Session in May 2019

1.1. General comments

1.2. Glossary

1.3. Criteria for listing species as susceptible to infection with a specific pathogen (Chapter 1.5.)

1.4. Amendments to fish disease-specific chapters regarding susceptible species

1.4.1. Article 10.5.2. – Infection with salmonid alphavirus (Chapter 10.5.)

1.4.2. Article 10.7.2. – Infection with koi herpesvirus (Chapter 10.7.)

1.4.3. Article 10.9.2. – Infection with spring viraemia of carp virus (Chapter 10.9.)

1.5. Infection with Ranavirus (Chapter 8.3.)

1.6. Acute hepatopancreatic necrosis disease (Chapter 9.1.)

1.7. Articles 10.2.1. and 10.2.2. – Infection with *Aphanomyces invadans* (Chapter 10.2.)

1.8. Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)

1.9. Model Article X.X.8.

2. Texts circulated for Member Country comments

2.1. New draft chapter on Biosecurity for Aquaculture Establishments (Chapter 4.X.)

2.2. Discussion paper on Approaches for determining periods required to demonstrate disease freedom

3. Other *Aquatic Code* topics

3.1. Disease listed by the OIE (Chapter 1.3.)

3.1.1. Infection with shrimp haemocyte iridescent virus (SIHV)

Annex 2 (contd)

3.2. Model Article 10.X.13.

3.2.1. Model Article 10.X.3.

3.2.2. Article 10.6.13. – Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)

3.3. Chapter 10.3. – Infection with *Gyrodactylus salaris*

3.4. Chapter 11.4. – Infection with *Marteilia refringens*

E. *AD HOC* GROUPS

4.1. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases

4.2. Electronic *ad hoc* Group on Tilapia lake virus

F. OIE *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS*

5. Texts to be proposed for adoption at the 2019 General Session

5.1. Scope and Sections 2.2.1. and 2.2.2. – Infection with yellow head virus genotype 1 (Chapter 2.2.9.)

5.2. Infectious haematopoietic necrosis (Chapter 2.3.4.)

5.3. Infection with salmonid alphavirus (Chapter 2.3.6.)

5.4. Scope and Sections 2.2.1. and 2.2.2. – Koi herpesvirus disease (Chapter 2.3.7.)

6. Other *Aquatic Manual* topics

6.1. Revision of disease-specific chapters using the new chapter template

6.1.1. Spring viraemia of carp (Chapter 2.3.9.)

6.1.2. Infection with *Batrachochytrium salamandrivorans* (Chapter 2.1.X.)

6.2. Infection with *Gyrodactylus salaris* (Chapter 2.3.3.)

6.3. Infection with *Marteilia refringens* (Chapter 2.4.4.)

6.4. Chapters that are being updated and reformatted using the new chapter template

G. OIE REFERENCE CENTRES

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

7.2. Review of annual reports of Reference Centre activities in 2018

7.3. Twinning

H. OTHER ISSUES

8.1. Technical disease cards

8.1.1. Tilapia lake virus

8.1.2. Infection with *Batrachochytrium salamandrivorans*

I. OIE GLOBAL CONFERENCE ON AQUATIC ANIMAL HEALTH

J. WORK PLAN OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION FOR 2018/2019

K. NEXT MEETING

Annex 3

GLOSSARY

BASIC BIOSECURITY CONDITIONS

means a minimum set of conditions required to ensure *biosecurity* applying to for a particular *disease,* and a particular *zone* or in a country, *zone* or *compartment* that should include required to ensure adequate disease security, such as:

a) compulsory notification of the *disease*, including or suspicion of the *disease*, is compulsorily notifiable to the *Competent Authority*; and

b) an *early detection system* is in place within the *zone* or country; and

c) import requirements to prevent the introduction of the *pathogenic agent* *disease* into the a *free* country *country*, or *zone* or *compartment*, or the spread within or from *infected zones* and *protection zones,* in accordance with the relevant disease-specific chapter as outlined in the *Aquatic Code*, are in place.

Annex 4 A (Tracked changes)

CHAPTER 1.5.

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

Article 1.5.1.

Purpose

In each disease-specific chapter, Article X.X.2. lists the *aquatic animal* species that have been found to be susceptible to *infection* with the relevant *pathogenic agent*. The recommendations of each disease-specific chapter apply only to the species listed in Article X.X.2.

The purpose of this chapter is to provide criteria for determining which species are listed as susceptible in Article ~~1.5.2.~~ X.X.2. of each disease-specific chapter in the *Aquatic Code*.

Article 1.5.2.

Scope

~~Susceptibility may include clinical or non-clinical *infection* but does not include species that may carry the *pathogenic agent* without replication~~.

Species of *aquatic animals* are considered susceptible to *infection* with a *pathogenic agent* when the presence of a multiplying~~,~~ or developing ~~or latent~~ *pathogenic agent* has been demonstrated by the occurrence of natural *cases* or by experimental exposure that mimics natural transmission pathways. Susceptibility includes clinical or non-clinical *infection.*

The decision to list an individual ~~a~~ species as susceptible in a disease-specific chapter~~s~~ should be based on a finding that the evidence is definite in accordance with Article 1.5.3. ~~All species in a taxonomic group may be listed as susceptible when certain criteria are met in accordance with Article 1.5.9.~~ A taxonomic ranking higher than species is listed when the criteria in Article 1.5.9. are met.

~~However,~~ ~~possible~~ Possible susceptibility of a species is also important information and, in accordance with Article 1.5.8., these species are ~~this~~ ~~should also be~~ included in Section ~~2.2.1.~~ *2.2.2. Species with incomplete evidence for susceptibility* ~~entitled «Susceptible host species »~~ of the relevant disease-specific chapter of the *Aquatic Manual*~~.~~ ~~in accordance with Article 1.5.8.~~

Article 1.5.3.

Approach

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

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

2) criteria to determine whether the *pathogenic agent* has been adequately identified (as described in Article 1.5.5.);

3) criteria to determine whether the evidence indicates that presence of the *pathogenic agent* constitutes an *infection* (as described in Article1.5.6.).

Annex 4A (Tracked changes) (contd)

Article 1.5.4.

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

The evidence should be classified as transmission through:

1) natural occurrence; includes situations where *infection* has occurred without experimental intervention e.g. *infection* in wild or farmed populations; or

2) non-invasive experimental procedures; includes cohabitation with infected hosts, *infection* by immersion or ingestion; or

3) invasive experimental procedures; includes injection, exposure to unnaturally ~~unnaturally~~ high loads of ~~pathogen~~ *pathogenic agent*, or exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment.

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

Article 1.5.5.

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

The *pathogenic agent* should be identified and confirmed in accordance with the methods described in Section ~~7~~ 4 (diagnostic methods) ~~(corroborative diagnostic criteria)~~ of the relevant disease-specific chapter in the *Aquatic Manual*, or other methods that have been demonstrated to be equivalent.

Article 1.5.6.

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

A combination of the following criteria should be used to determine *infection* (see Article 1.5.7.):

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

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

C. clinical or pathological changes are associated with the *infection*;

D. the specific location of the ~~pathogen~~ *pathogenic agent* corresponds with the expected target tissues.

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

Article 1.5.7.

Outcomes of the assessment

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

1) transmission has been obtained naturally or by experimental procedures that mimic natural pathways for the *infection* in accordance with Article 1.5.4.;

AND

2) the identity of the *pathogenic agent* has been confirmed in accordance with Article 1.5.5.;

AND

3) there is evidence of *infection* with the *pathogenic agent* in the suspect host species in accordance with criteria A to D in Article 1.5.6.. Evidence to support criterion A alone is sufficient to determine *infection*. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D would be required to determine *infection*.

Article 1.5.8.

Species for which there is incomplete evidence for susceptibility

The decision to list a species as susceptible in Article 1.5.2. of each disease-specific chapter should be based on a finding that the evidence is definite.

However, after application of Article 1.5.7., if where there is insufficient incomplete evidence to demonstrate susceptibility of a species through the approach described in Article 1.5.3. because transmission does not mimic natural pathways of *infection*, or the identity of the *pathogenic agent* has not been confirmed, or *infection* is only partially supported, but partial information is available, these species information will be included in Section 2.2.2. *Species with incomplete evidence for susceptibility* of the relevant disease-specific chapter in the *Aquatic Manual*.

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

Article 1.5.9.

Listing susceptible species at a taxonomic ranking of Genus or higher than species Pathogenic agents with a broad host range

Some *pathogenic agents* have low host species specificity and can infect numerous species across multiple taxa. These *pathogenic agents* are eligible for assessment using this article if they have at least one *susceptible species* in each of three or more taxa at the ranking of Family. The outcome of applying this article may be that *susceptible species* are listed in Article X.X.2. of each disease-specific chapter at a ranking of Genus or higher. For *pathogenic agents* with that have a broad host range, it may be appropriate for the outcome of the assessment of susceptibility to can be made at a taxonomic ranking higher than species (e.g. genus, family). For a *pathogenic agent* to be considered to have a broad host range, and thus be a potential candidate for listing *susceptible species* at a taxonomic ranking of genus or higher, there must be at least one *susceptible species* within each of three or more host families. It may be appropriate for the outcome of the assessment to be made at a taxonomic classification higher than species for a pathogenic agent that has a broad host range. A pathogenic agent will be considered to have a broad host range when it has been demonstrated as susceptible in at least three families.

1) For *pathogenic agents* that have a broad host range low host species specificity, 1)A a decision to conclude susceptibility of species at for a taxonomic ranking of Genus or higher level above species should only be made where:

A. susceptibility has been demonstrated in at least one species from within each of three or more families;

AND

BAa) after application of Article 1.5.7., more than one species within the family taxonomic ranking has been found to be susceptible in accordance with the approach described in Article 1.5.3. criteria above;

AND

CBb) no species within the taxonomic group ranking has been found to be refractory non-susceptible to *infection*;

AND

Cc) The the taxa taxonomic ranking is at chosen should be the lowest level supported by this evidence of points A a) and b)B.

22) Evidence that a of non-susceptibility of a species is refractory to *infection* may include includes:

a)A. absence of *infection* in a species exposed to the *pathogenic agent* in natural settings where the pathogen *pathogenic agent* is known to be present and it has causes caused *infection* in co-located *susceptible species*;

OR

b)B. absence of *infection* in a species exposed to the *pathogenic agent* through a controlled challenges appropriately designed experimental procedures.

<u>Annex 4B</u> (clean version)

CHAPTER 1.5.

CRITERIA FOR LISTING SPECIES AS SUSCEPTIBLE TO INFECTION WITH A SPECIFIC PATHOGENIC AGENT

Article 1.5.1.

Purpose

In each disease-specific chapter, Article X.X.2. lists the *aquatic animal* species that have been found to be susceptible to *infection* with the relevant *pathogenic agent*. The recommendations of each disease-specific chapter apply only to the species listed in Article X.X.2.

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

Article 1.5.2.

Scope

Species of *aquatic animal*s are considered susceptible to *infection* with a *pathogenic agent* when the presence of a multiplying or developing *pathogenic agent* has been demonstrated by the occurrence of natural *cases* or by experimental exposure that mimics natural transmission pathways. Susceptibility includes clinical or non-clinical *infection*.

The decision to list an individual species as susceptible in a disease-specific chapter should be based on a finding that the evidence is definite in accordance with Article 1.5.3. A taxonomic ranking higher than species is listed when the criteria in Article 1.5.9. are met.

Possible susceptibility of a species is also important information and, in accordance with Article 1.5.8., these species are included in Section *2.2.2. Species with incomplete evidence for susceptibility* of the relevant disease-specific chapter of the *Aquatic Manual*.

Article 1.5.3.

Approach

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

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

2) criteria to determine whether the *pathogenic agent* has been adequately identified (as described in Article 1.5.5.);

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

Article 1.5.4.

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

The evidence should be classified as transmission through:

1) natural occurrence: includes situations where *infection* has occurred without experimental intervention e.g. *infection* in wild or farmed populations; or

2) non-invasive experimental procedures: includes cohabitation with infected hosts, *infection* by immersion or ingestion; or

3) invasive experimental procedures: includes injection, exposure to unnaturally high loads of *pathogenic agent*, or exposure to stressors (e.g. temperature) not encountered in the host's natural or culture environment.

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

Article 1.5.5.

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

The *pathogenic agent* should be identified and confirmed in accordance with the methods described in Section 4 (diagnostic methods) of the relevant disease-specific chapter in the *Aquatic Manual*, or other methods that have been demonstrated to be equivalent.

Article 1.5.6.

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

A combination of the following criteria should be used to determine *infection* (see Article 1.5.7.):

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

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

C. clinical or pathological changes are associated with the *infection*;

D. the specific location of the *pathogenic agent* corresponds with the expected target tissues.

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

Article 1.5.7.

Outcomes of the assessment

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

1) transmission has been obtained naturally or by experimental procedures that mimic natural pathways for the *infection* in accordance with Article 1.5.4.;

AND

2) the identity of the *pathogenic agent* has been confirmed in accordance with Article 1.5.5.;

AND

3) there is evidence of *infection* with the *pathogenic agent* in the suspect host species in accordance with criteria A to D in Article 1.5.6. Evidence to support criterion A alone is sufficient to determine *infection*. In the absence of evidence to meet criterion A, satisfying at least two of criteria B, C or D would be required to determine *infection*.

Article 1.5.8.

Species for which there is incomplete evidence for susceptibility

The decision to list a species as susceptible in Article 1.5.2. of each disease-specific chapter should be based on a finding that the evidence is definite.

However, after application of Article 1.5.7., if there is incomplete evidence to demonstrate susceptibility of a species but partial information is available, these species will be included in Section 2.2.2. *Species with incomplete evidence for susceptibility* of the relevant disease-specific chapter in the *Aquatic Manual*.

If there is incomplete evidence to demonstrate susceptibility of a species, the *Competent Authority* should, prior to the implementation of any import health measures for the species, undertake a *risk assessment* for the *pathogenic agent* under consideration, in accordance with the recommendations in Chapter 2.1.

Article 1.5.9.

Listing susceptible species at a taxonomic ranking of Genus or higher

Some *pathogenic agents* have low host species specificity and can infect numerous species across multiple taxa. These *pathogenic agents* are eligible for assessment using this article if they have at least one *susceptible species* in each of three or more taxa at the ranking of Family. The outcome of applying this article may be that *susceptible species* are listed in Article X.X.2. of each disease-specific chapter at a ranking of Genus or higher.

1) For *pathogenic agents* that have a low host species specificity, a decision to conclude susceptibility of species at a taxonomic ranking of Genus or higher should only be made where:

   a) after application of Article 1.5.7., more than one species within the taxonomic ranking has been found to be susceptible;

   AND

   b) no species within the taxonomic ranking has been found to be non-susceptible to *infection*;

   AND

   c) the taxonomic ranking is at the lowest level supported by evidence of points *a)* and *b)*.

2) Evidence of non-susceptibility of a species to *infection* includes:

   a) absence of *infection* in a species exposed to the *pathogenic agent* in natural settings where the *pathogenic agent* is known to be present and has caused *infection* in co-located *susceptible species*;

OR

b) absence of *infection* in a species exposed to the *pathogenic agent* through appropriately designed experimental procedures.

CHAPTER 10.5.

INFECTION WITH SALMONID ALPHAVIRUS

Article 10.5.1.

General provisions

For the purposes of the *Aquatic Code*, infection with salmonid alphavirus means *infection* with any subtype genotype of the *pathogenic agent* salmonid alphavirus (SAV), of the Genus *Alphavirus* and Family *Togaviridae*.

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

Article 10.5.2.

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.: Arctic charr *(Salvelinus alpinus)*, Atlantic salmon *(Salmo salar)*, brown trout *(Salmo trutta)*, common dab *(Limanda limanda)* and rainbow trout *(Onchorynchus mykiss)*. These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

[…]

CHAPTER 10.7.

INFECTION WITH KOI HERPESVIRUS

[…]

Article 10.7.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: All varieties and subspecies of common carp (*Cyprinus carpio* ~~*carpio*~~)~~,~~ and common carp hybrids (e.g. *Cyprinus carpio* x *Carassius auratus*)~~, ghost carp (*Cyprinus carpio goi*)~~, ~~and koi carp (*Cyprinus carpio koi*) and common carp hybrids (e.g. *Cyprinus carpio* x *Carassius auratus*)~~. ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

[…]

Annex 7

CHAPTER 10.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[…]

Article 10.9.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: all varieties and subspecies of common carp *(Cyprinus carpio carpio)*, bighead carp *(Aristichthys nobilis)*, bream *(Abramis brama)*, Caspian white fish *(Rutilus frisiikutum)*, common carp *(Cyprinus carpio carpio)*, fathead minnow *(Pimephales promelas)*, golden shiner *(Notemigonus crysoleucas)*, goldfish *(Carassius auratus)*, grass carp (white amur)*(Ctenopharyngodon idellaidellus)*, and koi carp *(Cyprinus carpio koi)*, crucian carp *(Carassius carassius)*, roach *(Rutilus rutilus)*, and sheatfish (also known as European or wels catfishor wels) *(Silurus glanis)*, and silver carp *(Hypophthalmichthys molitrix)*, bighead carp *(Aristichthys nobilis)*, grass carp (white amur) *(Ctenopharyngodon idellus)*, goldfish *(Carassius auratus)*, orfe *(Leuciscus idus)* and zebrafish *(Sander vitreus)* *(Danio rerio)* tench *(Tinca tinca)*. These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

[…]

CHAPTER 8.3.

INFECTION WITH *RANAVIRUS* SPECIES

Article 8.3.1.

For the purposes of the *Aquatic Code*, infection with ranavirus*Ranavirus* species means *infection* with any member virus species of the Genus *Ranavirus* and Family *Iridoviridae* in amphibians with the exception of epizootic haematopoietic necrosis virus and European catfish virus.

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

Article 8.3.2.

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.: all species of the Orders *Anura*Anura (frogs and toads) and *Caudata* Caudata (salamanders and newts). The recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 8.3.3.

Importation or transit of aquatic animal products for any purpose regardless of the infection with ranavirus *Ranavirus* species status of the exporting country, zone or compartment

1) *Competent Authorities* should not require any conditions related to *Ranavirus* species, regardless of the infection with ranavirus *Ranavirus* species 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 8.3.2. that are intended for any purpose and comply with Article 5.4.1.:

   a) heat sterilised hermetically sealed amphibian 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 all virus species of the genus*Ranavirus* species [with the exception of epizootic haematopoietic necrosis virus and European catfish virus]);

   b) cooked amphibian products that have been subjected to heat treatment at 65°C for at least 30 minutes (or any time/temperature equivalent that has been demonstrated to inactivate all virus species of the genus *Ranavirus* specieswith the exception of epizootic haematopoietic necrosis virus and European catfish virus]);

   c) pasteurised amphibian 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 all virus species of the genus *Ranavirus* species [with the exception of epizootic haematopoietic necrosis virus and European catfish virus]);

   d) mechanically dried amphibian products (i.e. a heat treatment at 100°C for at least 30 minutes or any time/temperature equivalent that has been demonstrated to inactivateall virus species of the genus *Ranavirus* species [with the exception of epizootic haematopoietic necrosis virus and European catfish virus]).

2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.3.2., other than those referred to in point 1 of Article 8.3.3., *Competent Authorities* should require the conditions prescribed in Articles 8.3.7. to 8.3.12. relevant to the infection with ranavirus *Ranavirus* species 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 8.3.2. but which could reasonably be expected to pose a *risk* of transmission of ~~ranavirus~~ *Ranavirus* species, 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.

Article 8.3.4.

Country free from infection with ~~ranavirus~~ *Ranavirus* species

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with ~~ranavirus~~ *Ranavirus* species if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with ~~ranavirus~~ *Ranavirus* species (see Article 8.3.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from infection with ~~ranavirus~~ *Ranavirus* species if:

1) none of the *susceptible species* referred to in Article 8.3.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 8.3.2. are present and the following conditions have been met:

   a) there has been no occurrence of infection with ~~ranavirus~~ *Ranavirus* species for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last ten years;

OR

3) the infection with ~~ranavirus~~ *Ranavirus* species status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of *Ranavirus* species;

OR

4) it previously made a *self-declaration of freedom* from infection with ~~ranavirus~~ *Ranavirus* species and subsequently lost its free status due to the detection of ~~ranavirus~~ *Ranavirus* species but the following conditions have been met:

   a) on detection of *Ranavirus* species, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of *Ranavirus* species, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with ~~ranavirus~~ *Ranavirus* species; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of *Ranavirus* species.

In the meantime, part or all of the unaffected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 8.3.5.

Article 8.3.5.

Zone or compartment free from infection with ~~ranavirus~~ *Ranavirus* species

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

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from infection with ~~ranavirus~~ *Ranavirus* species may be declared free by the *Competent Authority* of the country concerned if:

1) none of the *susceptible species* referred to in Article 8.3.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 8.3.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a) there has been no occurrence of infection with ~~ranavirus~~ *Ranavirus* species for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last ten years;

OR

3) the infection with ~~ranavirus~~ *Ranavirus* species status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of *Ranavirus* species;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from infection with ~~ranavirus~~ *Ranavirus* species and subsequently lost its free status due to the detection of *Ranavirus* species in the *zone* but the following conditions have been met:

   a) on detection of *Ranavirus* species, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of *Ranavirus* species, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with ~~ranavirus~~ *Ranavirus* species; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of *Ranavirus* species.

Annex 8 (contd)

Article 8.3.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with ~~ranavirus~~ *Ranavirus* species following the provisions of points 1 or 2 of Articles 8.3.4. or 8.3.5. (as relevant) may maintain its status as free from infection with ~~ranavirus~~ *Ranavirus* species provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with ~~ranavirus~~ *Ranavirus* species following the provisions of point 3 of Articles 8.3.4. or 8.3.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions that are conducive to clinical expression of infection with ~~ranavirus~~ *Ranavirus* species, as described in the corresponding chapter of the *Aquatic Manual*, and *basic biosecurity conditions* are continuously maintained.

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

Article 8.3.7.

Importation of aquatic animals or aquatic animal products from a country, zone or compartment declared free from infection with ~~ranavirus~~ *Ranavirus* species

When importing *aquatic animals* of a species referred to in Article 8.3.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with ~~ranavirus~~ *Ranavirus* species, 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*. The *international aquatic animal health certificate* should state that, on the basis of the procedures described in Articles 8.3.4. or 8.3.5. (as applicable) and 8.3.6., the place of production of the *aquatic animals* or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with ~~ranavirus~~ *Ranavirus* species.

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal products* listed in point 1 of Article 8.3.3.

Article 8.3.8.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with ~~ranavirus~~ *Ranavirus* species

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 8.3.2. from a country, *zone* or *compartment* not declared free from infection with ~~ranavirus~~ *Ranavirus* species, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving the *quarantine* ~~facility~~ (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1~~)~~ of Article 8.3.3. or other products authorised by the *Competent Authority*; and

   c~~b~~*)* the treatment of all transport water, equipment, effluent and waste materials to inactivate *Ranavirus* species in accordance with Chapters 4.3., 4.7. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

   a) in the *exporting country*:

      i) identify potential source populations and evaluate their *aquatic animal* health records;

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with ~~ranavirus~~ *Ranavirus* species;

   b) in the *importing country*:

      i) import the F-0 population into a *quarantine* facility;

      ii) test the F-0 population for *Ranavirus* species in accordance with Chapter 1.4. to determine their suitability as broodstock;

      iii) produce a first generation (F-1) population in *quarantine*;

      iv) culture the F-1 population in *quarantine* under conditions that are conducive to the clinical expression of infection with ~~ranavirus~~ *Ranavirus* species, and sample and test for *Ranavirus* species in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter 2.1.2. of the *Aquatic Manual*;

      v) if *Ranavirus* species ~~is~~ are not detected in the F-1 population, it may be defined as free from infection with ~~ranavirus~~ *Ranavirus* species and may be released from *quarantine*;

      vi) if *Ranavirus* species are detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.7.

Article 8.3.9.

Importation of aquatic animals or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with ~~ranavirus~~ *Ranavirus* species

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 8.3.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from infection with ~~ranavirus~~ *Ranavirus* species, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 8.3.3. or in point 1 of Article 8.3.12., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of *Ranavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of *Ranavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

For these *aquatic animals* or *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product* being used for any purpose other than for human consumption.

Article 8.3.10.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption, including animal feed and agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with ~~ranavirus~~ *Ranavirus* species

When importing *aquatic animals* of a species referred to in Article 8.3.2., or *aquatic animal products* derived thereof, intended for uses other than human consumption, including animal *feed* and agricultural, industrial, research or pharmaceutical use, from a country, *zone* or *compartment* not declared free from infection with ~~ranavirus~~ *Ranavirus* species, the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in point 1 of Article 8.3.3. or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of *R~~r~~anavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of *R~~r~~anavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

Article 8.3.11.

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with ~~ranavirus~~ *Ranavirus* species

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 8.3.2. from a country, *zone* or *compartment* not declared free from infection with ~~ranavirus~~ *Ranavirus* species, the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of *R~~r~~anavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of *R~~r~~anavirus* species or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.; and

4) the carcasses are disposed of in accordance with Chapter 4.7.

Article 8.3.12.

Importation (or transit) of aquatic animal products for retail trade for human consumption regardless of the infection with ~~ranavirus~~ *Ranavirus* species status of the exporting country, zone or compartment

1) *Competent Authorities* should not require any conditions related to *R~~r~~anavirus* species, regardless of the infection with ~~ranavirus~~*Ranavirus* species status of the *exporting country*, *zone* or *compartment*, when authorising the importation (or transit) of the following *aquatic animal products* that have been prepared and packaged for retail trade and comply with Article 5.4.2.:

   – no *aquatic animal products* listed.

2) When importing *aquatic animal products*, other than those referred to in point 1 above, derived from a species referred to in Article 8.3.2. from a country, *zone* or *compartment* not declared free from infection with ~~ranavirus~~*Ranavirus* species, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

CHAPTER 9.1.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

Article 9.1.1.

For the purposes of the *Aquatic Code*, acute hepatopancreatic necrosis disease (AHPND) means *infection* with strains of *Vibrio parahaemolyticus (Vp_{AHPND})*, of the Family Vibrionaceae, that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB.

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

Article 9.1.2.

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.: giant tiger prawn *(Penaeus monodon)* and whiteleg shrimp *(Penaeus vannamei)*.

Article 9.1.3.

Importation or transit of aquatic animal products for any purpose regardless of the AHPND status of the exporting country, zone or compartment

1) *Competent Authorities* should not require any conditions related to AHPND, regardless of the AHPND 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 9.1.2., which are intended for any purpose and comply with Article 5.4.1.:

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

   b) cooked crustacean products that have been subjected to heat treatment at 100°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate Vp_{AHPND};

   c) crustacean oil;

   d) crustacean *meal*;

   e) chemically extracted chitin.

2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 9.1.2., other than those referred to in point 1 of Article 9.1.3., *Competent Authorities* should require the conditions prescribed in Articles 9.1.7. to 9.1.12. relevant to the AHPND 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 9.1.2. but which could reasonably be expected to pose a *risk* of transmission of Vp_{AHPND} ~~AHPND~~, 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.

Article 9.1.4.

Country free from AHPND

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from AHPND if all the areas covered by the shared water bodies are declared countries or *zones* free from AHPND (see Article 9.1.5.).

As described in Article 1.4.6., a country may make a *self-declaration of freedom* from AHPND if:

1) none of the *susceptible species* referred to in Article 9.1.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.1.2. are present and the following conditions have been met:

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

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the AHPND status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of Vp_{AHPND} ~~AHPND~~;

OR

4) it previously made a *self-declaration of freedom* from AHPND and subsequently lost its free status due to the detection of Vp_{AHPND} ~~AHPND~~ but the following conditions have been met:

   a) on detection of Vp_{AHPND} ~~AHPND~~, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of Vp_{AHPND} ~~AHPND~~, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of AHPND; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of Vp_{AHPND} ~~AHPND~~.

In the meantime, part or all of the unaffected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 9.1.5.

Article 9.1.5.

Zone or compartment free from AHPND

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

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from AHPND may be declared free by the *Competent Authority* of the country concerned if:

1) none of the *susceptible species* referred to in Article 9.1.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 9.1.2. are present in the *zone* or *compartment* and the following conditions have been met:

   a) there has not been any occurrence of AHPND for at least the last ten years despite conditions that are conducive to its clinical expression (as described in the corresponding chapter of the *Aquatic Manual*); and

   b) *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

3) the AHPND status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of Vp_{AHPND} ~~AHPND~~;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from AHPND and subsequently lost its free status due to the detection of Vp_{AHPND} ~~AHPND~~ in the zone but the following conditions have been met:

   a) on detection of Vp_{AHPND} ~~AHPND~~, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of Vp_{AHPND} ~~AHPND~~, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of AHPND; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of Vp_{AHPND} ~~AHPND~~.

Article 9.1.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from AHPND following the provisions of points 1 or 2 of Articles 9.1.4. or 9.1.5. (as relevant) may maintain its status as free from AHPND provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from AHPND following the provisions of point 3 of Articles 9.1.4. or 9.1.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions are conducive to clinical expression of AHPND, as described in the corresponding chapter of the *Aquatic Manual*, and *basic biosecurity conditions* are continuously maintained.

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

Annex 9 (contd)

Article 9.1.7.

Importation of aquatic animals or aquatic animal products from a country, zone or compartment declared free from AHPND

When importing *aquatic animals* of a species referred to in Article 9.1.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from AHPND, 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* or a *certifying official* approved by the *importing country*. The *international aquatic animal health certificate* should state that, on the basis of the procedures described in Articles 9.1.4. or 9.1.5. (as applicable) and 9.1.6., the place of production of the *aquatic animals* or *aquatic animal products* is a country, *zone* or *compartment* declared free from AHPND.

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal products* listed in point 1 of Article 9.1.3.

Article 9.1.8.

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

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving the *quarantine* ~~facility~~ (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1~~)~~ of Article 9.1.3. or other products authorised by the *Competent Authority*; and

   ~~*b)*~~*c)* the treatment of transport water, equipment, effluent and waste materials to inactivate Vp_{AHPND} in accordance with Chapters 4.3., 4.7. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following.

   a) In the *exporting country*:

      i) identify potential source populations and evaluate their *aquatic animal* health records;

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for AHPND.

   b) In the importing country:

      i) import the F-0 population into a *quarantine* facility;

      ii) test the F-0 population for Vp_{AHPND} in accordance with Chapter 1.4. to determine their suitability as broodstock;

      iii) produce a first generation (F-1) population in *quarantine*;

iv) culture F-1 population in *quarantine* under conditions that are conducive to the clinical expression of AHPND (as described in Chapter 2.2.1. of the *Aquatic Manual*) and test for Vp_{AHPND} in accordance with Chapter 1.4.;

v) if Vp_{AHPND} is not detected in the F-1 population, it may be defined as free from AHPND and may be released from *quarantine*;

vi) if Vp_{AHPND} is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.7.

Article 9.1.9.

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

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 9.1.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in point 1 of Article 9.1.3. or in point 1 of Article 9.1.11., or other products authorised by the *Competent Authority*; and

2) all containers and water used in transport are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all processing effluent and waste materials are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

For these *aquatic animals* or *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animals* or *aquatic animal products* being used for any purpose other than for human consumption.

Article 9.1.10.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption including animal feed, or for agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from AHPND

When importing, for use in animal feed or for agricultural, industrial, research or pharmaceutical use, *aquatic animals* of a species referred to in Article 9.1.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in point 1 of Article 9.1.3. or other products authorised by the *Competent Authority*; and

2) all containers and water used in transport are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all processing effluent and waste materials are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

Article 9.1.11.

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from AHPND

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of Vp_{AHPND} or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.; and

4) the carcasses are disposed of in accordance with Chapter 4.7.

Article 9.1.12.

Importation (or transit) of aquatic animal products for retail trade for human consumption regardless of the AHPND status of the exporting country, zone or compartment

1) *Competent Authorities* should not require any conditions related to Vp_{AHPND} ~~AHPND~~, regardless of the AHPND status of the *exporting country*, *zone* or *compartment*, when authorising the importation (or transit) of frozen peeled shrimp (shell off, head off) that have been prepared and packaged for retail trade and comply with Article 5.4.2.

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

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

2) When importing *aquatic animal products*, other than those referred to in point 1 above, derived from a species referred to in Article 9.1.2. from a country, *zone* or *compartment* not declared free from AHPND, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

CHAPTER 10.2.

INFECTION WITH *APHANOMYCES INVADANS* (EPIZOOTIC ULCERATIVE SYNDROME)

Article 10.2.1.

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

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

Article 10.2.2.

Scope

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

CHAPTER 10.6.

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

Article 10.6.1.

For the purposes of the *Aquatic Code*, infection with infectious haematopoietic necrosis virus means *infection* with the *pathogenic agent* Ssalmonid *Novirhabdovirus* novirhabdovirus (also commonly known as infectious haematopoietic necrosis virus ([IHNV]) of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*.

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

Article 10.6.2.

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.: Arctic charr *(Salvelinus alpinus)*, Atlantic salmon *(Salmo salar)*, brook trout *(Salvelinus fontinalis)*, brown trout *(Salmo trutta)*, chinook salmon *(Oncorhynchus tshawytscha)*, chum salmon *(Oncorhynchus keta)*, coho salmon *(Oncorhynchus kisutch)*, cutthroat trout *(Onchorynchus clarkii)*, lake trout *(Salvelinus namaycush)*, maseou salmon *(Oncorhynchus masou)*, marble trout *(Salmo marmoratus)*, pike *(Esox lucius)*, rainbow trout or steelhead *(Oncorhynchus mykiss)*, the Pacific salmon species (chinook [*Oncorhynchus tshawytscha*], sockeye [*Oncorhynchus nerka*], chum [*Oncorhynchus keta*], masou [*Oncorhynchus masou*], pink [*Oncorhynchus rhodurus*] and coho [*Oncorhynchus kisutch*]), and sockeye salmon (*Oncorhynchus nerka*) Atlantic salmon *(Salmo salar)*. These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.

Article 10.6.3.

Importation or transit of aquatic animal products for any purpose regardless of the infection with IHNV status of the exporting country, zone or compartment

1) *Competent Authorities* should not require any conditions related to IHNV, regardless of the infection with IHNV 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 10.6.2. that are intended for any purpose and comply with Article 5.4.1.:

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

   b) pasteurised fish products that have been subjected to a heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate IHNV);

   c) mechanically dried eviscerated fish (i.e. a heat treatment at 100˚C for at least 30 minutes or any time/temperature equivalent that has been demonstrated to inactivate IHNV);

   d) fish oil;

   e) fish *meal*;

   f) fish skin leather.

2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 10.6.2., other than those referred to in point 1 of Article 10.6.3., *Competent Authorities* should require the conditions prescribed in Articles 10.6.7. to 10.6.13. relevant to the infection with IHNV 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 10.6.2. but which could reasonably be expected to pose a *risk* of transmission of IHNV, 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.

Article 10.6.4.

Country free from infection with IHNV

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with IHNV if all the areas covered by the shared water bodies are declared countries or *zones* free from infection with IHNV (see Article 10.6.5.).

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

1) none of the *susceptible species* referred to in Article 10.6.2. are present and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 10.6.2. are present and the following conditions have been met:

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

   b) *basic biosecurity conditions* have been continuously met for at least the last ten years;

OR

3) the infection with IHNV status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

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

OR

4) it previously made a *self-declaration of freedom* from infection with IHNV and subsequently lost its free status due to the detection of IHNV but the following conditions have been met:

   a) on detection of IHNV, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of IHNV, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with IHNV; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of IHNV.

   In the meantime, part or all of the unaffected area may be declared a free *zone* provided that such a part meets the conditions in point 3 of Article 10.6.5.

Article 10.6.5.

Zone or compartment free from infection with IHNV

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

As described in Article 1.4.6., a *zone* or *compartment* within the *territory* of one or more countries not declared free from infection with IHNV may be declared free by the *Competent Authority* of the country concerned if:

1) none of the *susceptible species* referred to in Article 10.6.2. are present in the *zone* or *compartment* and *basic biosecurity conditions* have been continuously met for at least the last two years;

OR

2) any of the *susceptible species* referred to in Article 10.6.2. are present in the *zone* or *compartment* and the following conditions have been met:

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

   b) *basic biosecurity conditions* have been continuously met for at least the last ten years;

OR

3) the infection with IHNV status prior to *targeted surveillance* is unknown but the following conditions have been met:

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

   b) *targeted surveillance*, as described in Chapter 1.4., has been in place, in the *zone* or *compartment*, for at least the last two years without detection of IHNV;

OR

4) it previously made a *self-declaration of freedom* for a *zone* from infection with IHNV and subsequently lost its free status due to the detection of IHNV in the *zone* but the following conditions have been met:

   a) on detection of IHNV, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of IHNV, and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with IHNV; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last two years without detection of IHNV.

Annex 11 (contd)

Article 10.6.6.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with IHNV following the provisions of points 1 or 2 of Articles 10.6.4. or 10.6.5. (as relevant) may maintain its status as free from infection with IHNV provided that *basic biosecurity conditions* are continuously maintained.

A country, *zone* or *compartment* that is declared free from infection with IHNV following the provisions of point 3 of Articles 10.6.4. or 10.6.5. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions that are conducive to clinical expression of infection with IHNV, as described in the corresponding chapter of the *Aquatic Manual*, and *basic biosecurity conditions* are continuously maintained.

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

Article 10.6.7.

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

When importing *aquatic animals* of a species referred to in Article 10.6.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with IHNV, 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*. The *international aquatic animal health certificate* should state that, on the basis of the procedures described in Articles 10.6.4. or 10.6.5. (as applicable) and 10.6.6., the place of production of the *aquatic animals* or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with IHNV.

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal products* listed in point 1 of Article 10.6.3.

Article 10.6.8.

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

When importing for *aquaculture*, *aquatic animals* of a species referred to in Article 10.6.2. from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving the *quarantine* ~~facility~~ (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1~~)~~ of Article 10.6.3. or other products authorised by the *Competent Authority*; and

   ~~b~~c) the treatment of all transport water, equipment, effluent and waste materials to inactive IHNV in accordance with Chapters 4.3., 4.7. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

a) In the *exporting country*:

i) identify potential source populations and evaluate their *aquatic animal* health records;

ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with IHNV.

b) In the *importing country:*

i) import the F-0 population into a *quarantine* facility;

ii) test the F-0 population for IHNV in accordance with Chapter 1.4. to determine their suitability as broodstock;

iii) produce a first generation (F-1) population in *quarantine*;

iv) culture the F-1 population in *quarantine* for a duration sufficient for, and under conditions that are conducive to, the clinical expression of infection with IHNV, and sample and test for IHNV in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter 2.3.4. of the *Aquatic Manual*;

v) if IHNV is not detected in the F-1 population, it may be defined as free from infection with IHNV and may be released from *quarantine*;

vi) if IHNV is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.7.

Article 10.6.9.

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

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 10.6.2. or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 10.6.3. or in point 1 of Article 10.6.12., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials from the holding of the *aquatic animals* are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

For these *aquatic animals* or *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product* being used for any purpose other than for human consumption.

Article 10.6.10.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption, including animal feed and agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with IHNV

When importing *aquatic animals* of a species referred to in Article 10.6.2., or *aquatic animal products* derived thereof, intended for uses other than human consumption, including animal *feed* and agricultural, industrial**,** research or pharmaceutical use, from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in point 1 of Article 10.6.3. or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.

Article 10.6.11.

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with IHNV

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 10.6.2. from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3., 4.7. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of IHNV or disposed of in a biosecure manner in accordance with Chapters 4.3. and 4.7.; and

4) the carcasses are disposed of in accordance with Chapter 4.7.

Article 10.6.12.

Importation (or transit) of aquatic animal products for retail trade for human consumption regardless of the infection with IHNV status of the exporting country, zone or compartment

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

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

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

2) When importing *aquatic animal products*, other than those referred to in point 1 above, derived from a species referred to in Article 10.6.2. from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Model Article X.X.8. for all disease-specific chapters (or Article 10.4.12. for infection with infectious salmon anaemia virus)

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from 'infection with pathogen X'/ 'disease X'

When importing for *aquaculture*, *aquatic animals* of species referred to in Article X.X.2. from a country, *zone* or *compartment* not declared free from 'infection with pathogen X'/'*disease X'*, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1) and 2) below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving ~~the~~ *quarantine* ~~facility~~ (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1~~)~~ of Article X.X.3. or other products authorised by the *Competent Authority*; and

   ~~*b)*~~*c)* the treatment of all transport water, equipment, effluent and waste materials to inactive 'pathogen X' in accordance with Chapters 4.3., 4.7. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

   a) In the *exporting country*:

      i) identify potential source populations and evaluate their *aquatic animal* health records;

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for 'infection with pathogen X'/ 'disease X'.

   b) In the *importing country*:

      i) import the F-0 population into a *quarantine* facility;

      ii) test the F-0 population for 'pathogen X' in accordance with Chapter 1.4. to determine their suitability as broodstock;

      iii) produce a first generation (F-1) population in *quarantine*;

      iv) culture the F-1 population in *quarantine* for a duration sufficient for, and under conditions that are conducive to~~,~~ the clinical expression of 'infection with pathogen X'/'disease X' (as described in Chapter X.X.X. of the *Aquatic Manual*) and test for 'pathogen X' in accordance with Chapter 1.4.;

      v) if 'pathogen X' is not detected in the F-1 population, it may be defined as free from 'infection with pathogen X'/'disease X' and may be released from *quarantine*;

      vi) if 'pathogen X' is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.7.

Annex 13

CHAPTER 2.2.9.

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

1. Scope

Infection with yellow head virus genotype 1 means infection with the pathogenic agent yellow head virus genotype 1 (YHV1) of the Ggenus *Okavirus*, and Family *Roniviridae* and Order *Nidovirales*.

[…]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing a species as susceptible to infection with YHV1 according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) include: Blue shrimp (*Penaeus stylirostris*), dagger blade grass shrimp (*Palaemonetes pugio*), giant tiger prawn (*Penaeus monodon*), grass shrimp (*Palaemonetes pugio*), jinga shrimp (*Metapenaeus affinis*) and whiteleg shrimp (*Penaeus vannamei*).giant tiger prawn (*P. monodon*), white leg shrimp (*P. vannamei*), blue shrimp (*P. stylirostris*), daggerblade grass shrimp (*Palaemonetes pugio*), and Jinga shrimp (*Metapenaeus affinis*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing a species as for susceptibility susceptible to infection with YHV1 according to Chapter 1.5 of the *Aquatic Code* include: Banana prawn (*Penaeus merguiensis*), Carpenter prawn (*Palaemon serrifer*), kuruma prawn (*Penaeus japonicus*), northern brown shrimp (*Penaeus aztecus*), northern pink shrimp (*Penaeus duorarum*), northern white shrimp (*Penaeus setiferus*), Pacific blue prawn (*Palaemon styliferus*), red claw crayfish (*Cherax quadricarinatus*), Sunda river prawn (*Macrobrachium sintangense*) and yellow shrimp (*Metapenaeus brevicornis*). Sunda river prawn (*Macrobrachium sintangense*), yellow shrimp (*Metapenaeus brevicornis*), Carpenter prawn (*Palaemon serrifer*), Pacific blue prawn (*Palaemon styliferus*), northern brown shrimp (*Penaeus aztecus*), northern pink shrimp (*Penaeus duorarum*), kuruma prawn (*Penaeus japonicus*), banana prawn (*Penaeus merguiensis*), northern white shrimp (*Penaeus setiferus*) and red claw crayfish (*Cherax quadricarinatus*). Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is YHV1, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

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: Acorn barnacle (*Chelonibia patula*), blue crab (*Callinectes sapidus*), cyclopoid copepod (*Ergasilus manicatus*), gooseneck barnacle (*Octolasmis muelleri*), Gulf killifish (*Fundulus grandis*) and paste shrimp (*Acetes* sp.).

[…]

Annex 14

CHAPTER 2.3.4

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infectious haematopoietic necrosis (IHN) Infection with infectious haematopoietic necrosis virus means infection with the *pathogenic agent* Ssalmonid nN*ovirhabdovirus* (also known as infectious haematopoietic necrosis virus [IHNV]) of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*. is a viral disease affecting most species of salmonid fish reared in fresh water or sea water. Caused by the rhabdovirus, infectious haematopoietic necrosis virus (IHNV), the principal clinical and economic consequences of IHN occur on farms rearing rainbow trout where acute outbreaks can result in very high mortality. However, both Pacific and Atlantic salmon can be severely affected. For the purpose of this chapter, IHN is considered to be infection with IHNV.

2. Disease information

For detailed reviews of the disease, see Bootland & Leong (1999) or Wolf (1988).

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The fish rhabdovirus, IHNV, has a bullet-shaped virion containing a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides that encodes six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L). The presence of the unique NV gene and sequence similarity with certain other fish rhabdoviruses, such as viral haemorrhagic septicaemia virus, has resulted in the creation of the *Novirhabdovirus* genus of the family *Rhabdoviridae*, with IHNV as the type species. The type strain of IHNV is the Western Regional Aquaculture Center (WRAC) strain available from the American Type Culture Collection (ATCC VR-1392). The GenBank accession number of the genomic sequence of the WRAC strain is L40883 (Morzunov *et al*., 1995; Winton & Einer-Jensen, 2002).

Sequence analysis has been used to compare IHNV isolates from North America, Europe and Asia (Emmenegger *et al*., 2000; Enzmann *et al*., 2005; Enzmann *et al*., 2010; Johansson *et al*., 2009; Kim *et al*., 2007; Kolodziejek *et al*., 2008; Kurath *et al*., 2003; Nishizawa *et al*., 2006; Troyer & Kurath, 2003). Within the historical natural range of the virus in western North America, most isolates of IHNV from Pacific salmon form two genogroups that are related to geographical location and not to year of isolation or host species. The isolates within these two genogroups show a relatively low level of nucleotide diversity, suggesting evolutionary stasis or an older host-pathogen relationship. Conversely, isolates of IHNV from farmed rainbow trout in the USA form a third genogroup with more genetic diversity and an evolutionary pattern indicative of ongoing adaptation to a new host or rearing conditions. Isolates from farmed rainbow trout in Europe and Asia appear to have originated from North America, but show further, independent, divergence within their new geographical range (Enzmann *et al*., 2010; Kim *et al*., 2007; Nishizawa *et al*., 2006).

On the basis of antigenic studies using neutralising polyclonal rabbit antisera, IHNV isolates form a single serogroup (Engelking *et al.,* 1991), while mouse monoclonal antibodies have revealed a number of neutralising epitopes on the glycoprotein (Huang *et al.,* 1994; Ristow & Arnzen De Avila, 1991; Winton *et al.,* 1988), as well as the existence of a non-neutralising group epitope borne by the nucleoprotein (Ristow & Arnzen, 1989). However, there appears to be little or no correlation between genotypes and serotypes (Johansson *et al.,* 2009). Variations in the virulence and host preference of IHNV strains have been recorded during both natural cases of disease and in experimental infections (Garver *et al.,* 2006; LaPatra *et al.,* 1993a).

2.1.2. Survival outside the host

IHNV is heat, acid and ether labile. The virus will survive in fresh water for at least 1 month at cooler temperatures, especially if organic material is present.

2.1.3. Stability of the agent (effective inactivation methods)

IHNV is readily inactivated by common disinfectants and drying (Wolf, 1988).

2.1.4. Life cycle

Reservoirs of IHNV are clinically infected fish and covert carriers among cultured, feral or wild fish. Virus is shed via urine, sexual fluids and from external mucus, whereas kidney, spleen and other internal organs are the sites in which virus is most abundant during the course of overt infection (Bootland & Leong, 1999; Wolf, 1988).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5. of the Aquatic Animal Health Code (*Aquatic Code*) include:~~ The principal hosts for IHNV are members of the family *Salmonidae*. Species reported to be naturally infected with IHNV include~~ Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), chum salmon (*Oncorhynchus keta*), coho salmon (*Oncorhynchus kisutch*), cutthroat trout (*Onchorynchus clarkii*), lake trout (*Salvelinus namaycush*), mas~~o~~u salmon (*Oncorhynchus masou*), marble trout (*Salmo marmoratus*), Northern pike (*Esox lucius*), rainbow trout ~~or steelhead~~ (*Oncorhynchus mykiss*) ~~Chinook (*O. tshawytscha*), sockeye (*O. nerka*), chum (*O. keta*), amago (*O. rhodurus*), masou (*O. masou*), coho (*O. kisutch*),~~ and sockeye salmon (*Oncorhynchus nerka*)~~ Atlantic salmon (*Salmo salar*). Other salmonids including brown trout (*S. trutta*) and cutthroat trout (*O. clarki*), some chars (*Salvelinus namaycush*, *S. alpinus*, *S. fontinalis*, and *S. leucomaenis*), ayu (*Plecoglossus altivelis*) and non-salmonids including European eel (*Anguilla anguilla*), herring (*Clupea pallasi*), cod (*Gadus morhua*), sturgeon (*Acipenser transmontanus*), pike (*Esox lucius*), shiner perch (*Cymatogaster aggregata*) and tube-snout (*Aulorhychus flavidus*) have occasionally been found to be infected in the wild or shown to be susceptible by a natural route of infection (Bootland & Leong, 1999; EFSA, 2008; Wolf, 1988).~~

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence of susceptibility ~~to fulfil the criteria for listing as susceptible to infection with IHNV~~ according to Chapter 1.5. of the *Aquatic Code* include: ~~Northern pike (*Esox lucius*),~~ Pacific herring (*Clupea pallasii*), shiner perch (*Cymatogaster aggregate*), tube-snout (*Aulorhynchus flavidus*), burbot (*Lota* lota) and white sturgeon (*Acipenser transmontanus*).

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: all varieties and subspecies of common carp (*Cyprinus carpio*) *and* American yellow perch (*Perca flavescens*).

2.2.~~2~~3. Susceptible stages of the host

Infection with IHNV occurs among several species of salmonids with fry being the most highly susceptible stage. Older fish are typically more resistant to clinical disease, but among individuals, there is a high degree of variation in susceptibility to infection with IHNV. As with viral haemorrhagic septicaemia virus, good fish health condition seems to decrease susceptibility to overt infection with IHNV, while co-infections with bacterial diseases (e.g. bacterial coldwater disease), handling and other stressors can cause subclinical infections to become overt. Fish become increasingly resistant to infection with age until spawning, when they once again become highly susceptible and may shed large amounts of virus in sexual products. Survivors of infection with IHNV demonstrate a strong protective immunity with the synthesis of circulating antibodies to the virus (LaPatra *et al*., 1993b).

2.2.~~3~~4. Species or subpopulation predilection (probability of detection)

IHNV shows a strong phylogeographic signature (Enzmann *et al*., 2010; Kurath *et al*., 2003; Nishizawa *et al*., 2006) that reflects the host species from which the virus is most commonly isolated in various geographical areas (e.g. sockeye salmon in the Northeast Pacific – U genogroup; Chinook salmon in California, USA – L genogroup; and rainbow trout in Europe, Asia and Idaho, USA – E, J and M genogroups, respectively).

2.2.45. Target organs and infected tissue

Virus entry is thought to occur through the gills and at bases of fins while kidney, spleen and other internal organs are the sites in which virus is most abundant during the course of overt infection (Bootland & Leong, 1999; Wolf, 1988).

2.2.56. Persistent infection with lifelong carriers

Historically, the geographic range of infection with IHNV was limited to western North America, but the disease has spread to Europe and Asia via the importation of infected fish and eggs. Once IHNV is introduced into a farmed stock, the disease may become established among susceptible species of wild fish in the watershed. The length that individual fish are infected with IHNV varies with temperature; however, unlike infectious pancreatic necrosis virus (IPNV) or channel catfish virus (CCV), a true, life-long carrier state with IHNV appears to be a rare event at normal temperatures.

2.2.67. Vectors

Horizontal transmission of IHNV is typically by direct exposure, but invertebrate vectors have been proposed to play a role in some cases (Bootland & Leong, 1999).

Mayfly (*Callibaetis* sp.) (Shors & Winston, 1988) and salmon lice (*Lepeophtheirus salmonis*) (Jakob *et al.,* 2011) are potential vectors for IHNV.

2.2.78. Known or suspected wild aquatic animal carriers

IHNV is endemic among many populations of free-ranging salmonids. A marine reservoir has been proposed, but not confirmed.

2.3. Disease pattern

Infection with IHNV often leads to mortality due to the impairment of osmotic balance and occurs within a clinical context of oedema and haemorrhage. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues, and cells of the kidney underlies the clinical signs.

2.3.1. Transmission mechanisms

The transmission of IHNV between fish is primarily horizontal and high levels of virus are shed from infected juvenile fish, however, cases of vertical or egg-associated transmission have been recorded. Although egg-associated transmission is significantly reduced by the now common practice of surface disinfection of eggs with an iodophor solution, it is the only mechanism accounting for the occurrence of infection with IHNV in new geographical locations among alevins originating from eggs that were incubated and hatched in virus-free water (Winton, 1991).

2.3.2. Prevalence

Infection with IHNV is endemic and widely prevalent among populations of free-ranging salmonids throughout much of its historical range along the west coast of North America. The virus has also become established with a high prevalence of infection in major trout growing regions of North America, Europe and Asia where IHNV was introduced through the movement of infected fish or eggs.

2.3.3. Geographical distribution

Infection with IHNV has been detected in North America, Asia and Europe, but not in the Southern Hemisphere. Countries reporting confirmed or suspect cases of infection with IHNV to the OIE include: Austria, Belgium, Canada, China (People's Rep. of), Croatia, Czech Republic, France, Germany, Iran, Italy, Japan, Korea (Rep. of), Netherlands, Poland, Russia, Slovenia, Spain, Switzerland and United States of America. Infections and overt disease have been reported among fish reared in both fresh and sea water.

2.3.4. Mortality and morbidity

Depending on the species of fish, rearing conditions, temperature, and, to some extent, the virus strain, outbreaks of infection with IHNV may range from explosive to chronic. Losses in acute outbreaks will exceed several per cent of the population per day and cumulative mortality may reach 90–95% or more (Bootland & Leong, 1999). In chronic cases, losses are protracted and fish in various stages of disease can be observed in the pond.

2.3.5. Environmental factors

The most important environmental factor affecting the progress of infection with IHNV is water temperature. Experimental trials have demonstrated infection with IHNV can produce mortality from 3°C to 18°C (Bootland & Leong, 1999); however, clinical disease typically occurs between 8°C and 15°C under natural conditions.

2.4. Control and prevention

Control methods for infection with IHNV currently rely on avoidance of exposure to the virus through the implementation of strict control policies and sound hygiene practices (Winton, 1991). The thorough disinfection of fertilised eggs, the use of virus-free water supplies for incubation and rearing, and the operation of facilities under established biosecurity measures are all critical for preventing infection with IHNV at a fish production site.

2.4.1. Vaccination

Experimental vaccines to protect salmonids against infection with IHNV have been the subject of research for more than 40 years with some showing promise in both laboratory and field trials when delivered by immersion or injection (Kurath, 2008; Winton, 1991; Winton, 1997). Both autogenous, killed vaccines and a DNA vaccine have been licensed for commercial use in Atlantic salmon net-pen aquaculture on the west coast of North America where such vaccines can be delivered economically by injection. However, vaccines against infection with IHNV have not yet been licensed in other countries where the application of vaccines to millions of smaller fish will require additional research on novel mass delivery methods.

2.4.2. Chemotherapy

Although chemotherapeutic approaches for control of infection with IHNV have been studied, they have not found commercial use in aquaculture against ~~IHNV~~ the disease (Winton, 1991).

2.4.3. Immunostimulation

Immunostimulants are an active area of research, but have not found commercial use in aquaculture against infection with IHNV.

2.4.4. Resistance breeding

Experimental trials of triploid or inter-species hybrids have shown promise (Barroso *et al*., 2008; Winton, 1991) and the genetic basis of resistance to IHNV has been an active area of recent research (Miller *et al*., 2004; Purcell *et al*., 2010).

2.4.5. Restocking with resistant species

Within endemic areas, the use of less susceptible species has been used to reduce the impact of infection with IHNV in aquaculture.

2.4.6. Blocking agents

Natural compounds have been identified from aquatic microbes that have antiviral activity; however, these have not found commercial use in aquaculture against infection with IHNV (Winton, 1991).

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs is a highly effective method to block egg-associated transmission of IHNV in aquaculture settings (Bovo *et al*., 2005). The method is widely practiced in areas where the virus is endemic.

2.4.8. General husbandry practices

In addition to disinfection of eggs, use of a virus-free water supply has been shown to be a critical factor in the management of infection with IHNV within endemic areas. Several approaches include use of wells or springs that are free of fish or other sources of IHNV and disinfection of surface water sources using UV light or ozone (Winton, 1991).

3. Sampling

3.1. Selection of individual specimens

Clinical inspections are best carried out during a period whenever the water temperature is below 14°C. All production units (ponds, tanks, net-cages, etc.) must be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention must be paid to the water outlet area where weak fish tend to accumulate.

In farms with salmonids, if rainbow trout are present, only fish of that species are selected for sampling. If rainbow trout are not present, the sample has to be obtained from fish of all other infection with IHNV susceptible species present, as listed in Section 2.2.1. 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 risk status).

If more than one water source is used for fish production, fish from all water sources must be included in the sample. If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish are selected. If such fish are not present, the fish selected must 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. Preservation of samples for submission

Before shipment or transfer to the laboratory, parts of the organs to be examined must be removed from the fish with sterile dissection instruments and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10% foetal calf serum (FCS) and antibiotics. Addition 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.

3.3. Pooling of samples

Ovarian fluid or organ pieces from a maximum of ten fish may be collected in one sterile tube containing at least 4 ml transport medium and this represents one pooled sample. The tissue in each sample should weigh a minimum of 0.5 g. The tubes should be placed in insulated containers (for instance, thick-walled polystyrene boxes) together with sufficient ice or 'freezer blocks' to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided. The temperature of a sample during transit.

should never exceed 10°C and ice should still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen. Virological examination must be started as soon as possible and not later than 48 hours after collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after collection of the material, provided that the material to be examined is protected by transport medium and that the temperature requirements during transportation are fulfilled.

Whole fish may be sent to the laboratory if the temperature requirements during transportation can be fulfilled. Whole fish may be wrapped in paper with absorptive capacity and must be shipped in a plastic bag, chilled as mentioned above. Live fish can also be shipped. All packaging and labelling must be performed in accordance with present national and international transport regulations, as appropriate.

3.4. Best organs or tissues

The optimal tissue material to be examined is spleen, anterior kidney, and either heart or encephalon. In some cases, ovarian fluid and milt must be examined.

In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. If a sample consisted of whole fish less than 4 cm long, these should be minced with sterile scissors or a scalpel, after removal of the body behind the gut opening. If a sample consisted of whole fish with a body length between 4 cm and 6 cm, the viscera, including kidney, should be collected. If a sample consisted of whole fish more than 6 cm long, tissue specimens should be collected as described above. The tissue specimens should be minced with sterile scissors or a scalpel, homogenised and suspended in transport medium.

3.5. Samples/tissues that are not suitable

IHNV is very sensitive to degradation, therefore sampling tissues with high enzymatic activities or large numbers of contaminating bacteria such as the intestine or skin should be avoided when possible. Muscle tissue is also less useful as it typically contains a lower virus load.

4. Diagnostic methods

The "Gold Standard" for detection of IHNV is the isolation of the virus in cell culture followed by its immunological or molecular identification. While the other diagnostic methods listed below can be used for confirmation of the identity of virus isolated in cell culture or for confirmation of overt infections in fish, they are not approved for use as primary surveillance methods for obtaining or maintaining approved infection with IHNV-free status.

Due to substantial variation in the strength and duration of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the viral status of fish populations. In the future, validation of serological techniques for diagnosis of fish virus infections could render the use of fish serology more widely acceptable for diagnostic purposes. However, when present, a positive serological response is considered presumptive evidence of past exposure to infection with IHNV (Jorgensen *et al*., 1991).

4.1. Field diagnostic methods

4.1.1. Clinical signs

The disease is typically characterised by gross signs that include lethargy interspersed with bouts of frenzied, abnormal activity, darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally.

4.1.2. Behavioural changes

During outbreaks, fish are typically lethargic with bouts of frenzied, abnormal activity, such as spiral swimming and flashing. A trailing faecal cast is observed in some species. Spinal deformities are present among some of the surviving fish (Bootland & Leong, 1999).

4.2. Clinical methods

4.2.1. Gross pathology

Affected fish exhibit darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally. Internally, fish appear anaemic and lack food in the gut. The liver, kidney and spleen are pale. Ascitic fluid is present and petechiae are observed in the organs of the body cavity.

4.2.2. Clinical chemistry

The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leucocytes and thrombocytes, and large amounts of cellular debris. As with other haemorrhagic viraemias of fish, blood chemistry is altered in severe cases (Bootland & Leong, 1999).

4.2.3. Microscopic pathology

Histopathological findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. Necrosis of eosinophilic granular cells in the intestinal wall is pathognomonic of infection with IHNV ~~infection~~ (Bootland & Leong, 1999).

4.2.4. Wet mounts

Wet mounts have limited diagnostic value.

4.2.5. Tissue imprints and smears

Necrobiotic bodies and foamy macrophages, indicative of a clinical manifestation of infection with IHNV, can be best observed using tissue imprints obtained from the kidney and spleen rather than smears.

4.2.6. Electron microscopy/cytopathology

Electron microscopy of virus-infected cells reveals bullet-shaped virions of approximately 150–190 nm in length and 65–75 nm in width (Wolf, 1988). The virions are visible at the cell surface or within vacuoles or intracellular spaces after budding through cellular membranes. The virion possesses an outer envelope containing host lipids and the viral glycoprotein spikes that react with immunogold staining to decorate the virion surface.

4.3. Agent detection and identification methods

The traditional procedure for detection of IHNV is based on virus isolation in cell culture. Confirmatory identification may be achieved by use of immunological (neutralisation, indirect fluorescent antibody test or enzyme-linked immunosorbent assay), or molecular (polymerase chain reaction, DNA probe or sequencing) methods (Arakawa *et al*., 1990; Arnzen *et al*., 1991; Deering *et al*., 1991; Dixon & Hill, 1984; Jorgensen *et al*., 1991; LaPatra *et al*., 1989; Purcell *et al*., 2006; Winton & Einer-Jensen, 2002).

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Wet mounts are not appropriate for detection or identification of IHNV.

4.3.1.1.2. Smears

Smears are not appropriate for detection or identification of IHNV.

4.3.1.1.3. Fixed sections

Immunohistochemistry and *in-situ* hybridisation (ISH) methods have been used in research applications, but are not appropriate for detection or identification of IHNV in a diagnostic setting.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Cell lines to be used: EPC or FHM.

Detection of virus through the development of viral cytopathic effect (CPE) in cell culture would be followed by virus identification through either antibody-based tests or nucleic acid-based tests. Any antibody-based tests would require the use of antibodies validated for their sensitivity and specificity.

4.3.1.2.1.1. Virus extraction

In the laboratory the tissue in the tubes must be completely homogenised (either by stomacher, blender mortar and pestle with sterile sand or any other suitable and validated homogeniser) and subsequently suspended in the original transport medium. The final ratio between tissue material and transport medium must be adjusted in the laboratory to 1:10.

The homogenate is centrifuged in a refrigerated centrifuge at 2°C–5°C at 2000–4000 ***g*** for 15 minutes and the supernatant collected and treated for either four hours at 15°C or overnight at 4°C with antibiotics (e.g. 1 mg ml^{-1} gentamicin may be useful at this stage). 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 aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary.

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

Optional treatment of homogenate to inactivate competing virus: treatment of inocula with antiserum to IPNV (which in some parts of the world occurs in 50% of fish samples) aims at preventing CPE due to IPNV from confounding the ability to detect IHNV in cell culture. When samples come from production units, which are considered free from IPN, treatment of inocula with antiserum to IPNV should be omitted. Prior to the inoculation of the cells, the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubated with this for a minimum of one hour at 15°C or a maximum of 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

4.3.1.2.1.2. Inoculation of cell monolayers

EPC or FHM cells are grown at ~~20–30~~ 25°C in suitable medium, e.g. Eagle's MEM (or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris/HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2. Cell cultures to be used for inoculation with tissue material should be young (4-48 hours old) and actively growing (not confluent) at inoculation.

Antibiotic-treated organ suspension is inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively, (in order to prevent homologous interference). The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are acceptable as well.

4.3.1.2.1.3. Incubation of cell cultures

Inoculated cell cultures are incubated at 15°C for 7–10 days. If the colour of the cell culture medium changes from red to yellow, indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances has to be performed to maintain cell susceptibility to virus infection.

At least every six months or if decreased cell susceptibility is suspected, titration of frozen stocks of IHNV is performed to verify the susceptibility of the cell cultures to infection.

4.3.1.2.1.4. Microscopy

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

4.3.1.2.1.5. Subcultivation

If no CPE has developed after the primary incubation for 7–10 days, subcultivation is performed to fresh cell cultures utilising a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to the cell line 7–10 days after inoculation. The pools are then inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in Section 4.3.1.2.1.2 above.

Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into a well with fresh cell culture (well-to-well subcultivation). In case of salmonid samples, the inoculation may be preceded by preincubation of the dilutions with the antiserum to IPNV at an appropriate dilution as described above.

The inoculated cultures are then incubated for 7–10 days at 15°C with observation as in Section 4.3.1.2.1.4. If toxic CPE occurs within the first three days of incubation, subcultivation may be performed at that stage, but the cells must then be incubated for seven days and subcultivated again with a further seven days incubation. When toxic CPE develops after three days, the cells may be passed once and incubated to achieve the total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final seven days of incubation.

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

If no CPE occurs the test may be declared negative.

4.3.1.2.2. Antibody-based antigen detection methods

4.3.1.2.2.1. Neutralisation test (identification in cell culture)

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge an aliquot at 2000 ***g*** for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.

ii) Dilute virus-containing medium from 10^2–10^4.

iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of an IHNV antibody solution.

The neutralising antibody (Nab) solution must have a 50% plaque reduction titre of at least 2000. Likewise, treat a set of aliquots of each virus dilution with cell culture medium to provide a non-neutralised control.

iv) In parallel, a neutralisation test must be performed against a homologous IHNV strain (positive neutralisation test) to confirm the reactivity of the antiserum.

v) Incubate all the mixtures at 15°C for 1 hour.

vi) Transfer aliquots of each of the above mixtures on to 24-hour-old monolayers overlaid with cell culture medium containing 10% FBS (inoculate two wells per dilution) and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

vii) Check the cell cultures for the onset of CPE and read the results for each suspect IHNV sample as soon as it CPE occurs in non-neutralised controls. Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

viii) The tested virus is identified as IHNV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IHNV-specific antibody, whereas CPE is evident in all other cell cultures.

Other neutralisation tests of proven efficiency may be used alternatively.

4.3.1.2.2.2. Indirect fluorescent antibody test (IFAT)

Antibody-based antigen detection methods such as IFAT, ELISA and various immunohistochemical procedures for the detection of IHNV have been developed over the years. These techniques can provide detection and identification relatively quickly compared with virus isolation in cell culture. However, various parameters such as antibody sensitivity and specificity and sample preparation can influence the results; a negative result should be viewed with caution. These techniques should not be used in attempts to detect carrier fish.

4.3.1.2.2.2.1. Indirect fluorescent antibody test in cell cultures

i) Prepare monolayers of cells in 2 cm^2 wells of cell culture plastic plates or on cover slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FBS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of black 96-well plates for immunofluorescence is recommended.

ii) When the cell monolayers are ready for infection (i.e. on the same day or on the day after seeding) inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of IHNV in a similar way, in order to obtain a virus titre of about 5,000–10,000 plaque-forming units (PFU) per ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with a cold mixture of acetone 30%/ethanol 70% (v/v) (stored at –20°C).

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.

vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

viii) Prepare a solution of purified IHNV antibody or serum in 0.01 M PBS, pH 7.2, containing 0.05% Tween-80 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur (e.g. by adding a piece of wet cotton to the humid chamber). The volume of solution to be used is 0.25 ml 2 cm^{-2} well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of FITC- or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover slips using, for example, glycerol saline, pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with × 10 eye pieces and × 20–40 objective lens having numerical aperture >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.3.1.2.2.2.2. Indirect fluorescent antibody test on imprints

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces together with the other organs required for virus isolation in case this becomes necessary later.

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

v) Fix with acetone or ethanol/acetone and dry.

vi) Rehydrate the above preparations and block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C. vii) Rinse four times with PBST.

viii) Treat the imprints with the solution of antibody to IHNV and rinse.

ix) Block and rinse.

x) Reveal the reaction with suitable fluorescein isothiocyanate (FITC)-conjugated specific antibody, rinse and observe.

xi) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture, as described above.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of proven efficiency may be used alternatively.

4.3.1.2.2.3. Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IHNV, in 0.01 M PBS, pH 7.2 (200 µl/well).

ii) Incubate overnight at 4°C.

iii) Rinse four times with 0.01 M PBS containing 0.05% Tween-20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Rinse four times with PBST.

vi) Add 2% Triton X-100 to the virus suspension to be identified.

vii) Dispense 100 µl/well of two- or four-step dilutions of the virus to be identified and of IHNV control virus, and a heterologous virus control (e.g. viral haemorrhagic septicaemia virus). Allow the samples to react with the coated antibody to IHNV for 1 hour at 20°C.

viii) Rinse four times with PBST.

ix) Add to the wells either biotinylated polyclonal IHNV antiserum or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.

xiii) Rinse four times with PBST. Add the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

xiv) Interpretations of the results is according to the optical absorbencies achieved by negative and positive controls and must follow the guidelines for each test, e.g. absorbency at 450 nm of positive control must be minimum 5–10 × A450 of negative control.

The above biotin-avidin-based ELISA version is given as an example. Other ELISA versions of proven efficiency may be used instead.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Polymerase chain reaction

4.3.1.2.3.1.1. Viral RNA preparation

Total RNA from infected cells is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially-available RNA isolation kit used according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separation methods should be used for extraction of RNA from cell culture fluids.

4.3.1.2.3.1.2. Reverse-transcription (RT) and standard PCR protocol

i) Prepare a master mix for the number of samples to be analysed. Work under a hood and wear gloves.

ii) The master mix for one 50 µl reverse-transcription PCR is prepared as follows: 23.75 µl ribonuclease-free (DEPC-treated) or molecular biology grade water; 5 µl 10 × buffer; 5 µl 25 mM $MgCl_2$; 5 µl 2 mM dNTP; 2.5 µl (20 pmoles $µl^{-1}$) Upstream Primer

5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3'; 2.5 µl (20 pmoles $µl^{-1}$) Downstream Primer

5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'; 0.5 µl *Taq* polymerase (5 U $µl^{-1}$); 0.5 µl AMV reverse transcriptase (9 U $µl^{-1}$); 0.25 µl RNasin (39 U $µl^{-1}$).

iii) Centrifuge the tubes briefly (10 seconds) to make sure the contents are at the bottom.

iv) Place the tubes in the thermal cycler and start the following cycles – 1 cycle: 50°C for 30 minutes; 1 cycle: 95°C for 2 minutes; 30 cycles: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; 1 cycle: 72°C for 7 minutes and soak at 4°C.

v) Visualise the 693 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

NOTE: These PCR primers target a central region of the IHNV G gene (Emmenegger *et al*., 2000). While other primer sets can be used for amplification of portions of the N or G genes of IHNV (Winton & Einer-Jensen, 2002), the primer sequences listed above have been shown to be conserved among a broad range of IHNV isolates and are not present in the G gene of the related fish rhabdoviruses, viral haemorrhagic septicaemia virus or hirame rhabdovirus. Additionally, the new primers produce an amplicon that can be used as a template for sequence analysis of the 'mid-G' region of the IHNV genome for epidemiological purposes (Emmenegger *et al*., 2000; Kurath *et al*., 2003).

4.3.1.2.3.2. Other amplification-based assays

Other methods to detect IHNV based on amplification of target sequences of genomic or messenger RNA have been developed that use a loop-mediated isothermal amplification (LAMP) method (Gunimaladevi *et al*., 2005) or a highly sensitive quantitative reverse-transcriptase PCR assay (Overturf *et al*., 2001). However, these assays have not yet undergone sufficient laboratory validation using a panel of isolates representing the various IHNV genotypes to make them suitable for listing as a confirmatory method.

4.3.1.2.3.3. Sequencing

Sequence analysis of PCR amplicons has become much more rapid and less costly in recent years and is a good method for confirmation of IHNV (Winton & Einer-Jensen, 2002). In addition, sequence analysis provides one of the best approaches for identification of genetic strains and for epidemiological tracing of virus movement (Emmenegger *et al*., 2000; Kim *et al*., 2007; Kurath *et al*., 2003; Nishizawa *et al*., 2006).

5. Rating of tests against purpose of use

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

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Gametes	Fry	Juveniles	Adults		
Gross signs	d	c	c	d	b	d
Virus isolation	a	a	a	a	a	c
Direct LM	d	c	d	d	b	c
Histopathology	d	c	d	d	b	c
Transmission EM	d	d	d	d	b	c
Antibody-based assays	d	c	c	c	a	b
PCR assays	c	c	c	c	a	a
Sequencing	d	d	d	d	c	a

LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infectious haematopoietic necrosis

The method for targeted surveillance to declare freedom from infection with IHNV is isolation of virus in cell culture. For this purpose, the most susceptible stages of the most susceptible species should be examined. Reproductive fluids and tissues collected from adult fish of a susceptible species at spawning should be included in at least one of the sampling periods each year.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case is defined as the presence of typical, gross clinical signs of the disease in a population of susceptible fish, OR a typical internal histopathological presentation among susceptible species, OR detection of antibodies against IHNV in a susceptible species, OR typical cytopathic effect in cell culture without identification of the agent, OR a single positive result from one of the diagnostic assays ranked as ‘a’ or ‘b’ in Table 5.1.

7.2. Definition of confirmed case

A confirmed case is defined as a suspect case that has EITHER: 1) produced typical cytopathic effect in cell culture with subsequent identification of the agent by one of the antibody-based or molecular tests listed in Table 5.1., OR: 2) a second positive result from a different diagnostic assay ranked as ‘a’ or ‘b’ in the last column of Table 5.1.

8. References

ARAKAWA C.K., DEERING R.E., HIGMAN K.H., OSHIMA K.H., O’HARA P.J. & WINTON J.R. (1990). Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. *Dis. Aquat. Org*., **8**, 165–170.

ARNZEN J.M., RISTOW S.S., HESSON C.P. & LIENTZ J. (1991). Rapid fluorescent antibody tests for infectious hematopoietic necrosis virus (IHNV) utilizing monoclonal antibodies to the nucleoprotein and glycoprotein. *J. Aquat. Anim. Health*, **3**, 109–113.

BARROSO R.M., WHEELER P.A., LAPATRA S.E., DREW R.E. & THORGAARD G.H. (2008). QTL for IHNV resistance and growth identified in a rainbow trout (*Oncorhynchus mykiss*) X Yellowstone cutthroat (*Oncorhynchus clarki bouvieri*) trout cross. *Aquaculture,* **277**, 156–163.

BOOTLAND L.M. & LEONG J.C. (1999). Infectious hematopoietic necrosis virus. *In:* Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, Woo P.T.K. & Bruno D.W., eds. CAB International, Oxon, UK, 57–121.

BOVO G., HÅSTEIN T., HILL B., LAPATRA S., MICHEL C., OLESEN N.J., SHCHELKUNOV I., STORSET A., WOLLFROM T. & MIDTLYING P.J. (2005). Hazard identification for vertical transfer of fish disease agents. Available at: http://www.crl-fish.eu/upload/sites/crl-fish/reports/links/fisheggtrade%20wp1.pdf.

DEERING R.E., ARAKAWA C.K., OSHIMA K.H., O'HARA P.J., LANDOLT M.L. & WINTON J.R. (1991). Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. *Dis. Aquat. Org.,* **11**, 57–65.

DIXON P.F. & HILL B.J. (1984). Rapid detection of fish rhabdoviruses by the enzyme-linked immunosorbent assay (ELISA). *Aquaculture*, **42**, 1–12.

EMMENEGGER E.J., MEYERS T.R., BURTON T.O. & KURATH G. (2000). Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. *Dis. Aquat. Org.*, **40**, 163–176.

ENGELKING H.M., HARRY J.B. & LEONG J.C. (1991). Comparison of representative strains of infectious hematopoietic necrosis virus by serological neutralization and cross-protection assays. *Appl. Environ. Microbiol.,* **57**, 1372–1378.

ENZMANN P.-J., CASTRIC J., BOVO G., THIERY R., FICHTNER D., SCHÜTZE H. & WAHLI T. (2010). Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. *Dis. Aquat. Org.,* **89**, 9–15.

ENZMANN P.J., KURATH G., FICHTNER D. & BERGMANN S.M. (2005). Infectious hematopoietic necrosis virus: Monophyletic origin of European IHNV isolates from North-American genogroup M. *Dis. Aquat. Org*., **66**, 187–195.

EUROPEAN FOOD SAFETY AUTHORITY (2008). Scientific opinion of the panel on AHAW on a request from the European Commission on aquatic animal species susceptible to diseases listed in the Council Directive 2006/88/EC. *EFSA J.,* **808**, 1–144. Available at:
http://www.efsa.europa.eu/en/efsajournal/doc/ahaw_op_ej808_suscepspecies_opinion_en.pdf?ssbinary=true

GARVER, K.A., BATTS, W.N. & KURATH, G. (2006). Virulence comparisons of infectious hematopoietic necrosis virus (IHNV) U and M genogroups in sockeye salmon and rainbow trout. *J. Aquat. Anim. Health*, **18**, 232–243.

GUNIMALADEVI I., KONO T., LAPATRA S.E. & SAKAI M. (2005). A loop mediated isothermal amplification (LAMP) method for detection of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). *Arch. Virol*., **150**, 899–909.

HUANG C., CHIEN M-S., LANDOLT M. & WINTON J.R. (1994). Characterization of the infectious hematopoietic necrosis virus glycoprotein using neutralizing monoclonal antibodies. *Dis. Aquat. Org.,* **18**, 29–35.

JAKOB E., BARKER D.E. & GARVER K.A. (2011). Vector potential of the salmon louse *Lepeophtheirus salmonis* in the transmission of infectious haematopoietic necrosis virus (IHNV). *Dis. Aquat. Org.*, **97**, 155–165.

JOHANSSON T., EINER-JENSEN K., BATTS W., AHRENS P., BJÖRKBLOM C., KURATH G., BJÖRKLUND H. & LORENZEN N. (2009). Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. *Dis. Aquat. Org*., **86**, 213–221.

JORGENSEN P.E.V., OLESEN N.J., LORENZEN N., WINTON J.R. & RISTOW S.S. (1991). Infectious hematopoietic necrosis (IHN) and viral hemorrhagic septicemia (VHS): detection of trout antibodies to the causative viruses by means of plaque neutralization, immunofluorescence, and enzyme-linked immunosorbent assay. *J. Aquat. Anim. Health*, **3**, 100–108.

KIM W-S., OH M-J., NISHIZAWA T., PARK J-W., KURATH G. & YOSHIMIZU M. (2007). Genotyping of Korean isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. *Arch. Virol*., **152**, 2119–2124.

KOLODZIEJEK J., SCHACHNER O., DÜRRWALD R., LATIF M. & NOWOTNY N. (2008). “Mid-G” region sequences of the glycoprotein gene of Austrian infectious hematopoietic necrosis virus isolates form two lineages within European isolates and are distinct from American and Asian lineages. *J. Clin. Microbiol*., **46**, 22–30.

KURATH G. (2008). Biotechnology and DNA vaccines for aquatic animals. *Rev. Sci. Tech. Off. Int. Epiz.* **27**, 175–196.

KURATH G., GARVER K.A., TROYER R.M., EMMENEGGGER E.J., EINER-JENSEN K. & ANDERSON E.D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. *J. Gen. Virol.*, **84**, 803–814.

LAPATRA S.E., FRYER J.L. & ROHOVEC J.S. (1993a). Virulence comparison of different electropherotypes of infectious hematopoietic necrosis virus. *Dis. Aquat. Org.,* **16**, 115–120.

LAPATRA S.E., ROBERTI K.A., ROHOVEC J.S. & FRYER J.L. (1989). Fluorescent antibody test for the rapid diagnosis of infectious hematopoietic necrosis virus. *J. Aquat. Anim. Health,* **1**, 29–36.

LAPATRA S.E., TURNER T., LAUDA K.A., JONES G.R. & WALKER S. (1993b). Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. *J. Aquat. Anim. Health*, **5**, 165–171.

MILLER K.M., WINTON J.R., SCHULZE A.D., PURCELL M.K. & MING T.J. (2004). Major histocompatibility complex loci are associated with susceptibility of Atlantic salmon to infectious hematopoietic necrosis virus*. Environ. Biol. Fish*., **69**, 307–316.

MORZUNOV S.P., WINTON J.R. & NICHOL S.T. (1995). The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. *Vir. Res*., **38**, 175–192.

NISHIZAWA T., KINOSHITA S., KIM W-S., HIGASHI S. & YOSHIMIZU M. (2006). Nucleotide diversity of Japanese isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. *Dis. Aquat. Org*., **71**, 267–272.

OVERTURF K., LAPATRA S. & POWELL M. (2001). Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. *J. Fish Dis.,* **24**, 325–333.

PURCELL M.K., HART S.A., KURATH G. & WINTON J.R. (2006). Strand-specific, real-time RT-PCR assays for quantification of genomic and positive-sense RNAs of the fish rhabdovirus, infectious hematopoietic necrosis virus. *J. Virol. Methods*, **132**, 18–24.

PURCELL M.K., LAPATRA S.E., WOODSON J.C., KURATH G. & WINTON J.R. (2010). Early viral replication and induced or constitutive immunity in rainbow trout families with differential resistance to Infectious hematopieticnecrosis virus (IHNV). *Fish Shellfish Immunol*., **28**, 98–105.

RISTOW S.S. & ARNZEN J.M. (1989). Development of monoclonal antibodies that recognize a type 2 specific and a common epitope on the nucleoprotein of infectious hematopoietic necrosis virus. *J. Aquat. Anim. Health*, **1**, 119–125.

RISTOW S.S. & ARNZEN DE AVILA J.M. (1991). Monoclonal antibodies to the glycoprotein and nucleoprotein of infectious hematopoietic necrosis virus (IHNV) reveal differences among isolates of the virus by fluorescence, neutralization and electrophoresis. *Dis. Aquat. Org.*, **11**, 105–115.

SHORS S.T. & WINSTON V. (1989). Detection of infectious hematopoietic necrosis virus in an invertebrate (*Callibaetis* sp). *Am. J. Vet. Res.,* **50**, 1307–1309.

TROYER R.M. & KURATH G. (2003). Molecular epidemiology of infectious hematopoietic necrosis virus reveals complex virus traffic and evolution within southern Idaho aquaculture. *Dis. Aquat. Org*., **55**, 175–185.

WINTON J.R. (1991). Recent advances in the detection and control of infectious hematopoietic necrosis virus (IHNV) in aquaculture. *Ann. Rev. Fish Dis*., **1**, 83–93.

WINTON J.R. (1997). Immunization with viral antigens: Infectious haematopoietic necrosis. *Dev. Biol. Stand.*, **90**, 211–220.

WINTON J.R., ARAKAWA C.K., LANNAN C.N. & FRYER J.L. (1988). Neutralizing monoclonal antibodies recognize antigenic variants among isolates of infectious hematopoietic necrosis virus. *Dis. Aquat. Org.*, **4**, 199–204.

WINTON J.R. & EINER-JENSEN K. (2002). Molecular diagnosis of infectious hematopoietic necrosis and viral hemorrhagic septicemia. *In:* Molecular Diagnosis of Salmonid Diseases, Cunningham C.O., ed. Kluwer, Dordrecht, The Netherlands, pp. 49–79.

WOLF K. (1988). Infectious hematopoietic necrosis. *In:* Fish Viruses and Fish Viral Diseases. Cornell University Press, Ithaca, New York, USA, pp. 83–114

*
* *

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

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS HAEMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2012.

CHAPTER 2.3.6.

INFECTION WITH SALMONID ALPHAVIRUS

1. Scope

For the purpose of this chapter, iInfection with salmonid alphavirus (SAV) means infection with any subtype genotype of the pathogenic agent SAV, of the Genus *Alphavirus*, and Family *Togaviridae*.

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

SAV is an enveloped, spherical, single-stranded, positive-sense RNA virus, approximately 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 (SAV1–SAV6) 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).

Infection with SAV may cause pancreas disease (PD) or sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.), common dab (*Limanda limanda*), and rainbow trout (*Oncorhynchus mykiss*) McLoughlin & Graham, 2007) and Arctic charr (*Salvelinus alpinus*) (Lewisch *et al*., 2018). The disease is a systemic disease characterised microscopically by necrosis and loss of exocrine pancreatic tissue, and heart and skeletal muscle changes.

The genotype groups by susceptible species and environment and their geographical distributions are presented in the table below (abbreviations: SW = sea water, FW = fresh water, PD = pancreas disease, SD = sleeping disease):

Table 2.1. SAV genotypes by susceptible species and environment host, environment and geographic distribution

SAV subtype genotype	Host and environment Freshwater	Sea Wwater	Country
SAV 1 (PD)	Atlantic salmon (SW) Rainbow trout (FW)	Atlantic salmon (SW)	Ireland, UK (Northern Ireland, Scotland)
SAV 2 FW (SD)	Rainbow trout (FW) Atlantic salmon (SW) Atlantic salmon (FW) Arctic charr (FW)	Atlantic salmon (SW)	France, Germany, Italy, Spain, Switzerland, Poland, UK (England, Scotland) Scotland Austria

SAV subtype genotype	Host and environment Freshwater	Sea Wwater	Country
SAV 2 Marine (PD)	Atlantic salmon (SW)	Atlantic salmon (SW)	Norway, UK (Scotland)
SAV 3 (PD)	Rainbow trout (SW) Atlantic salmon (SW)	Rainbow trout (SW) Atlantic salmon (SW)	Norway
SAV 4 (PD)	Atlantic salmon (SW)	Atlantic salmon (SW)	Ireland, UK (Northern Ireland, Scotland)
SAV 5 (PD)	Atlantic salmon (SW) Common dab (SW)	Atlantic salmon (SW) Common dab (SW)	UK (Scotland) UK (Scotland), Ireland
SAV 6 (PD)	Atlantic salmon (SW)	Atlantic salmon (SW)	Ireland

2.1.2. Survival outside the host

Laboratory tests suggest that SAV would survive for extended periods in the aquatic environment. In these tests, virus survival was inversely related to temperature. In the presence of organic matter, marked longer survival times were observed in sea water compared with fresh water (Graham *et al.,* 2007c). SAV has been detected in fat leaking from dead fish, indicating that this may be a route for transmission. Fat droplets may accumulate at the sea water surface, contributing to long distance spread of the virus (Stene *et al.,* submitted2013).

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

2.1.3. Stability of the agent

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

2.1.4. Life cycle

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

Viraemia precedes both the onset of histological changes and clinical signs (McLoughlin & Graham, 2007). The route of shedding may be through natural excretions/secretions, supported by the detection of SAV by reverse-transcriptase polymerase chain reaction (RT-PCR) in the faeces and mucous of experimentally infected Atlantic salmon. These matrices may therefore play a role in the horizontal transmission of SAV through water (Graham *et al.,* 2012). Virus has been detected in water 4–13 days after post-infection, indicating that virus shedding coincides with the viraemic stage (Andersen *et al.,* 2010). An incubation period of 7–10 days at sea water temperatures of 12–15°C has been estimated based on analysis of antibody production in intraperitoneally infected fish and cohabitants in an experimental trial (McLoughlin & Graham, 2007). Several studies have shown that SAV RNA can be detected in fish for an extended period post-infection (Jansen *et al.,* 2010a; McLoughlin & Graham, 2007). Subclinical infection has been reported, suggesting that the severity of an outbreak may be influenced by several environmental factors (McLoughlin & Graham, 2007), and recent data show that seasonal increases in water temperature may trigger disease outbreaks in SAV-infected farms (Stene *et al.,* 2014).

2.2. Host factors

2.2.1. Susceptible host species

Disease outbreaks and infection experiments have shown that Atlantic salmon, rainbow trout and brown trout are susceptible (Boucher *et al.,* 1995; McLoughlin & Graham, 2007).

Species that fulfil the criteria for listing a species as susceptible to infection with SAV according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include: Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), common dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*).

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: long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*).

In addition, pathogen-specific positive PCR results have been reported in the following organisms species, but an active infection has not been demonstrated: Argentine hake (*Merluccius hubbsi*), bBallan wrasse (*Labrus bergylta*), brown trout (*Salmo trutta*), cod (*Gadus morhua*), European flounder (*Platichthys flesus*), haddock (*Melanogrammus aeglefinus*), herring (*Glupea harengus*), Norway pout (*Trisopterus esmarkii*), saithe (*Pollachius virens*), salmon louse (*Lepeophtheirus salmonis*), sculpin sp. (*Myoxocephalus octodecemspinosus*) and whiting (*Merlangius merlangus*).

2.2.23. Susceptible stages of the host

All life stages should be considered as susceptible to infection with SAV.

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

2.2.34. Species or subpopulation predilection (probability of detection)

There is no known species or subpopulation predilection.

2.2.45. Target organs and infected tissue

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

2.2.56. Persistent infection with lifelong carriers

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

2.2.67. Vectors

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

2.2.78. Known or Ssuspected wild aquatic animal carriers

In surveys of wild marine fish, SAV RNA has been detected in the flatfish species common dab (*Limanda limanda*), long rough dab (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*) (McCleary *et al.,* 2014; Snow *et al.,* 2010). The importance of wild marine or fresh water species as virus carriers needs to be determined clarified.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission of SAV occurs horizontally. This is supported by phylogenetic studies, successful transmission among fish in cohabitant studies, proven transmission between farming sites, studies on survival of SAV in sea water and the spread via water currents (Graham *et al.,* 2007c; 2011; Jansen *et al.,* 2010a; Kristoffersen *et al.,* 2009; Viljugrein *et al.,* 2009).

Long-distance transmission and thus introduction of SAV in a previously uninfected area is most likely assigned to movement of infected live fish (Kristoffersen *et al.,* 2009; Rodger & Mitchell, 2007). Once SAV has been introduced into an area, shared ownership and close site farm proximity and water currents are factors involved in local transmission (Aldrin *et al.,* 2010; Kristoffersen *et al.,* 2009; Viljugrein *et al.,* 2009). Risk factors for outbreaks 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).

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

2.3.2. Prevalence

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

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

2.3.3. Geographical distribution

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

2.3.4. Mortality and morbidity

Mortality rates due to infection with SAV may vary with genotype subtype, season, year, use of biosecurity measures and species of fish (Bang Jensen *et al.,* 2012; Graham *et al.,* 2011; Rodger & Mitchell, 2007; Stormoen *et al.,* 2013). The cumulative mortality at the farm level ranges from negligible to over 50% in severe cases (Bang Jensen *et al.,* 2012; Graham *et al.,* 2003; Rodger & Mitchell, 2007; Ruane *et al.,* 2008; Stene *et al.,* 2014).

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

2.3.5. Environmental factors

Clinical outbreaks and mortality 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.

2.4. Control and prevention

2.4.1. Vaccination

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

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

2.4.2. Chemotherapy

No chemotherapy is available.

2.4.3. Immunostimulation

No immunostimulation is available.

2.4.4. Resistance breeding

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

2.4.5. Restocking with resistant species

Not relevant.

2.4.6. Blocking agents

Not relevant.

2.4.7. Disinfection of eggs and larvae

Disinfection procedures were evaluated in fertilised ova from SAV genotype 3 positive broodstock (Kongtorp *et al.,* 2010). Nevertheless, further investigation is needed. (See Graham *et al.,* 2007b; Kongtorp *et al.,* 2010.)

2.4.8. General husbandry practices

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

3. Sampling

3.1. Selection of individual specimens

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

3.2. Preservation of samples for submission

Table 3.1. *Preservative used for each method*

Method	Preservative
Histology and immunohistochemistry	Fixation in neutral phosphate-buffered 10% formalin
Molecular biology (RT-PCR and sequencing)	Appropriate medium for preservation of RNA
Cell culture	Virus transport medium
Serology	Blood plasma or serum

3.3. Pooling of samples

For diagnostic purposes, pooling of samples from different individuals is not considered necessary or recommended as detection of SAV and characteristic histopathological changes in the same individual will strengthen the connection between the virus and the observed disease. For surveillance purposes, pooling of samples for virological examination (PCR or cell culture) may be accepted, but may decrease the sensitivity of the tests.

Pooling of samples may be acceptable, however, the impact on sensitivity and design prevalence must be considered.

3.4. Best organs or tissues

Heart and mid-kidney are the recommended organs for detection of SAV either by molecular biological methods or by cell culture. During the course of the disease, the heart usually contains more SAV than other tissues and should always be sampled. After disease outbreaks, gills and heart (Graham *et al.,* 2010) and pools of heart and mid-kidney (Jansen *et al.,* 2010a; 2010b) remained RT-PCR positive for months after initial detection.

During the initial viraemic phase, serum samples are also suitable for detection of SAV either by molecular biological methods or by cell culture. Serum sampling may therefore be used for early warning screening tests (Graham *et al*., 2010). From approximately 3 weeks after SAV infection, blood serum or plasma is suitable for a virus neutralisation test that identifies neutralising antibodies against SAV in fish exposed to SAV (Graham *et al.,* 2003).

Tissues for histological examinations should include gill, heart, pyloric caeca with attached pancreatic tissue, liver, kidney, spleen and skeletal muscle containing both red (aerobe) and white (anaerobe) muscle. Skin with associated skeletal muscle sample should be taken at the lateral line level and deep enough to include both red and white muscle.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

A sudden drop in appetite may be observed 1–2 weeks before the detection of ~~enhanced~~ 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 ~~in the water~~. However, it is important to ~~notice~~ note that clinical signs are not pathognomonic. ~~and that careful observation and examinations~~ Careful investigation of any dead, ~~weak~~ moribund or abnormally behaving fish is necessary to determine involvement of SAV and rule out other *pathogenic agents*.

Initially, nutritional status is usually normal, but in the months after an outbreak or in the later stages of disease, long slender fish (‘runts’) with ~~low~~ poor body condition are typically observed. The ~~development~~ presentation of long, slender fish can be caused by factors other than SAV.

4.2. Clinical methods

4.2.1. Gross pathology

Yellow mucoid gut contents are a usual post-mortem finding, as is typically seen in fish that are not eating. Occasionally signs of circulatory disturbances, such as petechial haemorrhages, small ascites or reddening of the pancreatic region between the pyloric caeca, may be seen. Some diseased fish may show pale hearts or heart ruptures. It is important to note that post-mortem findings are not pathognomonic.

4.2.2. Clinical chemistry

Not documented for diagnostic use.

4.2.3. Microscopic pathology

The changes most commonly found in clinically diseased fish are severe loss of exocrine pancreatic tissue, cardiomyocytic necrosis and inflammation, red (aerobe) skeletal muscle inflammation and white (anaerobe) skeletal muscle degeneration or inflammation. A less frequent but supporting finding is the detection of cells with many cytoplasmic eosinophilic granules along kidney sinusoids.

As the disease progresses, the development of these changes is not simultaneous in all organs: In a very short, early phase, the only lesion present can be necrosis of exocrine pancreatic tissue and a variable inflammatory reaction in the peripancreatic fat. Shortly thereafter, heart muscle cell degeneration and necrosis develops before the inflammation response in the heart becomes more pronounced. The pancreatic necrotic debris will seemingly disappear and the typical picture of severe loss of exocrine pancreatic tissue will soon appear simultaneously with the increasing inflammation in the heart. Somewhat later, skeletal muscle degeneration, inflammation and fibrosis develop. In a proportion of fish, severe fibrosis of the peri-acinar tissue may occur, and in this case the pancreas does not recover (runts) (Christie *et al.,* 2007; Kerbart Boscher *et al.,* 2006; McLoughlin & Graham, 2007; Taksdal *et al.,* 2007).

4.2.4. Wet mounts

Not relevant.

4.2.5. Smears

Not relevant.

4.2.6. Fixed sections, immunohistochemistry

~~The single~~ Immunohistochemical ~~method published~~ testing (Taksdal *et al.,* 2007) is only recommended for samples from fish with acute necrosis of exocrine pancreatic tissue.

4.2.6.1. Preparation of tissue sections

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

4.2.6.2. Staining procedure for immunohistochemistry

All incubations are carried out at room temperature and all washing steps are done with Tris-buffered saline (TBS).

i) Nonspecific antibody binding sites are first blocked in 5% bovine serum albumin (BSA) in TBS for 20 minutes. The solution is then poured off without washing.

ii) Sections are incubated with primary antibody (monoclonal mouse antibody 4H1 against E1 SAV glycoprotein [Todd *et al.,* 2001]), diluted 1/3000 in 2.5% BSA in TBS and then incubated overnight, followed by two wash out baths lasting a minimum of 5 minutes.

iii) Sections are incubated with secondary antibody (biotinylated rabbit anti-mouse Ig) diluted 1/300 for 30 minutes, followed by wash out baths as in step ii above.

iv) Sections are incubated with streptavidin with alkaline phosphatase 1/500 for 30 minutes followed by wash out baths as in step ii above.

v) For detection of bound antibodies, sections are incubated with Fast Red[1] (1 mg ml^{-1}) and Naphthol AS-MX phosphate (0.2 mg ml^{-1}) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) and allowed to develop for 20 minutes followed by one wash in tap water before counterstaining with Mayer's haematoxylin and mounting in aqueous mounting medium.

SAV-positive and SAV-negative tissue sections are included as controls in every setup (Taksdal *et al.,* 2007).

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

4.2.7. Electron microscopy/cytopathology

Not relevant for diagnostic use.

4.2.8. Differential diagnoses

4.2.8.1. Differential diagnoses relevant for microscopic pathology (Section 4.2.3)

Tissues that are changed by infection with SAV are also changed by heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and IPN. However, if all the main organs are examined by histopathology, the pattern of affected organs will usually appear different.

Table 4.1. Tissue changes associated with infection with SAV, HSMI, CMS and IPN

	Infection with SAV	HSMI	CMS	IPN
Heart*	+	+	+	–
Pancreas	+	–	–	+
Skeletal muscle	+	+	–	–

*Heart changes in CMS affects mainly the inner spongy layer of the ventricle and the atrium, whereas in Infection with SAV and HSMI, the compact layer of the ventricle is more severely affected. Although these three diseases induce epicarditis, HSMI causes the most severely inflamed epicardium.

In a very short, early acute stage of infection, when only necrosis of exocrine pancreas has developed, infection with SAV might be mistaken for IPN caused by infection with IPN virus (IPNV). In such cases, virological examination will clarify the causal agent.

Virological and serological examinations combined with histopathological examination of 5–10 clinically diseased fish will usually clarify the situation. HSMI and CMS have only been detected in Atlantic salmon.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Agent isolation and identification

4.3.1.1.1. Cell culture

Isolation of field isolates of SAV in cell culture may be challenging (Christie, 1998; Graham, 2007c; Petterson *et al.,* 2013). CHSE-214 are commonly used for primary SAV isolation, but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used. Variation in cell line susceptibility among different SAV field isolates has been reported (Graham *et al.,* 2008; Herath *et al.,* 2009), and it is therefore recommended that several cell lines are tested for initial cell culture isolation of SAV in a new laboratory or for a new virus strain.

The CHSE-214 cells are grown at 20°C in Eagle's minimal essential medium (EMEM) with non-essential amino acids and 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) buffer, or Leibovitz's L-15 cell culture medium, both supplemented with fetal bovine serum (FBS) (5% or 10%) and L-glutamine (4 mM).

For virus isolation, cells are grown in tissue culture flasks or multi-well cell culture plates. SAV-positive controls may be inoculated in parallel with the tissue samples as a test for cell susceptibility to SAV. When positive controls are included, measures must be taken to avoid contamination.

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 marine SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV ~~subtypes~~ 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 (the procedure can be repeated 1–2 times) to release virus from the infected cells.

Following centrifugation at 3000 ***g*** for 5 minutes, the supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 2–3 hours before addition of fresh medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]), as virus replication may occur without development of apparent CPE.

iv) Antibody-based verification of SAV growth in cell culture

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

a) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well plates), or on cover-slips, depending on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). The necessary monolayers for negative and positive controls must be included.

b) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 9–11 days.

c) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. If necessary, the fixed cell cultures may be stored dry for 14 days at 4°C until staining.

d) Incubate the cell monolayers with anti-SAV MAb in an appropriate dilution in phosphate-buffered saline (PBS) for 1 hour and rinse three times with PBS with 0.05% Tween 20.

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

Annex 15 (contd)

4.3.1.1.2. Reverse-transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, and genotyping by sequencing

The primers described below for real-time RT-PCR and RT-PCR with sequencing will detect all known subtypes 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 also the sensitivity of the test.

For genotyping, RT-PCR with subsequent sequencing of fragments from the E2 and nsP3 genes is recommended.

The primers and probe sequences for real-time RT-PCR from the nsP1 gene, as well as primers for genotyping, are listed in table 3.1. below. The E2-primers may also be used for conventional RT-PCR detection of SAV, if necessary. A variety of kits designed for RNA extraction/RT-PCR 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 3.1. Characteristic of pPrimers and probe sequences for RT-PCR and real time RT-PCR

RT-PCR: Primer and probe sequences	Named	Genomic segment	Product size	Reference
QnsP1F: 5'-CCG-GCC-CTG-AAC-CAG-TT-3' QnsP1R: 5'-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3' QnsP1probe: 5'FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3' (Taqman®probe)	forward primer reverse primer Taqman®probe	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'	forward primer reverse primer	E2	516 nt	Fringuelli *et al*., 2008
nsP3F: 5'-CGC-AGT-CCA-GCG-TCA-CCT-CAT-C-3' nsP3R: 5'-TCA-CGT-TGC-CCT-CTG-CGC-CG-3'	forward primer reverse primer	nsP3	490 nt	Fringuelli *et al*., 2008

4.3.2. Serological methods

4.3.2.1 Immunoperoxidase-based serum neutralisation assay (Graham *et al.,* 2003)

Experimental studies have shown that neutralising antibodies can first be detected 10–16 days post-infection (Graham *et al.,* 2003), and 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.

CHSE-214 cells are grown as described in Section 4.3.1.1.1 Cell culture. 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 4.3.1.1.1, step iv *Antibody-based verification of SAV growth in cell culture*, or using the following procedure: monolayers of CHSE-214 cells are fixed for 30 minutes at room temperature in 10% neutral buffered formalin. Following two washes with 0.01 M PBS, a MAb against SAV is added to the monolayers in an appropriate dilution. Bound MAb is visualised using a labelled streptavidin–biotin system according to the manufacturer's instructions.

iv) SN titres (ND_{50}) are then calculated according to the method of Karber (1931), with titres ≥ 1:20 being considered positive. Both serum controls (without virus added) and a virus control (without serum added) must always be included in the assay, to ensure valid results.

5. Rating of tests against purpose of use

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

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Fry	Juveniles	Adults		
Gross signs	d	d	d	c	d
Histopathology	c	c	c	~~a~~ b	~~a~~ d
Immunohistochemistry	d	d	d	b	b
Isolation in cell culture	d	d	d	c	c
Serum neutralisation assay	d	c	b	a	b
Real-time RT-PCR	b	b	b	b	b
RT-PCR with sequencing	d	b	b	b	a

RT-PCR = Reverse-transcriptase polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with SAV

The recommended test to be used in surveillance of susceptible fish populations for declaration of freedom from SAV is RT-PCR as described in Section 4.3.1.1.2 in this chapter.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspected case of infection with SAV is defined as:

i) Clinical signs consistent with infection with SAV (Section 4.1.1)

or

ii) Gross and microscopic~~ally~~ pathology consistent with the disease (Sections 4.2.1 and 4.2.3)

or

iii) Detection of antibodies against SAV (Section 4.3.2.1) or detection of SAV (Section 4.3.1.1.)

Annex 15 (contd)

or

iv) If epidemiological information of infectious contact with suspected or confirmed case(s) appears.

7.2. Definition of confirmed case

Evidence for the presence of SAV from two independent laboratory tests as microscopic pathology (Section 4.2.3), cell culture (Section 4.3.1.1.1), RT-PCR (Section 4.3.1.1.2) or serology (Section 4.3.2).

8. References

ALDRIN M., STORVIK B., FRIGESSI A., VILJUGREIN H. & JANSEN P.A. (2010). A stochastic model for the assessment of the transmission pathways of heart and skeleton muscle inflammation, pancreas disease and infectious salmon anaemia in marine fish farms in Norway. *Prev. Vet. Med.*, **93**, 51–61.

ANDERSEN L., BRATLAND A., HODNELAND K. & NYLUND A. (2007). Tissue tropism of salmonid alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon (*Salmon salar* L.). *Arch. Virol.,* **152**, 1871–1883.

ANDERSEN L., HODNELAND H. & NYLUND A. (2010). No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (*Salmon salar* L.). *Virol. J*., **7**, 198.

BANG JENSEN B., KRISTOFFERSEN A.B., MYR C. & BRUN E. (2012). Cohort study of effect of vaccination on pancreas disease in Norwegian salmon aquaculture. *Dis. Aquat. Org.*, **102**, 23–31.

~~BOUCHER P., RAYNARD R.S., HOUGHTON G. & BAUDIN LAURENCIN F. (1995). Comparative experimental transmission of pancreas disease in Atlantic salmon, rainbow trout and brown trout. *Dis. Aquat. Org.*, **22**, 19–24.~~

BRATLAND A. & NYLUND A. (2009). Studies on the possibility of vertical transmission of Norwegian salmonid Alphavirus in production of Atlantic salmon in Norway. *J. Aquat. Anim. Health*, **21**, 73–78.

CHRISTIE K.E., FYRAND K., HOLTET L. & ROWLEY H.M. (1998) Isolation of pancreas disease virus from farmed Atlantic salmon, *Salmo salar* L., in Norway. *J. Fish Dis*., **21**, 391–394.

CHRISTIE K.E., GRAHAM D.A., MCLOUGHLIN M. F., VILLOING S., TODD D. & KNAPPSKOG D. (2007). Experimental infection of Atlantic salmon *Salmo salar* pre-smolts by i.p. injection of new Irish and Norwegian salmonid alphavirus (SAV) isolates: a comparative study. *Dis. Aquat. Org.*, **75**, 13–22.

FRINGUELLI E., ROWLEY H.M., WILSON J.C., HUNTER R., RODGER H. & GRAHAM D.A. (2008). Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *J. Fish Dis*., **31**, 811–823.

GRAHAM D.A., BROWN A., SAVAGE P. & FROST P. (2012). Detection of salmon pancreas disease in the faeces and mucus of Atlantic salmon *Salmon salar* by real-time RT-PCR and cell culture following experimental challenge. *J. Fish Dis*., **35**, 949–951.

GRAHAM D.A., CHERRY K., WILSON C.J. & ROWLEY H.M. (2007a). Susceptibility of salmonid alphavirus to a range of chemical disinfectants. *J. Fish Dis*., **30**, 269–277.

GRAHAM D.A., FROST P., MCLAUGHLIN K., ROWLEY H.M., GABESTAD I., GORDON A. & MCLOUGHLIN M.F. (2011). A comparative study of marine salmonid alphavirus subtypes 1–6 using an experimental cohabitation challenge model. *J. Fish Dis*., **34**, 273–286.

GRAHAM D.A., FRINGUELLI E., WILSON C., ROWLEY H.M., BROWN, A., RODGER H., MCLOUGHLIN M.F., MCMANUS C., CASEY E., MCCARTHY L.J. & RUANE N.M. (2010). Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for virual persistence. *J. Fish Dis*., **33**, 123–135.

GRAHAM D.A., JEWHURST V.A., ROWLEY H.M., MCLOUGHLIN M.F. & TODD D. (2003). A rapid immunoperoxidase-basd neutralization assay for salmonid alphavirus used for a serological survey in Northern Ireland. *J. Fish Dis*., **26**, 407–413.

GRAHAM D.A., ROWLEY H.M., FRINGUELLI E., BOVO G., MANFRIN A., MCLOUGHLIN M.F., ZARZA C., KHALILI M. & TODD D. (2007b).First laboratory confirmation of salmonid alphavirus infection in Italy and Spain. *J. Fish Dis*., **30**, 569–572.

GRAHAM D.A., ROWLEY H.M. & FROST P. (2014). Cross-neutralization studies with salmonid alphavirus subtype 1–6 strains: results with sera from experimental studies and natural infections. *J. Fish Dis*., 37, 683–691.

GRAHAM D.A., STAPLES V., WILSON C.J., JEWHURST H., CHERRY K., GORDON A. & ROWLEY H.M. (2007c). Biophysical properties of salmonid alphaviruses: influences of temperature and pH on virus survival. *J. Fish Dis*., **30**, 533–543.

GRAHAM D.A., WILSON C., JEWHURST H. & ROWLEY H. (2008). Cultural characteristics of salmonid alphaviruses – influences of cell line and temperature. *J. Fish Dis*., **31**, 859–868.

HERATH T., COSTA J., THOMPSON K., ADAMS A. & RICHARDS R. (2009). Alternative cell line for the isolation of salmonid alphavirus-1. *Icelandic Agricultural Sci.,* **22**, 19–27.

HODNELAND K. & ENDRESEN C. (2006). Sensitive and specific detection of salmonid alphavirus using real-time PCR (TaqMan). *J. Virol. Methods,* **131**, 184–192.

JANSEN M.D., BANG JENSEN B. & BRUN E. (2014). Clinical manifestations of pancreas disease (PD) outbreaks in Norwegian marine salmon farming – variations due to salmonid alphavirus (SAV) subtype. (Accepted) *J. Fish Dis*., 24 March, doi: 10.1111/jfd.12238

JANSEN M.D., TAKSDAL T., WASMUTH M.A., GJERSET B., BRUN E., OLSEN A.B., BRECK O. & SANDBERG M. (2010a). Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon (*Salmo salar* L.) in freshwater and seawater sites in Norway from 2006 to 2008. *J. Fish Dis*., **33**, 391–402.

JANSEN M.D., WASWUTH M.A., OLSEN A.B., GJERSET B., MODAHL I., BRECK O., HALDORSEN R.N., HJELMELAND R., TAKSDAL T. (2010b). Pancreas disease (PD) in sea-reared Atlantic salmon, *Salmon salar* L., in Norway; a prospective, longitudinal study of disease development and agreement between diagnostic test results. *J. Fish Dis*., **33**, 723–736.

JEWHURST V.A., TODD D., ROWLEY H.M., WALKER I.W., WESTON J.H. MCLOUGHLIN M.F & GRAHAM D.A. (2004). Detection and antigenic characterization of salmonid alphavirus isolates fram sera obtained from farmed Atlantic salmon , *salmo salar* L., and farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis*., **27**, 143–149.

KERBART BOSCHER S., MCLOUGHLIN M., LE VEN A., CABON J., BAUD M. & CASTRIC J. (2006). Experimental transmission of sleeping disease in one-year-old rainbow trout *Onchorhynchus mykiss* (Walbaum), induced by sleeping disease virus. *J. Fish Dis*., **29**, 263–273.

KONGTORP R.T., STENE A., ANDREASSEN P.A., ASPEHAUG V., GRAHAM D.A., LYNGSTAD T.M., OLSEN A.B., OLSEN R.S., SANDBERG M., SANTI N., WALLACE C. & BRECK O. (2010). Lack of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, *salmo salar* L., exposed by natural and experimental routes. *J. Fish Dis*., **33**, 879–888.

KRISTOFFERSEN A.B., VILJUGREIN H., KONGTORP R.T., BRUN E. & JANSEN P.A. (2009). Risk factors for pancreas disease (PD) outbreaks in farmed Atlantic salmon and rainbow trout in Norway during 2003–2007. *Prev. Vet. Med.*, **90**, 127–136.

MCCLEARY S.J., GILTRAP M., HENSHILWOOD K. & RUANE N.M. (2014). Detection of salmonid alphavirus RNA in Celtic and Irish Sea flatfish. Submitted to *Dis. Aquat. Org*. (June 2013).

MCLOUGHLIN M.F. & GRAHAM D.A. (2007). Alphavirus infections in salmonids – a review. *J. Fish Dis*., **30**, 511–531.

MCVICAR A.H. (1990). Infection as a primary cause of pancreas disease in farmed Atlantic salmon. *Bull. Eur. Assoc. Fish Pathol*., **10** (3), 84–87.

PETTERSON E., SANDBERG M. & SANTI N. (2009). Salmonid alphavirus associated with *Lepeoptheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar* L. *J. Fish Dis*., **30**, 511–531.

PETTERSON E., STORMOEN, M., EVENSEN O., MIKALSEN A.B. & HAUGLAND O. (2013). Natural infection of Atlantic salmon (*Salmon salar*) with salmonid alphavirus 3 generates numerous viral deletion mutants. *J. Gen. Virol.,* **94**, 1945–1954.

RODGER H. & MITCHELL S. (2007). Epidemiological observations of pancreas disease of farmed Atlantic salmon, *Salmo salar* L., in Ireland. *J. Fish Dis*., **32**, 477–479.

RUANE N., GRAHAM D. & RODGER H. (2008). Pancreas disease in farmed salmon – health management and investigations at Irish farm sites 2005–2008. Marine Environments and Health Series, No. 34, Marine Institute. Available at http://oar.marine.ie/handle/10793/267

SNOW M., BLACK I., MCINTOSH R., BARETTO E., WALLACE I.S. & BRUNO D.W. (2010). Detection of salmonid alphavirus RNA in wild marine fish: implications for the origin of salmon pancreas disease in aquaculture. *Dis. Aquat. Org.*, **91**, 177–188.

STENE, A., VILJUGREIN H., YNDESTAD, H., TAVORNPANICH, S. & SKJERVE, E. (2013). Transmission dynamics of pancreas disease (PD) in a Norwegian fjord: aspects of water transport, contact networks and infection preassure among salmon farms. *J. Fish Dis*.,**37 (2)**, 123-134.

STENE A., BANG JENSEN B., KNUTSEN Ø., OLSEN A. & VILJUGREIN H. (2014). Seasonal increase in sea temperature triggers pancreas disease in Norwegian salmon farms. *J. Fish Dis*., **37**, 739–751.

Stene A., Hellebø A., Viljugrein H., Solevåg S.E., Devold M. & Aspehaug V. (2015). Liquid fat, a potential abiotic vector for horizontal transmission of salmonid alphavirus? *J. Fish Dis*., doi: 10.1111/jfd.12382. [Epub ahead of print].

STORMOEN M., KRISTOFFERSEN A.B. & JANSEN P.A. (2013). Mortality related to pancreas disease in Norwegian farmed salmonid fish, *Salmo salar* L. and *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis*., **36**, 639–645.

TAKSDAL T., OLSEN A.B., BJERKAAS I., HJORTAAS M.J.DANNEVIG B.H., GRAHAM D.A. & MCLOUGHLIN M.F. (2007). Pancreas disease in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Norway. *J. Fish Dis*., **30**, 545–558.

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for Mattrygghet) (2010). Risikovurdering - stamfiskovervåking og vertikal smitteoverføring. **01**, 1-44. Available at: HTTPS://VKM.NO/DOWNLOAD/18.A665C1015C865CC85BDFC47/1500464589864/VURDERING%20AV%20SANNSYNLIGHET%20FOR%20OG%20RISIKO%20VED%20VERTIKAL%20OVERF%C3%B8RING%20AV%20SMITTE.PDF

TODD D., JEWHURST V.A., WELSH M.D., BORGHMANS B.J., WESTON J.H., ROWLEY H.M., MACKIE D.P. & MCLOUGHLIN M.F. (2001). Production and characterisation of monoclonal antibodies to salmon pancreas disease virus. *Dis. Aquat. Org.*, **46**, 101–108.

VILJUGREIN H., STAALSTRØM A., MOLVÆR J., URKE H.A. & JANSEN P.A. (2009). Integration of hydrodynamics into a statistical mode on the spread of pancreas disease (PD) in salmon farming. *Dis. Aquat. Org.*, **88**, 35–44.

*

* *

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.

Annex 16

CHAPTER 2.3.7.

INFECTION WITH KOI HERPESVIRUS DISEASE

1. Scope

Infection with koi herpesvirus disease (KHVD) means infection with the pathogenic agent koi herpesvirus (KHV) of the Genus *Cyprinivirus* and Family *Alloherpesviridae* a herpesvirus infection (Hedrick *et al*., 2000) capable of inducing a contagious and acute viraemia in common carp (*Cyprinus carpio*) and varieties such as koi carp and ghost carp (Haenen *et al.,* 2004).

[…]

2.2. Host factors

2.2.1. Susceptible host species

Naturally occurring KHV infections have only been recorded from common carp (Cyprinus carpio) and varieties of this species (e.g. koi carp). Goldfish × common carp hybrids, produced by hybridising male goldfish with female carp, have been reported to show some susceptibility to KHV infections. Although mortality rate was low (5%), approximately 50% of these hybrids examined 25 days after intraperitoneal injection with a high dose of KHV possessed viral genomic DNA, as detected by polymerase chain reaction (PCR) (Hedrick et al., 2006). In a more recent study, infection by bath immersion with different KHV strains caused mortality of 35–42% in goldfish × koi carp hybrids and 91–100% in crucian carp × koi carp hybrids. The most marked clinical signs were large skin ulcers, excess mucus production and haemorrhages in the fins with the most extensive signs noted in the crucian carp × koi carp hybrids. Viral DNA was detected in all of the hybrid mortalities by PCR assay (Bergmann et al., 2010b).

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

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: Goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and Syberian crucian carp (*Carassius auratus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms species, but an active infection has not been demonstrated: Atlantic sturgeon (*Acipenser oxyrinchus gueldenstaedtii*), blue back ide (*Leuciscus idus*), common roach (*Rutilus rutilus*), Euraseas ruffe (*Gymnocephalus cernuus*), European perch (*Perca fluviatilis*), hybrid sterlet × beluga (*Acipenser ruthenus* × *Huso huso*), rainbow trout (*Oncorhynchus mykiss*), Russian sturgeon (*Acipencer gueldenstaedtii* *oxyrinchus*), scud (crustacean) (*Gammarus pulex*), silver carp (*Hypophthalmichthys militrix*), stone loach (*Barbatula barbatula*), swan mussel (*Anodonta cygnea*) and tench (*Tinca tinca*).

[…]

Annex 17

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 application of the standards in the *Aquatic Code* at the level of 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, the use of *risk analysis* 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*. This chapter describes *biosecurity* principles 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*. Staff, ~~and~~ service providers and *aquatic animal health professionals* or *veterinarians* should be engaged in developing and implementing the *biosecurity plan* to ensure it is practical and effective.

The outcome achieved through the implementation of *biosecurity* at *aquaculture establishments* is improved health status of *aquatic animals* throughout the production cycle. The benefits include market access and increased productivity, directly through improved survival, growth rates and *feed* conversion and indirectly through ~~the~~ reduction in treatments and associated production costs.

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~~ in an *aquaculture establishment*. Implementation of *biosecurity* within an *aquaculture establishment* requires planning to identify *risks* and ~~consider~~ cost effective measures to achieve the identified biosecurity objectives of the plan. The measures required will vary between *aquaculture establishments*, depending on factors such as *risk* of exposure to *pathogenic agents*, *aquatic animal* species, category of *aquaculture* production system, husbandry practices and geographic location. Although different approaches may be used to achieve an identified objective, the general principles for developing and implementing a *biosecurity plan* are described ~~as~~ below:

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

2) 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.*

3) *Risk analysis* should be undertaken to evaluate *biosecurity* threats and ensure 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 *disease risks*, as described in Article 4.X.7.

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

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

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

Article 4.X.5.

Categories of aquaculture production systems

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

Open systems

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

Semi-open

In a semi-open *aquaculture* production system, it is not possible to have control of water entering or exiting the system, or 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 and mollusc aquaculture in natural water bodies ~~and mollusc *aquaculture*~~, either suspended in the water column or on the ocean floor.

Semi-closed

In a semi-closed *aquaculture* production system, there is some control of water entering and exiting the system and of environmental conditions. *Aquatic animals* and *vectors* may 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

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

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 pathways that are likely to result in transmission of specific ~~may expose susceptible~~ ~~*aquatic animals*~~ ~~to high loads of~~ *pathogenic agents* should be prioritised.

The *risks* associated with introduction, spread, 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 ~~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 an *aquaculture establishment*, or moved within it, may include broodstock, juvenile stock for on-growing, and genetic material such as eggs. Both horizontal and vertical transmission mechanisms should be considered for *aquatic animals*. The *risk* of transmitting *pathogenic agents* via *aquatic animals* ~~can~~ should be managed; possible mitigation measures include the following ~~by~~:

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

b) ~~Quarantining~~ Placing introduced *aquatic animals* of unknown *disease* status into *quarantine* ~~from other farm populations in separate production units or dedicated~~ ~~*quarantine*~~ ~~facilities~~.

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

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

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

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

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

h) Where possible, preventing unintended movement of *aquatic animals* into, within or from the establishment.

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, within and 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 may include the entire body or parts of *aquatic animals* that have died or been killed for *disease* control purposes, as well as slaughtered *aquatic animals*, and their parts, that are not intended for human consumption.

Movement of *aquatic animal products* and *aquatic animal* waste into, within and out of *aquaculture establishments* may pose a *risk* of *pathogenic agent* transmission. This is particularly the case when a susceptible population is exposed to *aquatic animal products* and *aquatic animal* waste derived from clinically or sub-clinically infected *aquatic animals*. High *risk* waste includes *aquatic animal* waste that constitutes, or is suspected of constituting, a high health *risk* to *aquatic animals*.

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

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

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

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

c) ensuring systems are implemented for appropriate collection, treatment (inactivating *pathogenic agents*), transport, storage or disposal of *aquatic animal products* and *aquatic animal* waste to minimise 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 introduction of *pathogenic agents* into, spread within, and release from *aquaculture establishments.* The source of the water and how it 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 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 managed; possible mitigation measures include the following ~~can be managed by~~:

a) Where possible, choosing water sources that are 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 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 may present a *risk* of *pathogenic agent* transmission (e.g. oceans, streams or lakes). The level of treatment required will depend on the identified *risks*.

c) Ensuring 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.

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 of control on *aquatic animal feeds*. However, in semi-open production systems, *aquatic animals* may obtain food from their environment (e.g. filter feeding molluscs or wild fish which may be predated in net pens).

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

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

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

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

5. Fomites

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

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

a) Assessing any fomites brought into the *aquaculture establishment* for their *disease risk*.

b) Ensuring procedures and infrastructure are in place to clean and disinfect fomites, including at designated delivery and loading areas. Recommendations for the cleaning and 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.

6. Vectors

Vectors can transport *pathogenic agents* to susceptible *aquatic animals* in *aquaculture establishments*. These include wild *aquatic animals* entering via the water supply, predators, wild birds, ~~and~~ pest animals such as rodents and people. *Vectors* can 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 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:

a) netting (to prevent access by birds);

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

c) pest control and secure storage of feed and mortalities.

Article 4.X.7.

Risk analysis

Risk analysis is an accepted approach for evaluating *biosecurity* threats and 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* 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 the *biosecurity plan* are:

Step 1 – Hazard Identification

Hazard identification determines which *pathogenic agents* should be the subject of the *risk assessment*. A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents.* This step includes identifying and collecting relevant information on the *pathogenic agents* that have 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.

To complete the next steps of the *risk assessment*, required information on the identified *hazards* is 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 pathway. ~~A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents.*~~

Step 2 – Risk Assessment

A *risk assessment* can be initiated once it has been identified that a biological *hazard* exists. The aim of the *risk assessment* is to establish a *risk* estimate, which is the product of the likelihood and consequences of *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, transmission pathways will be assessed qualitatively but within a formal *risk assessment* framework. Examples of descriptors for 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, 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**
Insignificant	Impact not detectable or minimal.
Minor	Impact on *aquaculture establishment* productivity limited to some production units or short term only.
Moderate	Widespread impact on *aquaculture establishment* productivity due to increased mortality or decreased performance.
Major	Considerable impact on *aquaculture establishment* production resulting in serious supply constraints and financial impact.
Catastrophic	Complete ~~depopulation~~ <u>production loss in</u> ~~of~~ the *aquaculture establishment* and possibly barriers to resumption of production.

Table 3. Matrix for assessing 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	high	high	extreme	extreme

Results of *risk assessment* informs which biological *hazards* need to be addressed, which critical control points on the transmission pathway should be targeted, and the measures which are most likely to be effective in reducing *risk*.

Table 4. Interpretation of risk estimates

Risk* level	**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 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*.~~

**Risk* level determined by combination of likelihood and consequence score using the *risk* matrix (Table 3).

Step 3 – Risk Management

Risk management is used to determine the appropriate management response for the assessed level of *risk* as described in Table 4. The *risk assessment* process identifies the steps within transmission pathways necessary for a *risk* to be realised and thus allows the most effective mitigation measures to be determined. Many of the *hazards* will share the same pathways and thus mitigation measures may be effective against more than one *hazard*. Information on hazards and their pathways of introduction (step 1) should be combined with 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 different transmission pathways. The most appropriate mitigation measures for a specific *aquaculture establishment* will depend on the *risks* 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 develop 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 is recommended:

   a) objectives 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 epidemiologic units, if any, and the separation methods), loading/ unloading, unpacking, processing, feed storage, waste storage, reception areas, and maps showing major movements of *aquatic animals*, *aquatic animal products* and *aquatic animal* 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;

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

g~~h~~) internal and external communication procedures, and roles and responsibilities of personnel;

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

i~~j~~) performance evaluation;

j) standard operating procedures required to support all implementation of the mitigation measures, emergency procedures and the training requirements of personnel.

2. Key components of a biosecurity plan

a) Standard operating procedures (SOPs)

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

Staff 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 documentation necessary to provide evidence of compliance with the mitigation measures. 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*, visitors to the establishment, escapees, stocking densities, feeding and growth rates, records of staff 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 the producer, an *aquatic animal health professional* or a *veterinarian*), recording of clinical signs of *disease*, morbidity and mortality, 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 it continues to effectively address *biosecurity risks*.

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

Annex 18A

ASSESSMENT OF SHRIMP HAEMOCYTE IRIDESCENT VIRUS (SHIV) FOR LISTING IN CHAPTER 1.3. OF THE *AQUATIC ANIMAL HEALTH CODE*

Overall Assessment

The OIE Aquatic Animal Health Standards Commission assessed Shrimp haemocyte iridescent virus (SHIV) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code* and agreed that SHIV 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 SHIV

	Listing criteria						Conclusion
	1	2	3	4a	4b	4c	
Shrimp haemocyte iridescent virus	+	+	+	NA	+	-	The disease meets the criteria for listing

NA = not applicable.

Background

Shrimp haemocyte iridescent virus (SHIV) has so far only been detected in white-leg shrimp (*Penaeus vannamei*) and other crustacean species (*Fenneropenaeus chinensis*, *Macrobrachium rosenbergii*, *Procambarus clarkii* and *Cherax quadricarinatus*) in many coastal provinces in People's Republique of China. The Aquatic Animals Commission has recognised the potential significance of SHIV to many countries given the worldwide importance of crustacean farming and trade. At the moment SHIV is considered an "emerging disease" and, as such, should be reported in accordance with Article 1.1.4. of the *Aquatic Code*.

Historically, *P. vannamei* have been traded internationally as broodstock and postlarva for production in new geographic regions, and shrimp. *L. vannamei* products are traded internationally, thus the potential of international spread is likely.

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 in white-leg shrimp *(Penaeus vannamei)* and other crustacean species (*Fenneropenaeus chinensis*, *Macrobrachium rosenbergii*, *Procambarus clarkii* and *Cherax quadricarinatus*) in many coastal provinces in People's Republique of China. Historically, *P. vannamei* have been traded internationally as broodstock and postlarva for production in new geographic regions, and shrimp. *L. vannamei* products are traded internationally. Histopathology, visualization under TEM and *in-situ* hybridisation provide evidence that the virus can be found in haematopoietic tissue, gills, hepatopancreas, periopods and muscle (Qui *et al.*, 2017a). Quantitative PCR detection in artificially infected shrimp showed that haemolymph had the highest and muscle the lowest SHIV load (Qui *et al.*, 2018).

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, SHIV has only been detected in China but the distribution of the virus may be wider than what has been reported because mortalities have not been investigated. However, because of the broad distribution of *L. vannamei*, *M. rosenbergii*, and other susceptible species to SHIV, as well as extensive trade in these species, it is expected that expression of the disease would have been reported elsewhere if the virus had spread widely. It is, therefore, likely that at least one country may 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* exhibited empty stomach and guts in all diseased shrimp, slight loss of colour on the surface and section of hepatopancreas, and soft shell in partially infected shrimp. Some individuals had slightly reddish body. Moribund shrimp lost their swimming ability and sink to the bottom of pond. Diseased *M. rosenbergii* exhibited a significant white triangle inside the carapace at the base of rostrum which is the location of hematopoietic tissue.

To date, a nested PCR method (Qiu *et al.,* 2017a), a TaqMan probe based real-time PCR (TaqMan qPCR) method (Qiu *et al.,* 2018), and *in situ* hybridization method (Qiu *et al.,* 2017a) have been published and are available for SHIV detection. The primers and TaqMan probe have been shown to be specific for SHIV (no cross-reaction with other shrimp pathogens), with a low detection limit (4 copies per reaction) and high sensitivity and specificity (95.3% and 99.2%, respectively).

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

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%) have been observed in affected *L. vannamei* and *M. rosenbergii* populations in farms in the People's Republique of China. Laboratory infection tests mimicking the natural infection pathway (*per os* and reverse garvage) in *P. vannamei* has shown 100% cumulative mortality within 2 weeks (Qiu *et al.,* 2017a). Injection challenges in *L. vannamei*, *C. quadricarinatus*, and *P. clarkii* also exhibited 100% cumulative mortalities (Xu *et al.*, 2016; Qiu *et al.*, 2017a). Since 2014, some events with massive losses of *L. vannamei*, *F. chinensis* and *M. rosenbergii* in coastal provinces People's Republique of China have been associated with infection with SHIV (Qui *et al.*, 2017a). 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 SHIV have been shown to have a significant effect on the health of cultured shrimp, crayfish, or lobsters resulting in significant consequences including morbidity and mortality, and it is possible that the disease also would affect wild aquatic animals. However, there are to date no available data to demonstrating impact (e.g. morbidity or mortality) of the disease on wild aquatic animals at a population level.

Conclusion

Criterion is not met.

References

Li, F., Xu, L. & Yang, F. (2017). Genomic characterization of a novel iridovirus from redclaw crayfish *Cherax quadricarinatus*: evidence for a new genus within the family *Iridoviridae*. *J Gen Virol*, **98(10).**

Qiu, L., Chen, M. M. & Wan, X. Y., (2017a). Characterization of a new member of *Iridoviridae*, Shrimp hemocyte iridescent virus (SHIV), found in white leg shrimp (*Litopenaeus vannamei*). *Sci Rep*., **7(1)**:11834.

Qiu, L., Chen, M. M. & Wang, R. Y., (2017b). Complete genome sequence of shrimp hemocyte iridescent virus (SHIV) isolated from white leg shrimp, *Litopenaeus vannamei*. *Arch Virol*, , **130(9),** 1-5.

Qiu, L., Chen, M. M. & Wan, X. Y. (2018). Detection and quantification of Shrimp hemocyte iridescent virus by TaqMan probe based real-time PCR. *J Inverteb Pathol*,, **154.**

Xu, L., Wang, T. & Li, F. (2016). Isolation and preliminary characterization of a new pathogenic iridovirus from redclaw crayfish *Cherax quadricarinatus*. *Dis Aquat Org*, **120(1),**17.

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 shrimp haemocyte iridescent virus

– Infection with Taura syndrome virus

– Infection with white spot syndrome virus

– Infection with yellow head virus genotype 1.

Model Article 10.X.13. for the fish disease-specific Chapters 10.5., 10.6. and 10.10. (or Article 10.4.17. for infection with infectious salmon anaemia virus)

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 X, the *Competent Authority* of the *importing country* should assess ~~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 (ovarian fluid and milt); and

   c) the temperature and pH of the water to be used for *disinfection*.

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

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

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

   The *Competent Authority* may wish to 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 of this article have been fulfilled.

CHAPTER 10.6.

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

Article 10.6.13.

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

1) When importing disinfected eggs of the species referred to in Article 10.6.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with IHNV, the *Competent Authority* of the *importing country* should assess ~~the~~ ~~*risk* associated with~~ at least the following:

   a) the ~~infection with IHNV~~ likelihood that ~~status of the~~ water ~~to be~~ used during the *disinfection* of the eggs is contaminated with IHNV;

   b) the prevalence of infection with IHNV in broodstock (ovarian fluid and milt); and

   c) the temperature and pH of the water to be used for *disinfection*.

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

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

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

   The *Competent Authority* may wish to 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 10.6.2. for *aquaculture*, from a country, *zone* or *compartment* not declared free from infection with IHNV, 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 of this article have been fulfilled.

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

1. Scope

Infection with spring viraemia of carp virus means infection with the pathogenic agent *Carp sprivivirus* (also commonly known as spring viraemia of carp virus [SVCV]), in of the Genus *Sprivivirus* and the Family *Rhabdoviridae.* The current definition does not include viruses of the species *Pike fry sprivivirus.*

Spring viraemia of carp (SVC) is a rhabdovirus infection capable of inducing an acute haemorrhagic and contagious viraemia in several carp species and of some other cyprinid and ictalurid fish species. For the purpose of this chapter, SVC is considered to be infection with spring viraemia of carp virus (SVCV). Comprehensive references can be found in reviews by Wolf (1988), Ahne *et al*. (2002) and Dixon (2008).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent of SVC is Spring viraemia of carp virus (SVCV), a species in the genus *Vesiculovirus* in the virus family *Rhabdoviridae* (Carstens, 2010). The virus genome is a non-segmented, negative-sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (Ahne *et al.,* 2002). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Björklund *et al.* [1996] and Genbank accession AJ318079 by Hoffmann *et al.* [2002]). The complete genome sequence of isolates from China (People's Rep. of) has also been deposited in Genbank (Genbank accession DQ097384 by Teng *et al.* [2007] and Genbank accession EU177782 by Zhang *et al.* [2009]).

Stone *et al.* (2003) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 isolates from different fish species and geographical locations previously identified as SVCV or pike fry rhabdovirus (PFRV) by serology. The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to genogroup I, sharing <61% nucleotide identity with viruses in the other three genogroups. Re-analysis of the sequence data generated for viruses assigned to Genogroup I identified four subgroups (Ia–d). Those viruses originating in Asia were assigned to Subgroup Ia, those from Moldova, the Ukraine and Russia to Subgroups Ib and Ic, and those from the UK to Subgroup Id. Genogroup II comprised a single isolate from grass carp (GrCRV), previously identified by serology as PFRV, genogroup III comprised the reference PFRV isolate, and genogroup IV comprised a large number of unassigned isolates and isolates previously identified as PFRV. The latter genogroup was called the tench rhabdovirus (TenRV) group after the species from which the earliest member was isolated. Further analysis also showed that SVCV genogroup I could be further subdivided into at least four subgenogroups. Ahne et al. (1998) showed that the two viruses could also be differentiated by a ribonuclease protection assay using a G-gene probe, suggesting that genetic differences exist between the two viruses.

Antibodies directed against SVCV cross-react to various degrees with members of the other three genogroups, indicating that the viruses possess common antigens, whilst being genetically distinct. The viruses have been shown to share common antigenic determinants on the G, N and M proteins, but can be differentiated by neutralisation assays (Jørgensen *et al*., 1989).

2.1.2. Survival and stability in processed or stored samples

There are limited published data on the stability of the pathogen in host tissues. There is also limited information on the stability of the virus in the tissues after death of a diseased animal. Detection in the tissues of recently dead animals by both reverse-transcription polymerase chain reaction (RT-PCR) and culture should not be ruled out, and therefore, dead fish as well the moribund should be taken for analysis.

The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre for when stored for 1 month at –20°C, or for 6 months at –30 or –74°C (Ahne, 1976; Kinkelin & Le Berre, 1974). The virus is stable over four freeze (–30°C)–thaw cycles in medium containing 2% serum (Kinkelin & Le Berre, 1974).

2.1.3. Survival and stability outside the host

The virus has been shown to remain viable outside the host for 5 weeks in river water at 10°C, for more than 6 weeks in pond mud at 4°C, reducing to 4 days in pond mud at 10°C (Ahne, 1976). ~~The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants are also effective for inactivation: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre–1 chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al*., 2007). The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre for when stored for 1 month at –20°C, or for 6 months at –30 or –74°C (Ahne, 1976; Kinkelin & Le Berre, 1974). The virus is stable over four freeze (–30°C) thaw cycles in medium containing 2% serum (Kinkelin & Le Berre, 1974).~~

For inactivation methods, see Section 2.4.5.

~~2.1.4. Life cycle~~

~~The virus appears to enter the host via the gill. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine.~~

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: all varieties and subspecies of common carp (*Cyprinus carpio*), bighead carp (*Aristichthys nobilis*), bream (*Abramis brama*), Caspian white fish (*Rutilus kutum*), fathead minnow (*Pimephales promelas*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon Idella*), roach (*Rutilus rutilus*) and sheatfish (also known as European or wels catfish) (*Silurus glanis*).

~~Naturally occurring SVC infections have been recorded from the following cyprinid species: common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*), crucian carp (*Carassius carassius*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (white amur) (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), orfe (*Leuciscus idus*), and tench (*Tinca tinca*) and bream (*Abramis brama*) (Basic *et al*., 2009; Dixon, 2008). Three Indian carp species, merigal (*Cirrhinus merigala* [= *C.cirrhosus*]), rohu, (*Labeo rohita*) and catla (*Catla catla* [= *Gebelion catla*]) have been reported to be hosts of SVCV (Haghighi Khiabanian Asl *et al.* 2008a), but the nucleotide sequence data from a confirmatory reverse-transcription polymerase chain reaction (RT-PCR) deposited at Genbank does not align with known SVCV nucleotide sequence data (D.M Stone, pers. comm.). In addition, the deduced amino acid sequence shares only limited similarity with SVCV, and therefore further work is required to determine whether this virus is SVCV in origin. The virus has also been isolated from the non-cyprinid sheatfish (also known as European catfish or wels) (*Silurus glanis*) and from pike (*Esox lucius*); the viral nucleic acid has also been detected in pike by combined RT-PCR and nested PCR (Koutná *et al*., 2003).~~

SVCV has also been reported to have been isolated from Nile tilapia (*Sarotherodon niloticus*) (Soliman *et al*., 2008) and rainbow trout (*Oncorhynchus mykiss*) (Jeremic *et al*., 2006; Haghighi Khiabanian Asl *et al*. 2008b). Immunohistochemistry constituted the sole basis of identification of SVCV from Nile tilapia; electron microscopy purported to show virus in the nucleus, which is not a feature of SVCV infection. Haghighi Khiabanian Asl *et al.* (2008b) used the same RT-PCR to identify the virus in rainbow trout that produced equivocal results when used to type the virus in Indian carp described above, and so the identity of that virus in rainbow trout awaits confirmation. The virus isolated from rainbow trout by Jeremic *et al.* (2006) was subsequently confirmed to be SVCV by nucleotide sequence analysis, but attempts to infect rainbow trout with the virus by intraperitoneal injection were unsuccessful, although the virus was virulent for common carp (P.F. Dixon, J. Munro & D.M. Stone, unpublished data). Hence, the status of rainbow trout and tilapia as hosts for SVCV remains unresolved, and awaits further confirmatory data. Some serological tests do not distinguish SVCV from members of the other genogroups described by Stone *et al.* (2003), and it is imperative that sequence data are used to confirm the identity of putative SVCV isolates from new hosts.

Other cyprinid species have been shown to be susceptible to SVCV by experimental bath infection, including roach (*Rutilus rutilus*) (Haenen & Davidse, 1993) whilst zebra fish (*Danio rerio*) and the golden shiner (*Notemigonus crysoleucas*) have been infected with SVCV by intraperitoneal injection (see Dixon, 2008). It is reasonable to assume that other cyprinid species in temperate waters may be susceptible to infection. Other species can also be infected experimentally, e.g. guppy (*Lebistes reticulatus*). The pumpkinseed (*Lepomis gibbosus*) has been reported to have been experimentally infected with SVCV, but there are no supporting data.

The nucleotide sequence of the G gene of a rhabdovirus isolated from the Pacific white shrimp, *Litopenaeus* (*Penaeus*) *vannamei*, in Hawaii is over 99% identical to that of SVCV (Johnson *et al*., 1999), and is serologically related to SVCV. The virus caused mortality in juvenile Pacific blue shrimp, *L. stylirostris*, fed food pellets soaked in the virus (Lu & Loh, 1994).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: Crucian carp (*Carassius carassius*), pike (*Esox lucius*), firebelly newt (*Cynops orientalis*), silver carp (*Hypophthalmichthys molitrix*), Yellow perch (*Perca flavescens*), *and* zebrafish (*Danio rerio*). Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is SCVC, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

2.2.2. Susceptible stages of the host

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations Species or subpopulation predilection (probability of detection)

Common carp varieties are the principal hosts for SVCV and are considered to be most susceptible to infection with SVCV infection followed, in order of susceptibility, by other carp species (including hybrids), other susceptible cyprinid species and finally susceptible non-cyprinid fish species. When sampling during surveillance programmes for SVCV, common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp), then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Should these species not be available then other known susceptible species should be sampled. in the following preferential order: tench, orfe, wels catfish and, finally, any other cyprinid species present. Cyprinid species are increasingly mixed together in polyculture systems and the risk of transmission of SVCV between species during disease outbreaks is high (Billard & Berni, 2004).

Generally, young fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Moreover, there is a high variability in the degree of susceptibility to infection with SVCV among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age or the age-related status of innate immunity appears to be extremely important: the younger the fish, the higher the susceptibility to overt disease, although even adult broodfish can be susceptible to infection.

Fish that have separated from the shoal and found at the water inlet or sides of a pond are more likely to be infected.

2.2.4. Distribution of the pathogen in the host

The transmission of SVCV is horizontal (Fijan, 1988). SVCV appears to enter via the gills and then spreads to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks high titres of virus occur in the liver and kidney of infected fish, but much lower titres occur in the spleen, gills and brain (Dixon, 2008). The virus has been detected in ovarian fluid (Békési & Csontos, 1985), but vertical transmission has yet to be demonstrated.

2.2.5. Persistent infection with lifelong carriers

The reservoirs of SVCV are clinically infected fish and covert virus carriers among cultured, feral or wild fish. Factors affecting persistence and duration of the carrier state have not been studied.

2.2.5. Reservoirs of infection

Liu *et al.* (2004) isolated SVCV in China (People's Rep. of) from common and koi carp exhibiting no external or internal signs of disease, and similarly, the virus was isolated from apparently healthy wild carp in Canada (Garver *et al*., 2007).

2.2.6. Vectors

Among animate vectors, The parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola geometra* (Annelida, Hirudinea) have been demonstrated to transfer transferred SVCV from diseased to healthy fish under experimental conditions and the virus has been isolated from *A. foliaceus* removed from infected carp (Ahne *et al.*, 2002; Dixon, 2008). It has been demonstrated experimentally that virus can be isolated from fish tissues regurgitated by herons (*Ardea cinerea*) 120 minutes after being fed with SVCV-infected carp, suggesting a potential route for SVCV transmission, but is not known whether such transmission has occurred in nature (Peters & Neukirch, 1986). Herons (*Ardea cinerea*) were fed SVCV-infected carp and made to regurgitate the fish at intervals post-feeding. Virus was isolated from fish regurgitated 120 minutes after feeding.

2.2.7. Known or suspected wild aquatic animal carriers

Most reports of SVC have been from cultured fish, but the virus has been isolated from both diseased and apparently healthy feral carp in lakes.

It has been suggested that a possible mode of transmission of the virus is by the movement of baitfish, but there are no data to show that this has occurred (Goodwin *et al.*, 2004). The main mode of transmission of the virus from one area to another is by movements of infected fish. The virus is often found in ornamental fish such as goldfish and koi carp, which are regularly transported around the world.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence Transmission mechanisms

The mode of transmission for SVCV is horizontal, but 'egg-associated' transmission (usually called 'vertical' transmission) cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid although there have been no further such reports. Horizontal transmission may be direct or vectorial, water being the major abiotic vector (Fijan, 1988). Animate vectors (Section 2.2.6.) and fomites may also be involved in transmission of SVCV (Fijan, 1988). Once SVCV is established in pond stock or pond farm stock, it may be very difficult to eradicate without destroying all types of life at the fish production site.

During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Co-infections with koi herpesvirus or carp oedema virus can increase levels of mortality.

Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both nonspecific (e.g. interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Poor physiological condition of over-wintered fish may be a contributory factor to disease susceptibility. In European aquaculture, losses can be up to 70% in young carp (Ahne *et al.*, 2002), but are usually from 1 to 40%. Approximately 20% of the carp population in a lake in the USA died from SVC during a disease outbreak.

In one survey from Serbia, the virus was isolated by culture in samples collected from 12 of the 38 hatcheries screened over the 10-year period (1992–2002). The virus occurred sporadically in different ponds on one site, and sporadically from year to year at different sites (Svetlana *et al.,* 2004). In another study, 18 of 30 tissue pools (five fish/pool) of wild common carp sampled in Canada in 2006 were positive for SVCV by culture (Garver *et al.,* 2007). The isolation of SVCV in the latter case was from asymptomatic common carp which correlates with observations that SVCV infection can often be clinically inapparent (Fijan, 1999).

2.3.2. Prevalence

There are very few data on the prevalence of SVC, although there have been a small number of surveys of prevalence of antibody against the virus. In one such survey, carp in 19 of 20 hatcheries surveyed were positive for antibody against the virus. Data collected over the 10-year period 1992–2002 from Serbia showed that the virus had been isolated from carp at 12 of 38 hatcheries. The virus can occur sporadically in different ponds on one site, and sporadically from year to year at different sites.

2.3.2. Clinical signs, including behavioural changes

Fish can become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Clinical signs of infection with SVCV are nonspecific, and not all fish will exhibit all of the signs. Two of the most obvious and consistent features are abdominal distension and haemorrhages. The latter may occur on the skin, fin bases, eyes and gills, which may be pale. The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Diseased fish usually appear darker in colour. There may be no clinical signs in cases with a sudden onset of mortality.

2.3.3 Gross pathology

There are no pathognomonic gross lesions. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucous instead of food. Oedema and haemorrhage of the visceral organs is commonly observed (the spleen is often enlarged), and organs adhere to each other and to the peritoneum. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder (see Dixon, 2008). However, petechial haemorrhages are uncommon in cases caused by Asian strains of SVCV (Dikkeboom *et al*., 2004; Goodwin, 2003).

2.3.4. Modes of transmission and life cycle

The transmission of SVCV is horizontal (Fijan, 1988). The virus appears to enter the host via the gill. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine (Ahne 1977, 1979, 1982).

Vertical or ‘egg-associated’ transmission cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid, although there have been no further reports (Békési & Csontos, 1985).

Horizontal transmission may be direct or vectorial, water being the major abiotic vector (Fijan, 1988). Animate vectors (Section 2.2.6.) and fomites may also be involved in transmission of SVCV (Fijan, 1988). Once SVCV is established in populations, it may be very difficult to eradicate without destroying all types of life at the site.

2.3.5. Environmental and management factors

Disease outbreaks in carp generally occur between 11 and 17°C. They rarely occur below 10°C, and mortalities, particularly in older fish, decline as the temperature exceeds 22°C (Fijan, 1988). However, the virus was isolated from apparently healthy fish from a lake in Canada that had been sampled over a 13-day period during which the water temperature varied between 24.2°C and 27.3°C (Garver *et al,* 2007). Secondary and concomitant bacterial and/or parasitic infections can affect the mortality rate and display of signs. In carp, the disease is often observed in springtime (hence the common name for the disease), particularly in countries having cold winters. It is believed that the poor condition of the over-wintered fish may be a contributory factor in disease occurrence. The disease can occur in fish in quarantine following the stress of transportation, even though there has been no evidence of virus in the fish prior to transportation.

2.3.6. Geographical distribution

For a long time, the geographical range of SVC was limited to countries of the European continent that experience low water temperatures during winter. Consequently, the disease has been recorded from most European countries and from certain of the western Independent States of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine) (see Dixon 2008 for references to these and the following locations). However, in 1998, the disease was recorded South America (in goldfish in a lake in Brazil), in 2002 in the USA, and in 2006 in Canada. Detection of the virus in carp in China (People's Rep. of) was confirmed in 2004.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

A safe and effective vaccine is not currently available. However, a number of experimental inactivated preparations, live attenuated vaccines and DNA vaccines have given encouraging results (Dixon, 2008, Emmenegger & Kurath, 2008). The use of live attenuated vaccines or the DNA vaccines might affect diagnostic performance. A number of studies have reported the efficacy of vaccination, and vaccination trials in the field have been reported from the former Yugoslavia, Austria and the former Czechoslovakia (Fijan, 1988); a vaccine was once marketed in the latter country, but is no longer available. Laboratory trials have shown that DNA vaccination can protect fish (Dixon, 2008; Emmenegger & Kurath, 2008), but further developmental work is required.

2.4.2. Chemotherapy including blocking agents

Methisoprinol inhibits the replication of SVCV *in vitro*, but has not been tested under carp culture conditions.

2.4.3. Immunostimulation

Injection into carp of single-stranded and double-stranded RNA (which is an interferon inducer) protected carp for longer than 3 weeks, but the treatment is not effective by bath administration (Alikin *et al.*, 1996; Masycheva *et al.*, 1995).

2.4.4. Breeding resistant strains

The "Krasnodar" strain of common carp has been bred for increased resistance to SVCV (Kirpichnikov *et al*., 1993).

The wide host range of the virus means that rigorous selection procedures would have to be applied to prospective alternative species.

2.4.5. Inactivation methods

The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants are also effective for inactivation: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre^{-1} chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al.*, 2007).

2.4.6. Disinfection of eggs and larvae

Eggs can be disinfected by iodophor treatment (Ahne & Held, 1980).

2.4.7. General husbandry

Methods to control of infection with SVCV SVC disease mainly rely relies on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. Hygiene measures should include disinfection of eggs by iodophor treatment (Ahne & Held, 1980), until it has been confirmed unequivocally that vertical transmission does not occur, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish. Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C may stop or prevent outbreaks of infection with SVCV SVC outbreaks.

Ponds should be disinfected regularly and effective disease biosecurity practices should be used. Equipment, particularly nets, should not be used in different ponds unless first disinfected. Practices that might cause stress should be minimised, and high stocking densities should be avoided.

3. Specimen selection, sample collection, transportation and handling

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

3.1. Selection of populations and individual specimens

For disease investigations, moribund fish or fish exhibiting clinical signs of the disease infection with SVC should be collected fish should be alive when collected. Ideally fish should be alive when collected, however recently dead fish can be collected for diagnostic purposes. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. However, There may be no pathognomonic gross lesions and no clinical signs in cases of sudden mortality (see Section 4.1.1.).

Histopathological changes can be observed lesions and there may be no clinical signs in cases of sudden mortality (see Section 4.1.1). An identification label that includes information on the place, time, date, species, number of samples collected, dead/moribund state on collection, and the name and contact information of the individual collecting the sample(s) should be attached to the sample(s). A general approach to surveillance and sampling is provided in the *Aquatic Animal Health Code,* Chapter 1.4 *Aquatic animal health surveillance*. See also the OIE *Guide for Aquatic Animal Health Surveillance* (Corsin *et al.,* 2009).

Fish collection should encompass a statistically significant number of specimens, but it is obvious that failure to detect certain pathogens from the sample does not guarantee the absence of these agents in the specimen examined or in the stock. This is particularly true of free-ranging or feral stocks from which it is difficult to collect a representative and random sample. However, the likelihood of not detecting the pathogen when present is lower in fish farms whose fish stocks have been inspected and checked for pathogens for several years (at least two), insofar as they are not exposed to possible recontamination by feral fish.

Samples should comprise all susceptible species on the site with each lot of a group species being represented in the sample. A group lot is defined as a group population of the same fish species that shares a common water supply and that originates from the same broodfish or spawning population. Generally, young fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Any moribund fish present in the fish population to be sampled should be selected first for sample collection and the remainder of the sample should comprise randomly selected live fish from all rearing units that represent the lot being examined.

3.2. Selection of organs or tissues

Subclinically infected fish (apparently healthy fish): kidney, spleen, gill and encephalon (any size fish).

For clinically affected fish: whole alevin (body length ≤ 4 cm), entire viscera including kidney and encephalon (> 4 cm body length ≤ 6 cm) or, for larger sized fish, liver, kidney, spleen and encephalon should be selected.

3.3. Samples or tissues not suitable for pathogen detection

It can be difficult to isolate virus from subclinically infected carrier fish and, in particular, from fish surviving a disease outbreak with increasing time after the disease occurrence. Likewise, isolation of virus from such fish at temperatures outside the clinical range for the disease is problematic. It may be possible to detect antibody against the virus in such fish (Dixon, 2008), but see the caveat in Section 4 below. Virus isolation may not be possible from decomposed clinical samples, so the presence of signs of SVC disease and a positive indirect immunofluorescent antibody test (IFAT) or enzyme-linked immunosorbent assay (ELISA) may be considered sufficient to initiate control measures. A number of studies in which attempts were made to isolate virus from reproductive fluids were unsuccessful, although the virus has been isolated at low frequency from ovarian, but not seminal, fluids.

Virus isolation may also not be possible from decomposed clinical samples. A number of studies in which attempts were made to isolate virus from reproductive fluids were unsuccessful, although the virus has been isolated at low frequency from ovarian, but not seminal, fluids.

3.4. Non-lethal sampling

Serological assays for antibodies can be undertaken on blood samples; the cross reactivity of anti-SVCV antibodies with viruses of the species pike fry sprivivirus allows for a presumptive indication of infection with SVCV.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0. or 2.3.0. or 2.4.0.

Samples for virus isolation should be transported to the laboratory at 4°C using refrigerated containers or on ice, preferably in virus transport medium (Chapter 2.3.0 *General information* [on diseases of fish], Section A.2.2.1.), and tested within 24 hours or, in exceptional circumstances, 48 hours. The shipment of organ samples is preferred, but live or whole dead fish can be submitted to the testing laboratory if necessary. If this is not possible, samples can be frozen, but there may be loss of virus viability on thawing the samples. Repeated freeze–thawing of the sample must be avoided.

3.5.1. Samples for pathogen isolation

Samples for virus isolation (Section 3.2.) should be transported to the laboratory at 4°C using refrigerated containers or on ice, preferably in virus transport medium and tested within 24 hours or, in exceptional circumstances, 48 hours. The shipment of organ samples is preferred, but live or whole dead fish can be submitted to the testing laboratory if necessary. If this is not possible, samples can be frozen, but there may be loss of virus viability on thawing the samples. Repeated freeze–thawing of the sample must be avoided.

3.5.2. Fixed samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. [Alternatives to ethanol can be mentioned if they can be referenced.]

The material collected for virus culture is generally used for the molecular diagnostic assays, but additional tissue samples for RT-PCR can be preserved in commercially available RNA preservation solutions according to the manufacturers' recommendations, or, alternatively, samples can be preserved in 80–90% (v/v) analytical grade (absolute) ethanol at the recommended ratio of ethanol to tissue of 10:1.

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

Histology samples from each individual fish must be taken into 10% neutral buffered formalin (NBF) immediately after collection to prevent sample deterioration. The recommended ratio of fixative to tissue is 10:1 and each sample should be no thicker than approximately 4 mm to allow the fixative to penetrate the material and should be cut cleanly.

3.5.4. Fixed samples for electron microscopy

EM sampling is not required as standard, and the material is collected only where it is considered beneficial to facilitate potential further diagnostic work. From each fish sampled a 2 mm cubed (approximately) 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

Tubes for the separation of serum are available commercially. After collection of the blood is allowed to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Serum is clarified by centrifuging at 1000–2000 ***g*** for 10 minutes in a refrigerated centrifuge.

It is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette and maintain the samples at 2–8°C while handling. If the serum is not analysed immediately, it should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze–thaw cycles because this is detrimental to many serum components. Samples that are haemolysed, icteric or lipaemic can invalidate certain tests.

3.6. Pooling of samples

Traditionally pools of five animals have been used and more recently this has been increased to pools of ten animals for virus culture. However, no published data on the effect of pooling on test characteristics has been published.

4. Diagnostic methods

Diagnosis of SVC in clinically affected fish may be achieved by virus isolation or, more rapidly, by IFAT or ELISA on infected tissues. Ideally, direct diagnosis by IFAT or ELISA should be confirmed by virus isolation followed by a virus neutralisation (VN) test or RT-PCR and sequence analysis.

The detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations because of insufficient knowledge of the serological responses of fish to virus infections. However, the validation of some serological techniques for certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for health screening purposes. As SVCV cannot be detected at all times of the year, or with confidence from all carrier fish, there are occasions when detection of fish antibody may provide useful information for epidemiological studies or risk assessments. However, it must be borne in mind that the presence of specific antibody only indicates previous exposure to the virus, and is not an indicator of the current presence of virus in a fish. Antibody surveys are best used at the population level, rather than the individual level as an indicator of previous exposure to the virus.

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.

Annex 21A (contd)

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
Wet mounts												
Cytopathology[3]												
Histopathology[3]												
Cell or artificial media culture		+++	+++	1		+++	+++	1		+++	+++	1
Real-time PCR												
Conventional PCR		++	++	1		+++	+++	1				
Amplicon sequencing[4]										+++	+++	1
In-situ hybridisation						±	±	1		±	±	1
Bioassay												
LAMP		±	±	1		++	±	1				
Ab ELISA												
Ag ELISA						++	++	1				
Other antigen detection methods												
Other serological method												

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

⇒ Technical procedure

▪ How to use positive/negative controls

⇒ Interpretation of results

⇒ Availability of test (from Reference Laboratories, commercial sources or easily synthesised)

4.1. Wet mounts

Not applicable.

4.1. Field diagnostic methods

4.1.1. Clinical signs

During an outbreak of SVC there will be a noticeable increase in mortality in the population. Diseased fish usually appear darker in colour. Typical clinical signs include exophthalmia, pale gills, haemorrhages on the skin, base of the fins and the vent, abdominal distension or dropsy and a protruding vent (anus), often with trailing mucoid faecal casts. All these clinical signs may not be present in individual fish, and they may not all be present in the affected population. Some of these signs may be present in diseases caused by other pathogens. There may be no clinical signs in cases with a sudden onset of mortality.

4.1.2. Behavioural changes

Generally, young fish up to 1 year are most susceptible to clinical disease, but all age groups can be affected. Fish become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium.

4.2. Clinical methods

4.2.1. Gross pathology

There are no pathognomonic gross lesions. Final diagnosis must await direct detection of viral antigen or nucleic acid in tissues or virus isolation and identification. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucus instead of food. Oedema and haemorrhage of the visceral organs is commonly observed. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder.

4.2.2. Clinical chemistry

In the absence of large-scale studies, clinical chemistry is an unreliable means of indicating SVC disease. The data presented below are only indicative of nonspecific disease processes.

Some groups of sheatfish experimentally infected with the virus exhibited lowered haematocrit values, but in other groups the values remained unchanged. Transaminase activity increased in all groups.

During 3 months following an outbreak of SVC in carp in ponds there was an increase in neutrophils, monocytes, eosinophils, and basophils. The numbers of lymphocytes declined then rose back to the starting levels. Over the same period, fish with signs of SVC had an increase in plasma levels of Ca^{2+}, inorganic phosphate levels, total bilirubin, alanine aminotransferase activity, lactic acid dehydrogenase activity and α-hydroxybutyryl dehydrogenase activity. Levels of total protein, cholesterol and alkaline phosphatase activity decreased.

4.2.3. Microscopic pathology

4.2. Cyto- and histopathology

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivasculitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necrosis and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres, and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed and lymph vessels are filled with detritus and macrophages. In the swim bladder, the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the submucosa are dilated with nearby lymphocyte infiltration.

As the histopathological picture is not specific for the disease. and not all fish will exhibit each feature (Dixon & Stone 2016; Gaafar *et al.,* 2011; Misk *et al.,* 2016), microscopic methods by themselves are not recommended for diagnosis of SVC as the histopathological picture is not specific for the disease. They may, however, provide supporting evidence, particularly, when immunohistological (IHC) or DNA based *in-situ* hybridisation methods are used (see the relevant Sections below)

Fixed sections can also be used for histoimmunochemistry immunohistochemical procedures (but see caveats in Section 4.6.).

4.2.4. Wet mounts

Not relevant.

4.2.5. Smears

Only of value if an immunohistological procedure such as the IFAT (see Section 4.3.1.2.2.1) or immunoperoxidase procedure is used but see caveats in Section 4.3.1.2.

4.2.6. Fixed sections

See Section 4.2.3. Fixed sections can also be used for immunohistochemical procedures as in 4.2.5, but see caveats in Section 4.3.1.2.

4.2.7. Electron microscopy/cytopathology

The virus has the typical bullet shape of a rhabdovirus and is approximately 60–90 nm wide by 90–180 nm long, following negative staining. The virus comprises a nucleocapsid surrounded by an envelope.

4.3. Agent detection and identification methods

See the following sections in Chapter 2.3.0:

- Section A.2.2.1 for further details of transportation.
- Section A.2.2.2 for virus extraction and obtaining organ homogenates.

4.3.1. Direct detection methods

The virus can be observed directly by electron microscopy, but this will only indicate the presence of a rhabdovirus and further identification will be needed. Virus antigen and nucleic acid can potentially be identified in extracts of tissues from clinically infected fish, and virus can usually be isolated from those fish. However, it is much less likely that virus antigen or nucleic acid will be detected directly from tissues from subclinically infected carrier fish. Virus isolation is the preferred method for detecting such fish, but is not 100% effective.

4.3.1.1. Microscopic methods

Microscopic methods by themselves are not recommended for diagnosis of SVC as the histopathological picture is not specific for the disease. They may, however, provide supporting evidence, particularly, when immunohistological methods are used, but see caveats in Section 4.3.1.2.

4.3.1.1.1. Wet mounts

Not relevant.

4.3.1.1.2. Smears/tissue imprints

Only of value if an immunohistological procedure such as the IFAT (see Section 4.3.1.2.2.1) or immunoperoxidase procedure is used but see caveats in Section 4.3.1.2.

4.3.1.1.3. Fixed sections

Only of value if an immunohistological procedure such as the IFAT (see Section 4.3.1.2.1.2) or immunoperoxidase procedure is used but see caveats in Section 4.3.1.2. See Chapter 2.3.0, Section B.3.3.1 for details of fixation of specimens.

4.3.1.2. Agent isolation and identification

Following isolation, the virus must be identified and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur (Section 2.1.1 and Section 5). Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al*., 2007) to confirm the identity of the virus.

4.3.1.2.1. Cell culture/artificial media

4.3. Cell or artificial media culture for isolation

If culturing viruses, cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

Cell culture

Cell line to be used: EPC, FHM or GCO (Chapter 2.3.0, Section B.1.1).

Virus extraction: Use the procedure described in Chapter 2.3.0., Section A.2.2.2.

Inoculation of cell monolayers: make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium (i.e. the homogenate supernatants will be 1/100 and 1/1000 dilutions of the original organ material) and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers drained of their culture medium. Alternatively, make a single tenfold dilution of the 1/10 organ homogenate (i.e. a 1/100 dilution of the original organ material) and add an appropriate volume of both the 1/10 and 1/100 dilutions directly to undrained 24-hour-old cell monolayers, to effect 1/100 and 1/1000 final dilutions of the organ homogenate. Should toxicity of the sample be a problem, make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium as described above and inoculate at least 2 cm^2 of drained cell monolayer with 100 µl of each dilution. Allow to adsorb for 0.5–1 hour at 10–15°C, withdraw the inoculum and add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml well–1 for 24-well cell culture plates). Incubate at 20°C.

Monitoring incubation: Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

Maintain the pH of the cell culture medium at between 7.3. and 7.6. during incubation. This can be achieved by the addition to the inoculated medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris (Tris [hydroxymethy]) aminomethane)/HCl buffer solution (for cell culture plates).

If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately (see Section 4.6.2. Sections 4.3.1.2.1.1, 4.3.1.2.1.2, 4.3.1.2.1.3 and 4.3.1.2.3.1 below).

Subcultivation procedures: using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24-hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above. If no CPE occurs, the test may be declared negative.

If no CPE occurs the test may be declared negative. However, if undertaking surveillance to demonstrate freedom from SVCV it would be advisable to screen the cells at the end of the 14 days using an SVCV-specific RT-PCR or real-time RT-PCR (Section 4.4.). Following a positive result culture should be re-attempted.

If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

Subcultivation procedures: using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24-hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above. If no CPE occurs, the test may be declared negative.

4.3.1.2.1.1. Confirmation of virus identity by neutralisation

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge at 2000 ***g*** for 15 minutes at 4°C, or filter through a 0.45 µm pore membrane to remove cell debris.

ii) Dilute the virus-containing medium from 10^{-2} to 10^{-4}.

iii) Mix aliquots of each dilution with equal volumes of an antibody solution against SVCV, and similarly treat aliquots of each virus dilution with cell culture medium. The neutralising antibody (NAb) solution must have a 50% plaque reduction titre of at least 2000 based on neutralisation of 50–100 plaque-forming units (PFU) of SVCV.

iv) In parallel, other neutralisation tests must be performed against:

- a homologous virus strain (positive neutralisation test)
- a heterologous virus strain (negative neutralisation test).

v) Incubate all the mixtures at 20°C for 1 hour.

vi) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 15–20°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

vii) When adsorption is completed, add cell culture medium, supplemented with 2% FCS and buffered at pH 7.4–7.6, to each well and incubate at 20°C.

viii) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase-contrast preferable) or after discarding the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

ix) The tested virus is identified as SVCV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the SVCV-specific antibody, whereas CPE is evident in all other cell cultures.

NOTE: Presumptive SVCV isolates identified by the ELISA or the IFAT may not be neutralised by NAb to SVCV. Also, some SVCV subgenogroups may not be completely neutralised by NAb prepared against an isolate from a different subgenogroup. Where neutralisation by NAb to SVCV is absent or incomplete, confirmation by the RT-PCR and nucleotide sequence analysis of RT-PCR products is recommended to confirm the presence of SVCV.

Following isolation, the virus must be identified, and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods are generally regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur. Commercially available kits using polyclonal antibodies may also lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al*., 2007) to confirm the identity of the virus.

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

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

Real-time RT-PCR assays are available to detect and confirm infection with SVCV (Yue *et al*., 2008; Zhang *et al*., 2009), however, they are not currently recommended as they have not been sufficiently validated.

4.4.2. Conventional PCR (PCR)

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

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

Reverse-transcription polymerase chain reaction (*RT-PCR*) (*confirmation of virus identity or directly from fish tissue extracts*)

The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).

i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 50µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.

   A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR. Examples are Trizol ReagentT (RL, Life Technologies, Paisley, UK), SV Total RNA isolation system (Promega) and Nucleospin® RNA (AB gene), EZ virus mini kit, Ez RNA tissue mini kit (Qiagen).

ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or an equivalent reverse transcriptase system and 1/10 of the total RNA extracted above.

iii) PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is ~~overlaid with mineral oil and~~ subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.

iv) If the CPE in culture is not extensive it is possible that a visible product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).

v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.

vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.

vii) In cases where the CPE is extensive and the virus replicates to a high titre, or where a semi-nested RT-PCR assay was used, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

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

NOTE: SVCV primer-annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (Björklund *et al*., 1996; Genbank accession U18101), and the vesicular stomatitis virus (VSV) New Jersey (Gallione & Rose, 1983, Genbank accession V01214), and Piry strains (Genbank accession D26175). Primers were then designed to anneal to the regions encoding the conserved amino acids using the published sequence for SVCV (Björklund *et al*., 1996) as a skeleton, and introducing degenerate bases at the 3' termini to allow for potential differences in codon usage. The appropriate IUB codes have been used where appropriate and are indicated by an asterisk (*).

Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutná *et al*., 2003; Shimahara *et al*., 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al*., 2014). With the exception of the conventional PCR assay developed by Shimahara *et al*. (2016) the other assays were not fully validated against representatives from each of the recognized SVCV genogroups and may they fail to detect the full range of SVCV genotypes.

4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification assays are available to detect and confirm SVCV infections (Shivappa *et al.,* 2008), however, they are currently not recommended as they are not sufficiently validated.

Infection with SVCV has also been confirmed using RT-PCR and hybridisation with non-radioactive probes (Oreshkova *et al*., 1999; Sheppard *et al*., 2007).

4.5. Amplicon sequencing of the amplicon

See above (Section 4.4.2.). All RT-PCR amplicons should be sequenced to confirm that they are SVCV in origin. SVCV-specific products will share higher degree of nucleotide identity to one of the published sequences for SVCV (Genbank accession U18101, AJ318079, DQ097384 and EU177782) compared to the published sequences for the *Pike sprivivirus*es (GenBank FJ872827, KC113518 and KC113517).

4.6. *In-situ* hybridisation (and histoimmunochemistry)

Although *in-situ* hybridisation can be used to locate the virus in different tissues on known positive animals, but it has not been well validated for SVCV as a diagnostic tool.

SVCV can be detected by immunohistochemistry, however, care must be taken with interpreting the results of serological tests for SVCV, and positive results from antibody-based assays should be confirmed by RT-PCR and sequencing (see Section 4.8.)

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store and transport the kidney pieces as indicated in Chapter 2.3.0., Section A.2.2.1.) together with the other organs required for virus isolation.

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

v) Fix with cold acetone (stored at –20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBST, and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

x) Rinse four times with PBST.

xi) Incubate the imprints with a solution of FITC-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xii) Rinse four times with PBST.

xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.

xiv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical aperture of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.7. Bioassay

Not available.

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

Serological methods must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses closely related spriviruses (PFRV, GrCRV and TenRV) may occur. Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005).

Confirmation of virus identity by the indirect fluorescent antibody test (IFAT)

i) Prepare monolayers of cells in 2 cm^2 wells of plastic cell culture plates, flasks or on cover-slips or glass slides in order to reach approximately 80% confluency within 24 hours of incubation at 25°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. For tests using cells cultured on glass cover-slips or slides, the dilutions are made in sterile containers and then used to inoculate the cells.

iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5000–10,000 PFU ml^{-1} in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for slides or cover-slips or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for cells on plastic substrates. Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.

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

vii) Rehydrate the dried cell monolayers, if they have been stored frozen, by four rinsing steps with PBS containing 0.05% Tween 20 (PBST) and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

viii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

ix) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

x) Incubate the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

xi) Rinse four times with PBST.

xii) Incubate the cell monolayers with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) View the treated cell monolayers on plastic substrates immediately, or mount the slides or cover-slips using glycerol saline at pH 8.5, or a commercially available mountant.

xv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

Virus identification by enzyme-linked immunosorbent assay (ELISA)

~~Confirmation of virus identity by enzyme-linked immunosorbent assay (ELISA)~~

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl $well^{-1}$). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.

vii) Dispense 100 µl well^{-1} of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control). Also include SVCV positive control virus. Incubate for 1 hour at 37°C.

viii) Rinse four times with PBST.

ix) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. An MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

x) Rinse four times with PBST.

xi) If HRPO-conjugated antibody has been used, go to step xiii. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xii) Rinse four times with PBST.

xiii) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

~~*4.3.1.2.2. Antibody-based antigen detection methods directly on fish tissues*~~

~~*4.3.1.2.2.1. Indirect fluorescent antibody test*~~

~~i) Bleed the fish thoroughly.~~

~~ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.~~

~~iii) Store and transport the kidney pieces as indicated in Chapter 2.3.0, Section A.2.2.1.) together with the other organs required for virus isolation.~~

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

~~v) Fix with cold acetone (stored at –20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.~~

~~vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBST, and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.~~

~~vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.~~

~~viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).~~

~~ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.~~

~~x) Rinse four times with PBST.~~

~~xi) Incubate the imprints with a solution of FITC-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.~~

~~xii) Rinse four times with PBST.~~

~~xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.~~

~~xiv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical aperture of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.~~

Enzyme-linked immunosorbent assay (*ELISA*) *using tissue homogenates*

See Chapter 2.3.0, Section A.2.2.2 for obtaining organ homogenates.

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well^{-1}). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Store a 1/4 aliquot of each homogenate at 4°C, in case the test is negative and virus isolation in cell culture is required.

vii) Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulphonide fluoride; mix gently.

viii) Dispense 100 µl well–1 of two- or four-step dilutions of the sample to be identified, and of negative control tissues. Also include an SVCV positive control virus. Incubate for 1 hour at 37°C.

ix) Rinse four times with PBST.

x) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. A MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) If HRPO-conjugated antibody has been used, go to step xiv. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xiii) Rinse four times with PBST.

xiv) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

xv) If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 4.5.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Reverse transcription polymerase chain reaction (RT PCR) (confirmation of virus identity or directly from fish tissue extracts)

The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).

i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 100 µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.

   A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR. Examples are Trizol ReagentT (RL, Life Technologies, Paisley, UK), SV Total RNA isolation system (Promega) and Nucleospin® RNA AB gene).

ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or equivalent reverse transcriptase and 1/10 of the total RNA extracted above.

iii) PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.

iv) If the CPE in culture is not extensive it is possible that a product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).

v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.

vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.

vii) In some cases where the CPE is extensive and the virus replicates to a high titre, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4- TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

NOTE: SVCV primer-annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (Björklund *et al*., 1996; Genbank accession U18101), and the vesicular stomatitis virus (VSV) New Jersey (Gallione & Rose, 1983, Genbank accession V01214), and Piry strains (Genbank accession D26175). Primers were then designed to anneal to the regions encoding the conserved amino acids using the published sequence for SVCV (Björklund *et al*., 1996) as a skeleton, and introducing degenerate bases at the 3' termini to allow for potential differences in codon usage. The appropriate IUB codes have been used where appropriate and are indicated by an asterisk (*).

4.3.1.2.4. Agent purification

The virus can be purified as described by Hill *et al*. (1975).

i) Harvest medium from infected cell cultures.

ii) Clarify by centrifugation at 2000 ***g*** for 15 minutes at 4°C. Remove the supernatant.

iii) Sediment the virus by centrifuging the supernatant at 40,000 ***g*** for 1 hour.

iv) Remove and discard the supernatant. Resuspend the pellet in a small volume of PBS or TNE (0.01 M Tris, 0.1 M NaCl, 1 mM ethylenediaminetetra-acetic acid, pH 7.2). The volume will depend on the original amount of cell culture medium and the size of tube used to make the gradient. If the supernatant fluids have been centrifuged in several tubes, combine the resuspended pellets.

v) Prepare a 15–45% sucrose gradient made up in the buffer used in step iv.

vi) Gently overlay the resuspended pellets and centrifuge at 40,000 ***g*** for 2 hours at 4°C.

vii) An opalescent band of virus should be visible in the gradient. Harvest the band and dilute at least tenfold with the buffer in use.

viii) Centrifuge at 40,000 ***g*** for 2 hours at 4°C.

ix) Resuspend the pellet in the buffer in use.

4.9. Other serological methods

Not applicable

Fish produce an immune response following infection with SVCV, and this has been studied mainly by following antibody development. The antibody response is influenced by water temperature. Following SVCV infection at low temperatures such as 10°C, antibody may not be detected, or may be present at low titre and may take several weeks to develop, whereas at 20°C, antibody develops sooner (after 1 week), and high titres can be present (Dixon, 2008). The duration of the antibody response is not known, but antibody has been detected 1 year after a natural infection in a fishery, and after over 2 years following experimental infection (unpublished observations). Detection of neutralising antibody has been used in many surveys for the virus, but ELISA methods are more sensitive. Dixon *et al*. (1994) developed a competitive ELISA, which is applicable to detection of antibody in a wide range of hosts.

5. Rating of tests against purpose of use

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

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance		Presumptive diagnosis	Confirmatory diagnosis
	Juveniles	Adults		
Gross signs	d	d	b	d
Histopathology	d	d	b	c
Transmission EM	d	d	d	d

~~Isolation in cell culture~~	~~a~~	~~a~~	~~a~~	~~a~~
~~Test for virus antigen~~	~~d~~	~~d~~	~~a~~	~~c~~
~~Test for fish antibody against the virus~~	~~c~~	~~c~~	~~c~~	~~d~~
~~RT-PCR~~	~~c~~	~~c~~	~~a~~	~~a~~
~~Sequence~~	~~n/a~~	~~n/a~~	~~a~~	~~a~~

~~EM = electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction; n/a = not applicable.~~

~~NOTE: Isolation in cell culture can only be regarded as presumptive until the identity of the isolated virus is confirmed by a suitable method.~~

~~Four genogroups of piscine rhabdoviruses have been described (Stone *et al.*, 2003): genogroup I (SVCV), genogroup II (grass carp rhabdovirus), genogroup III (pike fry rhabdovirus) and genogroup IV (tench rhabdovirus). Further analysis also showed that the SVCV genogroup could be further subdivided into at least four subgenogroups. Antibodies directed against SVCV cross-react to various degrees with all of the rhabdoviruses in the other three genogroups. The ability to confirm SVCV based on results from serological tests, such as ELISA, IFAT and serum neutralisation, relies on the specificity of the antibodies used in the tests. Results from those serological tests can only be accepted as confirming the presence of SVCV if the antisera used have been validated as detecting viruses in all four subgenogroups of genogroup I and do not cross-react with isolates from the other three genogroups.~~

~~Many diagnostic laboratories have encountered difficulties in obtaining antibodies against SVCV that are suitable for use in serological tests and have turned to commercially available test kits. Two commercial test kits are available for identification of SVCV, the TestLine ELISA kit (TestLine, Brno, Czech Republic) and the Bio-X IFAT kit (Bio-X Diagnostics, Jemelle, Belgium). Recently the tests have been assessed for their specificity against virus isolates from genogroups I, II, III and IV by Dixon & Longshaw (2005) who found that the TestLine ELISA, which uses a polyclonal rabbit antibody, was nonspecific and could not distinguish SVCV from viruses in the other three genogroups. Conversely, the Bio-X IFAT, which uses a monoclonal mouse antibody, was too specific and could only detect SVCV isolates from one of the four SVCV subgenogroups. These commercial test kits can be applied for presumptive diagnosis of SVC, but the problems of specificity severely limit their application for confirmatory diagnosis.~~

~~It is recommended that RT-PCR and nucleotide sequence analysis of the PCR products are used for confirmatory identification of SVCV.~~

5. Test(s) recommended for ~~targeted~~ surveillance to demonstrate ~~declare~~ freedom ~~from spring viraemia of carp~~ in apparently healthy populations

The method for surveillance of susceptible fish populations for declaration of freedom from infection with SVCV is inoculation of cell culture with tissue extracts (as described in Section 4.5.) to demonstrate absence of the virus.

6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status[2]

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

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection 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) Positive result by a recommended molecular or antigen or antibody detection test

ii) Cytopathic effect in cell culture (viruses)

SVC should be considered as a cause of disease when rapid mortalities and significant numbers of mortalities occur in a population of susceptible fish species, particularly if accompanied by clinical signs of SVC.

A suspect case of SVC disease is defined as the presence of typical clinical signs of the disease in a population of susceptible fish OR presentation of typical histopathology in tissue sections OR typical CPE in cell cultures without identification of the causative agent OR a single positive result from one of the diagnostic assays described above.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection shall be confirmed if positive results has been obtained on at least one animal from two test used in the following combination:

i) Pathogen isolation AND Conventional PCR test and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

The first case of the disease in a new area, or in an area where SVC has occurred before but has not been identified over a 2-year surveillance period is described as the index case. A confirmed index case is defined as a suspect case that has produced a typical CPE in cell culture with subsequent identification of the causative agent by one of the serological tests using validated antisera or RT-PCR plus sequencing described above OR a second positive result from a separate and different diagnostic assay described above. If a serological test is used, the antisera must be "fit for purpose" as indicated in Section 5. If RT-PCR is used, the product obtained must be sequenced in order to confirm SVCV; if not the case is suspect SVCV.

During follow-up investigations after a confirmed index case, a case can be confirmed on the basis of RT-PCR plus sequencing alone.

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses. [For many diseases, especially those affecting mollusc, 'clinical signs' are extremely limited and mortality may be the only or most dominant observation.]

6.2.1. Definition of suspect case in clinically affected animals

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

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

2 For example transboundary commodities.

ii) Positive result by a recommended molecular or antigen or antibody detection test on at least one animal

iii) Cytopathic effect in cell culture.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection shall be confirmed if positive results has been obtained on at least one animal from two test used in the following combination:

i) Pathogen isolation AND Conventional PCR test and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

Test type	Test purpose	Source population	Tissue/ sample type	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
RT-LAMP*	Surveillance	Live imported fish	Spleen, kidney and brain homogenate	Common carp, koi, goldfish	92.6 (27)	98.2 (445)	Virus isolation	Liu *et al.*, 2008

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.
RT-LAMP: = real-time loop mediated isothermal amplification. *Listed as suitable test

7. References

AHNE W. (1976). Untersuchungen über die Stabilität des karpfenpathogenen Virusstammes 10/3. *Fisch und Umwelt*, **2**, 121–127.

AHNE W. (1982). Vergleichende Untersuchungen über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zbl. Vet. Med. B*, **29**, 457–476

AHNE W. (1986). Unterschiedliche biologische Eigenschaften 4 cyprinidenpathogener Rhabdovirusisolate. *J. Vet. Med. B*, **33**, 253–259.

AHNE W., BJORKLUND H.V., ESSBAUER S., FIJAN N., KURATH G. & WINTON J.R. (2002). Spring viremia of carp (SVC). *Dis. aquat. Org.*, **52**, 261–272.

AHNE W. & HELD C. (1980). Untersuchungen über die viruzide Wirkung von Actomar® K_{30} auf fischpathogene Viren. *Tierärztl. Umsch.*, **35**, 308–318.

AHNE W., KURATH G. & WINTON J. (1998). A ribonuclease protection assay can distinguish spring viremia of carp virus from pike fry rhabdovirus. *Bull. Eur. Ass. Fish Pathol.*, **18**, 220–224.

ALIKIN Y.S., SHCHELKUNOV I.S., SHCHELKUNOVA T.I., KUPINSKAYA O.A., MASYCHEVA V.I., KLIMENKO V.P. & FADINA V.A. (1996). Prophylactic treatment of viral diseases in fish using native RNA linked to soluble and corpuscular carriers. *J. Fish Biol.*, **49**, 195–205.

BASIC A., SCHACHNER O., BILIC I. & HESS M. (2009). Phylogenetic analysis of spring viraemia of carp virus isolates from Austria indicates the existence of at least two subgroups within genogroup Id. *Dis. aquat. Org.*, **85**, 31–40.

BJÖRKLUND H.V., HIGMAN K.H. & KURATH G. (1996). The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame rhabdovirus – analysis of relationships with other rhabdoviruses. *Virus Res.*, **42**, 65–80.

BÉKÉSI L. & CSONTOS L. (1985). Isolation of spring viraemia of carp virus from asymptomatic broodstock carp, cyprinus carpio l. J. Fish Dis., **8**, 471–472.

CARSTENS E.B. (2010). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). *Arch. Virol.*, **155**, 133–146.

CORSIN F., GEORGIADIS M., HAMMELL K.L. & HILL B. (2009). Guide for Aquatic Animal Health Surveillance. The World Organisation for Animal Health (OIE), Paris, France, 114 p.

DIKKEBOOM A.L., RADI C., TOOHEY-KURTH K., MARCQUENSKI S., ENGEL M., GOODWIN A.E., WAY K., STONE D.M. & LONGSHAW C. (2004). First Report of Spring Viremia of Carp Virus (SVCV) in Wild Common Carp in North America. *J. Aquat. Anim. Health*, **16**, 169–178.

DIXON P.F. (2008). Virus diseases of cyprinids. *In*: Fish Diseases, Vol. 1. Eiras J.C., Segner H., Wahli, T. & Kapoor, B.G. eds. Science Publishers, Enfield, New Hampshire, USA, 87–184.

DIXON P.F., HATTENBERGER-BAUDOUY A.-M. & WAY K. (1994). Detection of carp antibodies to spring viraemia of carp virus by a competitive immunoassay. *Dis. Aquat. Org.*, **19**, 181–186.

DIXON P.F. & LONGSHAW C.B. (2005). Assessment of commercial test kits for identification of spring viraemia of carp virus. *Dis. Aquat. Org.*, **67**, 25–29.

EMMENEGGER E.J. & KURATH G. (2008). DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. *Vaccine*, **26**, 6415–6421.

FIJAN N. (1988). Vaccination against spring viraemia of carp. *In*: Fish Vaccination, Ellis A.E., ed. Academic Press, London, UK, 204–215.

FIJAN N. (1999) Spring viraemia of carp and other viral diseases and agents of warm-water fish. *In:* Fish Diseases and Disorders Vol. 3: Viral, Bacterial, and Fungal Infections, Woo P.T.K. & Bruno D.W., eds. CAB International, London, UK, pp. 177–244.

GALLIONE C.J. & ROSE J.K. (1983). Nucleotide sequence of a clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. *J. Virol.*, **46**, 162–169.

GARVER K.A., DWILOW A.G., RICHARD J., BOOTH T.F., BENIAC D.R. & SOUTER B.W. (2007). First detection and confirmation of spring viraemia of carp virus in common carp, *Cyprinus carpio* L., from Hamilton Harbour, Lake Ontario, Canada. *J. Fish Dis.,* **30**, 665–671.

GOODWIN A.E., PETERSON J.E., MEYERS T.R. & MONEY D.J. (2004). Transmission of exotic fish viruses: The relative risks of wild and cultured bait. *Fisheries*, **29**, 19–23.

HAENEN O.L.M. & DAVIDSE A. (1993). Comparative pathogenicity of two strains of pike fry rhabdovirus and spring viremia of carp virus for young roach, common carp, grass carp and rainbow trout. *Dis. Aquat. Org.*, **15**, 87–92.

HAGHIGHI KHIABANIAN ASL A., AZIZZADEH M., BANDEHPOUR M., SHARIFNIA Z. & KAZEMI B. (2008a). The first report of SVC from Indian carp species by PCR and histopathologic methods in Iran. *Pakistan J. Biol. Sci.*, **11**, 2675–2678.

HAGHIGHI KHIABANIAN ASL A., BANDEHPOUR M., SHARIFNIA Z. & KAZEMI B. (2008b). The first report of spring viraemia of carp in some rainbow trout propagation and breeding by pathology and molecular techniques in Iran. *Asian J. Anim. Vet. Adv.*, **3**, 263–268.

HILL B.J., UNDERWOOD B.O., SMALE C.J. & BROWN F. (1975). Physico-chemical and serological characterization of five rhabdoviruses infecting fish. *J. Gen. Virol.*, **27**, 369–378.

HOFFMANN B., SCHÜTZE H. & METTENLEITER T.C. (2002). Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. *Virus Res.*, **84**, 89–100.

JEREMIC S., DOBRILA J.-D. & RADOSAVLJEVIC V. (2004). Dissemination of spring viraemia of carp (SVC) in Serbia during the period 1992–2002. *Acta Vet. (Beograd)*, **54**, 289–299.

JOHNSON M.C., MAXWELL J.M., LOH P.C. & LEONG J.-A.C. (1999). Molecular characterization of the glycoproteins from two warm water rhabdoviruses: snakehead rhabdovirus (SHRV) and rhabdovirus of penaeid shrimp (RPS)/spring viremia of carp virus (SVCV). *Virus Res.*, **64**, 95–106.

JØRGENSEN P.E.V., OLESEN N.J., AHNE W. & LORENZEN N. (1989). SVCV and PFR viruses: Serological examination of 22 isolates indicates close relationship between the two fish rhabdoviruses. *In*: Viruses of Lower Vertebrates, Ahne W. & Kurstak E., eds. Springer, Berlin, Germany, 349–366.

KINKELIN P.DE & LE BERRE M. (1974). Rhabdovirus des poissons II. Propriétés *in vitro* du virus de la virémie printanière de la carpe. *Ann. Microbiol.* (*Inst. Pasteur*), **125 A**, 113–124.

KIRPICHNIKOV V.S., ILYASOV I., SHART L.A., VIKHMAN A.A., GANCHENKO M.V, OSTASHEVSKY A.L., SIMONOV V.M., TIKHONOV G.F. & TJURIN V.V. (1993). Selection of Krasnodar common carp (*Cyprinus carpio* L.) for resistance to dropsy: principal results and prospect. *In:* Genetics in Aquatculture, Proceedings of the Fourth International Symposium on Genetics in Aquaculture, Graham A.E. & Gall G.A.E., eds. Elsevier, Amsterdam, Netherlands, 7–20.

KIRYU I., SAKAI T., KURITA J. & IIDA T. (2007). Virucidal effect of disinfectants on spring viremia of carp virus. *Fish Pathol.*, **42**, 111–113.

KOUTNÁ M., VESELY T., PSIKAL I. & HULOVÁ J. (2003). Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. *Dis. Aquat. Org.*, **55**, 229–235.

LU Y. & LOH P.C. (1994). Infectivity studies of rhabdovirus in the penaeid blue shrimp. *Aquacult. Int.*, **2**, 123–127.

LIU H., GAO L., SHI X., GU T., JIANG Y. & CHEN H. (2004). Isolation of spring viraemia of carp virus (SVCV) from cultured koi (*Cyprinus carpio koi*) and common carp (*C. carpio carpio*) in PR *China. Bull. Eur. Assoc. Fish Pathol.*, **24**, 194–202.

LIU Z., TENG Y., XIE X., LI H., LV J., GAO L., TIAN F., JIANG Y., CHU Z., XIE C. & LIU H. (2008). Development and evaluation of a one-step loop-mediated isothermal amplification for detection of spring viraemia of carp virus. *J. Appl. Microbiol*., **105**, 1220–1226.

MISK E., GARVER K., NAGY E., ISAAC S., TUBBS L., HUBER P., AL-HUSSINEE L. & LUMSDEN J.S. (2016). Pathogenesis of spring viremia of carp virus in emerald shiner *Notropis atherinoides* Rafinesque, fathead minnow *Pimephales promelas* Rafinesque and white sucker *Catostomus commersonii* (Lacepede). *J. Fish Dis*., **39**, 729–739.

ORESHKOVA S.F., SHCHELKUNOV I.S., TIKUNOVA N. V, SHCHELKUNOVA T.I., PUZYREV A.T. & ILYICHEV A.A. (1999). Detection of spring viremia of carp virus isolates by hybridization with non-radioactive probes and amplification by polymerase chain reaction. *Virus Res*., **63**, 3–10.

PETERS F. & NEUKIRCH M. (1986) Transmission of some fish pathogenic viruses by the heron, *Ardea cinerea.J. Fish Dis.,* **9**, 539–544.

RUANE N.M., RODGER H.D., MCCARTHY L.J., SWORDS D., DODGE M., KERR R.C., HENSHILWOOD K. & STONE D.M. (2014). Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland. *Dis. Aquat. Org*., **112**, 121–130.

SHEPPARD A.M., LEDEUFF R.-M., MARTIN P.D., WOOLFORD G., WAY K. & STONE D.M. (2007). Genotyping spring viraemia of carp virus and other piscine vesiculo-like viruses using reverse hybridisation. *Dis. Aquat. Org.*, **76**, 163–168.

SHIMAHARA Y., KURITA J., NISHIOKA T., KIRYU I., YUASA K., SAKAI T., OSEKO N., SANO M. & DIXON P. (2016). Development of an improved RT-PCR for specific detection of spring viraemia of carp virus. *J. Fish Dis*., **39**, 269–275.

SHIVAPPA R.B., SAVAN R., KONO T., SAKAI M., EMMENEGGER E., KURATH G. & LEVINE J F (2008). Detection of spring viraemia of carp virus (SVCV) by loop-mediated isothermal amplification (LAMP) in koi carp, *Cyprinus carpio* L *J. Fish Dis.,* **31**, 249–258.

SOLIMAN M.K., ABOEISA M.M., MOHAMED S.G. & SALEH W.D. (2008). First record of isolation and identification of spring viraemia of carp virus from *Oreochromis niloticus* in Egypt. 8th International Symposium on Tilapia in Aquaculture 2008, 1287–1306.

STONE D.M., AHNE W., DENHAM K.D., DIXON P.F., LIU C.T.Y., SHEPPARD A.M., TAYLOR G.R. & WAY K. (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Dis. Aquat. Org.*, **53**, 203–210.

SVETLANA J., DOBRILA J.-D. & RADOSAVLJEVI V. (2004). Dissemination of spring viraemia of carp (svc) in serbia during the period 1992-2002. *Acta Veterinaria* (Beograd), **54**, 289-299.

TENG Y., LIU H., LV J.Q., FAN W.H., ZHANG Q.Y. & QIN Q.W. (2007). Characterization of complete genome sequence of the spring viremia of carp virus isolated from common carp (*Cyprinus carpio*) in China. *Arch. Virol.*, **152**, 1457–1465.

WOLF K. (1988). Spring viraemia of carp. *In*: Fish viruses and fish viral diseases. Cornell University Press, Ithaca, USA, 191–216.

YUE Z., TENG Y., LIANG C., XIE X., XU B., ZHU L., LEI Z., HE J., LIU Z., JIANG Y., LIU H. & QIN Q. (2008). Development of a sensitive and quantitative assay for spring viremia of carp virus based on real-time PCR. J. Virol. Methods, 152, 43–48.

ZHANG N.Z., ZHANG L.F., JIANG Y.N., ZHANG T. & XIA C. (2009). Molecular analysis of spring viraemia of carp virus in China: A fatal aquatic viral disease that might spread in East Asian. *PLoS ONE*, **4**, pp 1–9

*
* *

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

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 20xx.

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

1. Scope

Infection with spring viraemia of carp virus means infection with the pathogenic agent *Carp sprivivirus* (common known as spring viraemia of carp virus [SVCV]), of the Genus *Sprivivirus* and the Family *Rhabdoviridae.*

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The virus genome is a non-segmented, negative-sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (Ahne *et al.,* 2002). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Björklund *et al.* [1996] and Genbank accession AJ318079 by Hoffmann *et al.* [2002]). The complete genome sequence of isolates from China (People's Rep. of) has also been deposited in Genbank (Genbank accession DQ097384 by Teng *et al.* [2007] and Genbank accession EU177782 by Zhang *et al.* [2009]).

Stone *et al.* (2003) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 isolates from different fish species and geographical locations previously identified as SVCV or pike fry rhabdovirus (PFRV) by serology. The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to genogroup I, sharing <61% nucleotide identity with viruses in the other three genogroups. Re-analysis of the sequence data generated for viruses assigned to Genogroup I identified four subgroups (Ia–d). Those viruses originating in Asia were assigned to Subgroup Ia, those from Moldova, the Ukraine and Russia to Subgroups Ib and Ic, and those from the UK to Subgroup Id.

2.1.2. Survival and stability in processed or stored samples

There are limited published data on the stability of the pathogen in host tissues. There is also limited information on the stability of the virus in the tissues after death of a diseased animal. Detection in the tissues of recently dead animals by both reverse-transcription polymerase chain reaction (RT-PCR) and culture should not be ruled out, and therefore, dead fish as well the moribund should be taken for analysis.

The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre for when stored for 1 month at –20°C, or for 6 months at –30 or –74°C (Ahne, 1976; Kinkelin & Le Berre, 1974). The virus is stable over four freeze (–30°C)–thaw cycles in medium containing 2% serum (Kinkelin & Le Berre, 1974).

2.1.3. Survival and stability outside the host

The virus has been shown to remain viable outside the host for 5 weeks in river water at 10°C, for more than 6 weeks in pond mud at 4°C, reducing to 4 days in pond mud at 10°C (Ahne, 1976).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: all varieties and subspecies of common carp (*Cyprinus carpio*), bighead carp *(Aristichthys nobilis*), bream (*Abramis brama*), Caspian white fish (*Rutilus kutum*)*,* fathead minnow (*Pimephales promelas*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon Idella*)*,* roach (*Rutilus rutilus*) and sheatfish (also known as European or wels catfish) (*Silurus glanis*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: Crucian carp (*Carassius carassius*), pike (*Esox lucius*), firebelly newt (*Cynops orientalis*), silver carp (*Hypophthalmichthys molitrix*), Yellow perch (*Perca flavescens*), *and* zebrafish (*Danio rerio*). Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is SCVC, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

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

Common carp varieties are the principal hosts for SVCV and are considered to be most susceptible to infection with SVCV followed, in order of susceptibility, by other carp species (including hybrids), other susceptible cyprinid species and finally susceptible non-cyprinid fish species. When sampling during surveillance programmes for SVCV, common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp), then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Should these species not be available then other known susceptible species should be sampled. . Cyprinid species are increasingly mixed together in polyculture systems and the risk of transmission of SVCV between species during disease outbreaks is high (Billard & Berni, 2004).

Generally, young fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Moreover, there is a high variability in the degree of susceptibility to infection with SVCV among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age or the age-related status of innate immunity appears to be extremely important: the younger the fish, the higher the susceptibility to overt disease, although even adult broodfish can be susceptible to infection.

Fish that have separated from the shoal and found at the water inlet or sides of a pond are more likely to be infected.

2.2.4. Distribution of the pathogen in the host

The transmission of SVCV is horizontal (Fijan, 1988). SVCV appears to enter via the gills and then spreads to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks high titres of virus occur in the liver and kidney of infected fish, but much lower titres occur in the spleen, gills and brain (Dixon, 2008). The virus has been detected in ovarian fluid (Békési & Csontos, 1985), but vertical transmission has yet to be demonstrated.

2.2.5. Reservoirs of infection

Liu *et al.* (2004) isolated SVCV in China (People's Rep. of) from common and koi carp exhibiting no external or internal signs of disease, and similarly, the virus was isolated from apparently healthy wild carp in Canada (Garver *et al*., 2007).

2.2.6. Vectors

The parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola geometra* (Annelida, Hirudinea) have been demonstrated to transfer SVCV from diseased to healthy fish under experimental conditions and the virus has been isolated from *A. foliaceus* removed from infected carp (Ahne *et al.*, 2002; Dixon, 2008). It has been demonstrated experimentally that virus can be isolated from fish tissues regurgitated by herons (*Ardea cinerea*) 120 minutes after being fed with SVCV-infected carp, suggesting a potential route for SVCV transmission, but is not known whether such transmission has occurred in nature (Peters & Neukirch, 1986).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Co-infections with koi herpesvirus or carp oedema virus can increase levels of mortality.

Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both nonspecific (e.g. interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Poor physiological condition of over-wintered fish may be a contributory factor to disease susceptibility. In European aquaculture, losses can be up to 70% in young carp (Ahne *et al.*, 2002), but are usually from 1 to 40%.

In one survey from Serbia, the virus was isolated by culture in samples collected from 12 of the 38 hatcheries screened over the 10-year period (1992–2002). The virus occurred sporadically in different ponds on one site, and sporadically from year to year at different sites (Svetlana *et al.,* 2004). In another study, 18 of 30 tissue pools (five fish/pool) of wild common carp sampled in Canada in 2006 were positive for SVCV by culture (Garver *et al.,* 2007). The isolation of SVCV in the latter case was from asymptomatic common carp which correlates with observations that SVCV infection can often be clinically inapparent (Fijan, 1999).

2.3.2. Clinical signs, including behavioural changes

Fish can become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Clinical signs of infection with SVCV are nonspecific, and not all fish will exhibit all of the signs. Two of the most obvious and consistent features are abdominal distension and haemorrhages. The latter may occur on the skin, fin bases, eyes and gills, which may be pale. The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Diseased fish usually appear darker in colour. There may be no clinical signs in cases with a sudden onset of mortality.

2.3.3 Gross pathology

There are no pathognomonic gross lesions. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucous instead of food. Oedema and haemorrhage of the visceral organs is commonly observed (the spleen is often enlarged), and organs adhere to each other and to the peritoneum. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder (see Dixon, 2008). However, petechial haemorrhages are uncommon in cases caused by Asian strains of SVCV (Dikkeboom *et al*., 2004.

2.3.4. Modes of transmission and life cycle

The transmission of SVCV is horizontal (Fijan, 1988). The virus appears to enter the host via the gill. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine (Ahne, 1982).

Vertical or ‘egg-associated’ transmission cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid, although there have been no further reports (Békési & Csontos, 1985).

Horizontal transmission may be direct or vectorial, water being the major abiotic vector (Fijan, 1988). Animate vectors (Section 2.2.6.) and fomites may also be involved in transmission of SVCV (Fijan, 1988). Once SVCV is established in populations, it may be very difficult to eradicate without destroying all types of life at the site.

2.3.5. Environmental and management factors

Disease outbreaks in carp generally occur between 11 and 17°C. They rarely occur below 10°C, and mortalities, particularly in older fish, decline as the temperature exceeds 22°C (Fijan, 1988). However, the virus was isolated from apparently healthy fish from a lake in Canada that had been sampled over a 13-day period during which the water temperature varied between 24.2°C and 27.3°C (Garver *et al,* 2007). Secondary and concomitant bacterial and/or parasitic infections can affect the mortality rate and display of signs. In carp, the disease is often observed in springtime (hence the common name for the disease), particularly in countries having cold winters. It is believed that the poor condition of the over-wintered fish may be a contributory factor in disease occurrence. The disease can occur in fish in quarantine following the stress of transportation, even though there has been no evidence of virus in the fish prior to transportation.

2.3.6. Geographical distribution

For a long time, the geographical range of SVC was limited to countries of the European continent that experience low water temperatures during winter. Consequently, the disease has been recorded from most European countries and from certain of the western Independent States of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine) (see Dixon 2008 for references to these and the following locations). However, in 1998, the disease was recorded South America (in goldfish in a lake in Brazil), in 2002 in the USA, and in 2006 in Canada. Detection of the virus in carp in China (People’s Rep. of) was confirmed in 2004.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

A safe and effective vaccine is not currently available. However, a number of experimental inactivated preparations, live attenuated vaccines and DNA vaccines have given encouraging results (Dixon, 2008, Emmenegger & Kurath, 2008). The use of live attenuated vaccines or the DNA vaccines might affect diagnostic performance.

2.4.2. Chemotherapy including blocking agents

Methisoprinol inhibits the replication of SVCV *in vitro*, but has not been tested under carp culture conditions.

2.4.3. Immunostimulation

Injection into carp of single-stranded and double-stranded RNA (which is an interferon inducer) protected carp for longer than 3 weeks, but the treatment is not effective by bath administration (Alikin *et al.,* 1996).

2.4.4. Breeding resistant strains

The “Krasnodar” strain of common carp has been bred for increased resistance to SVCV (Kirpichnikov *et al*., 1993).

2.4.5. Inactivation methods

The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants are also effective for inactivation: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre^{-1} chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al.,* 2007).

2.4.6. Disinfection of eggs and larvae

Eggs can be disinfected by iodophor treatment (Ahne & Held, 1980).

2.4.7. General husbandry

Methods to control of infection with SVCV relies on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. Hygiene measures should include disinfection of eggs by iodophor treatment (Ahne & Held, 1980), until it has been confirmed unequivocally that vertical transmission does not occur, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish. Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C may stop or prevent outbreaks of infection with SVCV.

3. Specimen selection, sample collection, transportation and handling

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

3.1. Selection of populations and individual specimens

For disease investigations, moribund fish or fish exhibiting clinical signs of infection with SVC should be collected. Ideally fish should be alive when collected, however recently dead fish can be collected for diagnostic purposes. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no pathognomonic gross lesions and no clinical signs in cases of sudden mortality (see Section 4.1.1.).

Samples should comprise all susceptible species on the site with each group being represented in the sample. A group is defined as a population of the same fish species that shares a common water supply and that originates from the same broodfish or spawning population. Generally, young fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Any moribund fish present in the fish population to be sampled should be selected first for sample collection and the remainder of the sample should comprise randomly selected live fish from all rearing units that represent the lot being examined.

3.2. Selection of organs or tissues

For clinically affected fish: whole alevin (body length ≤ 4 cm), entire viscera including kidney and encephalon (> 4 cm body length ≤ 6 cm) or, for larger sized fish, liver, kidney, spleen and encephalon should be selected.

3.3. Samples or tissues not suitable for pathogen detection

Virus isolation may also not be possible from decomposed clinical samples. A number of studies in which attempts were made to isolate virus from reproductive fluids were unsuccessful, although the virus has been isolated at low frequency from ovarian, but not seminal, fluids.

3.4. Non-lethal sampling

Serological assays for antibodies can be undertaken on blood samples; the cross reactivity of anti-SVCV antibodies with viruses of the species pike fry sprivivirus allows for a presumptive indication of infection with SVCV.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0. or 2.3.0. or 2.4.0.

3.5.1. Samples for pathogen isolation

Samples for virus isolation (Section 3.2.) should be transported to the laboratory at 4°C using refrigerated containers or on ice, preferably in virus transport medium and tested within 24 hours or, in exceptional circumstances, 48 hours. The shipment of organ samples is preferred, but live or whole dead fish can be submitted to the testing laboratory if necessary. If this is not possible, samples can be frozen, but there may be loss of virus viability on thawing the samples. Repeated freeze–thawing of the sample must be avoided.

3.5.2. Fixed samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. [Alternatives to ethanol can be mentioned if they can be referenced.]

The material collected for virus culture is generally used for the molecular diagnostic assays, but additional tissue samples for RT-PCR can be preserved in commercially available RNA preservation solutions according to the manufacturers' recommendations, or, alternatively, samples can be preserved in 80–90% (v/v) analytical grade (absolute) ethanol at the recommended ratio of ethanol to tissue of 10:1.

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

Histology samples from each individual fish must be taken into 10% neutral buffered formalin (NBF) immediately after collection to prevent sample deterioration. The recommended ratio of fixative to tissue is 10:1 and each sample should be no thicker than approximately 4 mm to allow the fixative to penetrate the material and should be cut cleanly.

3.5.4. Fixed samples for electron microscopy

EM sampling is not required as standard, and the material is collected only where it is considered beneficial to facilitate potential further diagnostic work. From each fish sampled a 2 mm cubed (approximately) 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

Tubes for the separation of serum are available commercially. After collection of the blood is allowed to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Serum is clarified by centrifuging at 1000–2000 ***g*** for 10 minutes in a refrigerated centrifuge.

It is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette and maintain the samples at 2–8°C while handling. If the serum is not analysed immediately, it should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze–thaw cycles because this is detrimental to many serum components. Samples that are haemolysed, icteric or lipaemic can invalidate certain tests.

3.6. Pooling of samples

Traditionally pools of five animals have been used and more recently this has been increased to pools of ten animals for virus culture. However, no published data on the effect of pooling on test characteristics has been published.

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
Wet mounts												
Cytopathology[3]												
Histopathology[3]												
Cell or artificial media culture		+++	+++	1		+++	+++	1		+++	+++	1
Real-time PCR												
Conventional PCR		++	++	1		+++	+++	1				
Amplicon sequencing[4]										+++	+++	1
In-situ hybridisation						+	+	1		+	+	1
Bioassay												
LAMP		+	+	1		++	+	1				
Ab ELISA												
Ag ELISA						++	++	1				
Other antigen detection methods												
Other serological method												

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

> ⇒ Technical procedure
>
> ▪ How to use positive/negative controls
>
> ⇒ Interpretation of results
>
> ⇒ Availability of test (from Reference Laboratories, commercial sources or easily synthesised)

4.1. Wet mounts

Not applicable.

4.2. Cyto- and histopathology

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivasculitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necrosis and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres, and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed and lymph vessels are filled with detritus and macrophages. In the swim bladder, the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the submucosa are dilated with nearby lymphocyte infiltration.

As the histopathological picture is not specific for the disease. and not all fish will exhibit each feature (Misk *et al.,* 2016), microscopic methods by themselves are not recommended for diagnosis of SVC as the histopathological picture is not specific for the disease. They may, however, provide supporting evidence, particularly, when immunohistological (IHC) or DNA based *in-situ* hybridisation methods are used (see the relevant Sections below)

Fixed sections can also be used for histoimmunochemistry (but see caveats in Section 4.6.).

4.3. Cell or artificial media culture for isolation

If culturing viruses, cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

Cell culture

Cell line to be used: EPC, FHM or GCO.

Virus extraction: Use the procedure described in Chapter 2.3.0., Section A.2.2.2.

Inoculation of cell monolayers: make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium (i.e. the homogenate supernatants will be 1/100 and 1/1000 dilutions of the original organ material) and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers drained of their culture medium. Alternatively, make a single tenfold dilution of the 1/10 organ homogenate (i.e. a 1/100 dilution of the original organ material) and add an appropriate volume of both the 1/10 and 1/100 dilutions directly to undrained 24-hour-old cell monolayers, to effect 1/100 and 1/1000 final dilutions of the organ homogenate. Should toxicity of the sample be a problem, make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium as described above and inoculate at least 2 cm^2 of drained cell monolayer with 100 µl of each dilution. Allow to adsorb for 0.5–1 hour at 10–15°C, withdraw the inoculum and add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml well–1 for 24-well cell culture plates). Incubate at 20°C.

Monitoring incubation: Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris (Tris [hydroxymethy]) aminomethane)/HCl buffer solution (for cell culture plates).

If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately (see Section 4.6.2.).

Subcultivation procedures: using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24-hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above. If no CPE occurs, the test may be declared negative.

If no CPE occurs the test may be declared negative. However, if undertaking surveillance to demonstrate freedom from SVCV it would be advisable to screen the cells at the end of the 14 days using an SVCV-specific RT-PCR or real-time RT-PCR (Section 4.4). Following a positive result culture should be re-attempted.

Following isolation, the virus must be identified, and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods are generally regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur. Commercially available kits using polyclonal antibodies may also lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al*., 2007) to confirm the identity of the virus.

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

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

Real-time RT-PCR assays are available to detect and confirm infection with SVCV (Yue *et al*., 2008; Zhang *et al*., 2009), however, they are not currently recommended as they have not been sufficiently validated.

4.4.2. Conventional PCR (PCR)

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

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

Reverse-transcription polymerase chain reaction (*RT-PCR*) (*confirmation of virus identity or directly from fish tissue extracts*)

The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).

i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 50µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.

A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR. Examples are Trizol ReagentT (RL, Life Technologies, Paisley, UK), SV Total RNA isolation system (Promega) and Nucleospin® RNA (AB gene), EZ virus mini kit, Ez RNA tissue mini kit (Qiagen).

ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or an equivalent reverse transcriptase system and 1/10 of the total RNA extracted above.

iii) PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.

iv) If the CPE in culture is not extensive it is possible that a visible product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).

v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.

vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.

vii) In cases where the CPE is extensive and the virus replicates to a high titre, or where a semi-nested RT-PCR assay was used, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

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

NOTE: The appropriate IUB codes have been used where appropriate and are indicated by an asterisk (*).

Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutná *et al*., 2003; Shimahara *et al*., 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al*., 2014). With the exception of the conventional PCR assay developed by Shimahara *et al*. (2016) the other assays were not fully validated against representatives from each of the recognized SVCV genogroups and may they fail to detect the full range of SVCV genotypes.

4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification assays are available to detect and confirm SVCV infections (Shivappa *et al.,* 2008), however, they are currently not recommended as they are not sufficiently validated.

Infection with SVCV has also been confirmed using RT-PCR and hybridisation with non-radioactive probes (Oreshkova *et al.,* 1999; Sheppard *et al*., 2007).

4.5. Amplicon sequencing of the amplicon

See above (Section 4.4.2). All RT-PCR amplicons should be sequenced to confirm that they are SVCV in origin. SVCV-specific products will share higher degree of nucleotide identity to one of the published sequences for SVCV (Genbank accession U18101, AJ318079, DQ097384 and EU177782) compared to the published sequences for the *Pike sprivivirus*es (GenBank FJ872827, KC113518 and KC113517).

4.6. *In-situ* hybridisation (and histoimmunochemistry)

Although *in-situ* hybridisation can be used to locate the virus in different tissues on known positive animals, but it has not been well validated for SVCV as a diagnostic tool. SVCV can be detected by immunohistochemistry, however, care must be taken with interpreting the results of serological tests for SVCV, and positive results from antibody-based assays should be confirmed by RT-PCR and sequencing (see Section 4.8.)

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store and transport the kidney pieces as indicated in Chapter 2.3.0., Section A.2.2.1.) together with the other organs required for virus isolation.

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

v) Fix with cold acetone (stored at –20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBST, and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

x) Rinse four times with PBST.

xi) Incubate the imprints with a solution of FITC-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xii) Rinse four times with PBST.

xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.

xiv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical aperture of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.7. Bioassay

Not available.

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

Serological methods must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses closely related spriviviruses (PFRV, GrCRV and TenRV) may occur. Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005).

Confirmation of virus identity by the indirect fluorescent antibody test (IFAT)

i) Prepare monolayers of cells in 2 cm^2 wells of plastic cell culture plates, flasks or on cover-slips or glass slides in order to reach approximately 80% confluency within 24 hours of incubation at 25°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. For tests using cells cultured on glass cover-slips or slides, the dilutions are made in sterile containers and then used to inoculate the cells.

iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5000–10,000 PFU ml^{-1} in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for slides or cover-slips or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for cells on plastic substrates. Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.

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

vii) Rehydrate the dried cell monolayers, if they have been stored frozen, by four rinsing steps with PBS containing 0.05% Tween 20 (PBST) and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

viii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.

ix) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).

x) Incubate the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.

xi) Rinse four times with PBST.

xii) Incubate the cell monolayers with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) View the treated cell monolayers on plastic substrates immediately, or mount the slides or cover-slips using glycerol saline at pH 8.5, or a commercially available mountant.

xv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

Virus identification by enzyme-linked immunosorbent assay (*ELISA*)

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well^{-1}). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.

vii) Dispense 100 µl well^{-1} of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control). Also include SVCV positive control virus. Incubate for 1 hour at 37°C.

viii) Rinse four times with PBST.

ix) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. An MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

x) Rinse four times with PBST.

xi) If HRPO-conjugated antibody has been used, go to step xiii. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xii) Rinse four times with PBST.

xiii) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

Enzyme-linked immunosorbent assay (*ELISA*) *using tissue homogenates*

See Chapter 2.3.0., Section A.2.2.2. for obtaining organ homogenates.

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well^{-1}). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with PBST.

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well^{-1}).

v) Rinse four times with PBST.

vi) Store a 1/4 aliquot of each homogenate at 4°C, in case the test is negative and virus isolation in cell culture is required.

vii) Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulphonide fluoride; mix gently.

viii) Dispense 100 µl well–1 of two- or four-step dilutions of the sample to be identified, and of negative control tissues. Also include an SVCV positive control virus. Incubate for 1 hour at 37°C.

ix) Rinse four times with PBST.

x) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. A MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) If HRPO-conjugated antibody has been used, go to step xiv. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xiii) Rinse four times with PBST.

xiv) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

xv) If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 4.5.

4.9. Other serological methods

Not applicable

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

The method for surveillance of susceptible fish populations for declaration of freedom from infection with SVCV is inoculation of cell culture with tissue extracts (as described in Section 4.5.) to demonstrate absence of the virus.

6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status[3]

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

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection 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) Positive result by a recommended molecular or antigen or antibody detection test

ii) Cytopathic effect in cell culture (viruses)

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection shall be confirmed if positive results has been obtained on at least one animal from two test used in the following combination:

i) Pathogen isolation AND Conventional PCR test and amplicon sequencing

[3] 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. [For many diseases, especially those affecting mollusc, 'clinical signs' are extremely limited and mortality may be the only or most dominant observation.]

6.2.1. Definition of suspect case in clinically affected animals

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

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

ii) Positive result by a recommended molecular or antigen or antibody detection test on at least one animal

iii) Cytopathic effect in cell culture.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection shall be confirmed if positive results has been obtained on at least one animal from two test used in the following combination:

i) Pathogen isolation AND Conventional PCR test and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

Test type	Test purpose	Source population	Tissue/ sample type	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
RT-LAMP*	Surveillance	Live imported fish	Spleen, kidney and brain homogenate	Common carp, koi, goldfish	92.6 (27)	98.2 (445)	Virus isolation	Liu *et al.*, 2008

DSe: = diagnostic sensitivity, DSp = diagnostic specificity,
RT-LAMP: = real-time loop mediated isothermal amplification. *Listed as suitable test

7. References

AHNE W. (1976). Untersuchungen über die Stabilität des karpfenpathogenen Virusstammes 10/3. *Fisch und Umwelt*, **2**, 121–127.

AHNE W. (1982). Vergleichende Untersuchungen über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zbl. Vet. Med. B*, **29**, 457–476

AHNE W. (1986). Unterschiedliche biologische Eigenschaften 4 cyprinidenpathogener Rhabdovirusisolate. *J. Vet. Med. B*, **33**, 253–259.

AHNE W., BJORKLUND H.V., ESSBAUER S., FIJAN N., KURATH G. & WINTON J.R. (2002). Spring viremia of carp (SVC). *Dis. aquat. Org.*, **52**, 261–272.

AHNE W. & HELD C. (1980). Untersuchungen über die viruzide Wirkung von Actomar® K_{30} auf fischpathogene Viren. *Tierärztl. Umsch.*, **35**, 308–318.

Annex 21B (contd)

ALIKIN Y.S., SHCHELKUNOV I.S., SHCHELKUNOVA T.I., KUPINSKAYA O.A., MASYCHEVA V.I., KLIMENKO V.P. & FADINA V.A. (1996). Prophylactic treatment of viral diseases in fish using native RNA linked to soluble and corpuscular carriers. *J. Fish Biol*., **49**, 195–205.

BJÖRKLUND H.V., HIGMAN K.H. & KURATH G. (1996). The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame rhabdovirus – analysis of relationships with other rhabdoviruses. *Virus Res.*, **42**, 65–80.

BÉKÉSI L. & CSONTOS L. (1985). Isolation of spring viraemia of carp virus from asymptomatic broodstock carp, cyprinus carpio l. J. Fish Dis., **8**, 471–472.

DIKKEBOOM A.L., RADI C., TOOHEY-KURTH K., MARCQUENSKI S., ENGEL M., GOODWIN A.E., WAY K., STONE D.M. & LONGSHAW C. (2004). First Report of Spring Viremia of Carp Virus (SVCV) in Wild Common Carp in North America. *J. Aquat. Anim. Health*, **16**, 169–178.

DIXON P.F. (2008). Virus diseases of cyprinids. *In*: Fish Diseases, Vol. 1. Eiras J.C., Segner H., Wahli, T. & Kapoor, B.G. eds. Science Publishers, Enfield, New Hampshire, USA, 87–184.

DIXON P.F. & LONGSHAW C.B. (2005). Assessment of commercial test kits for identification of spring viraemia of carp virus. *Dis. Aquat. Org.*, **67**, 25–29.

EMMENEGGER E.J. & KURATH G. (2008). DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. *Vaccine*, **26**, 6415–6421.

FIJAN N. (1988). Vaccination against spring viraemia of carp. *In*: Fish Vaccination, Ellis A.E., ed. Academic Press, London, UK, 204–215.

FIJAN N. (1999) Spring viraemia of carp and other viral diseases and agents of warm-water fish. *In:* Fish Diseases and Disorders Vol. 3: Viral, Bacterial, and Fungal Infections, Woo P.T.K. & Bruno D.W., eds. CAB International, London, UK, pp. 177–244.

GARVER K.A., DWILOW A.G., RICHARD J., BOOTH T.F., BENIAC D.R. & SOUTER B.W. (2007). First detection and confirmation of spring viraemia of carp virus in common carp, *Cyprinus carpio* L., from Hamilton Harbour, Lake Ontario, Canada. *J. Fish Dis.,* **30**, 665–671.

HOFFMANN B., SCHÜTZE H. & METTENLEITER T.C. (2002). Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. *Virus Res.*, **84**, 89–100.

KINKELIN P.DE & LE BERRE M. (1974). Rhabdovirus des poissons II. Propriétés *in vitro* du virus de la virémie printanière de la carpe. *Ann. Microbiol.* (*Inst. Pasteur*), **125 A**, 113–124.

KIRPICHNIKOV V.S., ILYASOV I., SHART L.A., VIKHMAN A.A., GANCHENKO M.V, OSTASHEVSKY A.L., SIMONOV V.M., TIKHONOV G.F. & TJURIN V.V. (1993). Selection of Krasnodar common carp (*Cyprinus carpio* L.) for resistance to dropsy: principal results and prospect. *In:* Genetics in Aquatculture, Proceedings of the Fourth International Symposium on Genetics in Aquaculture, Graham A.E. & Gall G.A.E., eds. Elsevier, Amsterdam, Netherlands, 7–20.

KIRYU I., SAKAI T., KURITA J. & IIDA T. (2007). Virucidal effect of disinfectants on spring viremia of carp virus. *Fish Pathol.*, **42**, 111–113.

KOUTNÁ M., VESELY T., PSIKAL I. & HULOVÁ J. (2003). Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. *Dis. Aquat. Org.*, **55**, 229–235.

LIU H., GAO L., SHI X., GU T., JIANG Y. & CHEN H. (2004). Isolation of spring viraemia of carp virus (SVCV) from cultured koi (*Cyprinus carpio koi*) and common carp (*C. carpio carpio*) in PR *China*. *Bull. Eur. Assoc. Fish Pathol.,* **24**, 194–202.

LIU Z., TENG Y., XIE X., LI H., LV J., GAO L., TIAN F., JIANG Y., CHU Z., XIE C. & LIU H. (2008). Development and evaluation of a one-step loop-mediated isothermal amplification for detection of spring viraemia of carp virus. *J. Appl. Microbiol*., **105**, 1220–1226.

MISK E., GARVER K., NAGY E., ISAAC S., TUBBS L., HUBER P., AL-HUSSINEE L. & LUMSDEN J.S. (2016). Pathogenesis of spring viremia of carp virus in emerald shiner *Notropis atherinoides* Rafinesque, fathead minnow *Pimephales promelas* Rafinesque and white sucker *Catostomus commersonii* (Lacepede). *J. Fish Dis*., **39**, 729–739.

ORESHKOVA S.F., SHCHELKUNOV I.S., TIKUNOVA N. V, SHCHELKUNOVA T.I., PUZYREV A.T. & ILYICHEV A.A. (1999). Detection of spring viremia of carp virus isolates by hybridization with non-radioactive probes and amplification by polymerase chain reaction. *Virus Res*., **63**, 3–10.

PETERS F. & NEUKIRCH M. (1986) Transmission of some fish pathogenic viruses by the heron, *Ardea cinerea*.*J. Fish Dis.,* **9**, 539–544.

RUANE N.M., RODGER H.D., MCCARTHY L.J., SWORDS D., DODGE M., KERR R.C., HENSHILWOOD K. & STONE D.M. (2014). Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland. *Dis. Aquat. Org*., **112**, 121–130.

SHEPPARD A.M., LEDEUFF R.-M., MARTIN P.D., WOOLFORD G., WAY K. & STONE D.M. (2007). Genotyping spring viraemia of carp virus and other piscine vesiculo-like viruses using reverse hybridisation. *Dis. Aquat. Org.*, **76**, 163–168.

SHIMAHARA Y., KURITA J., NISHIOKA T., KIRYU I., YUASA K., SAKAI T., OSEKO N., SANO M. & DIXON P. (2016). Development of an improved RT-PCR for specific detection of spring viraemia of carp virus. *J. Fish Dis*., **39**, 269–275.

SHIVAPPA R.B., SAVAN R., KONO T., SAKAI M., EMMENEGGER E., KURATH G. & LEVINE J F (2008). Detection of spring viraemia of carp virus (SVCV) by loop-mediated isothermal amplification (LAMP) in koi carp, *Cyprinus carpio* L *J. Fish Dis.,* **31**, 249–258.

STONE D.M., AHNE W., DENHAM K.D., DIXON P.F., LIU C.T.Y., SHEPPARD A.M., TAYLOR G.R. & WAY K. (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Dis. Aquat. Org.*, **53**, 203–210.

SVETLANA J,, DOBRILA J.-D. & RADOSAVLJEVI V. (2004). Dissemination of spring viraemia of carp (svc) in serbia during the period 1992-2002. *Acta Veterinaria* (Beograd), **54**, 289-299.

TENG Y., LIU H., LV J.Q., FAN W.H., ZHANG Q.Y. & QIN Q.W. (2007). Characterization of complete genome sequence of the spring viremia of carp virus isolated from common carp (*Cyprinus carpio*) in China. *Arch. Virol.*, **152**, 1457–1465.

YUE Z., TENG Y., LIANG C., XIE X., XU B., ZHU L., LEI Z., HE J., LIU Z., JIANG Y., LIU H. & QIN Q. (2008). Development of a sensitive and quantitative assay for spring viremia of carp virus based on real-time PCR. J. Virol. Methods, 152, 43–48.

ZHANG N.Z., ZHANG L.F., JIANG Y.N., ZHANG T. & XIA C. (2009). Molecular analysis of spring viraemia of carp virus in China: A fatal aquatic viral disease that might spread in East Asian. *PLoS ONE*, **4**, pp 1–9

*
* *

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

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 20xx.

CHAPTER 2.1.X.

INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

1. Scope

Infection with *Batrachochytrium salamandrivorans* (Bsal) means infection of amphibians with the pathogenic agent *Batrachochytrium salamandrivorans*, of the Genus *Batrachochytrium* and Family *Incertae sedis*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The *Batrachochytrium salamandrivorans* (Bsal) type strain is AMFP13/1. Three more isolates have been described (Martel *et al*., 2014) but no information is available on genetic structuring or phenotypic variation. Phylogenetic analyses show that Bsal forms a clade with its sister species *B. dendrobatidis* (Martel *et al*., 2013). The genome size of the type strain was determined at 32.6 Mb with 10.138 protein-coding genes predicted (Farrer *et al*., 2017). The contribution of these proteins to virulence is currently not clear.

2.1.2. Survival and stability inside the host tissues

Bsal is an intracellular pathogen that develops inside epidermal cells. The presence of Bsal could be demonstrated using real-time polymerase chain reaction (qPCR) on dorsal skin swabs up to 7 days on average post-mortem and using histopathology of dorsal skin tissue up to 3 days on average post-mortem (Thomas *et al*., 2018). It is not clear how long Bsal can survive inside tissues of a dead host and how long a dead host remains infectious. Storage of tissues or skin swabs in 70% ethanol or at –20°C allows detection of Bsal using qPCR for more than 150 years as demonstrated by analysis of museum specimens (Martel *et al*., 2014).

2.1.3. Survival and stability outside the host

Encysted spores have been shown to remain infectious in pond water up to at least 31 days (Stegen *et al*., 2017) and are considered more environmentally resistant compared with zoospores. Experimentally inoculated forest soil was demonstrated to remain infectious to fire salamanders for 48 hours (Stegen *et al.,* 2017). However, Bsal DNA was detected up to 28 weeks in contaminated forest soil (Stegen *et al*., 2017). Whether this reflects the presence of viable Bsal organisms is not clear. The effect of dessication on Bsal survival has not been studied.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with Bsal according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include: [alpine newt *(Ichthyosaura alpestris)*, blue-tailed fire-bellied newt *(Cynops cyanurus)*, fire salamander *(Salamandra salamandra)*, eastern newt *(Nothophthalmus viridescens)*, French cave salamander *(Hydromantes strinatii)*, Italian newt *(Lissotriton italicus)*, yellow spotted newt *(Neurergus crocatus)*, Japanese fire-bellied newt *(Cynops pyrrhogaster)*, northern spectacle salamander *(Salamandrina perspicillata)*, Tam Dao salamander *(Paramesotriton deloustali)*, rough-skinned newt *(Taricha granulosa)*, sardinian brook salamander *(Euproctus platycephalus)* and Spanish ribbed newt *(Pleurodeles waltl)*] (under study).

2.2.2. Species with incomplete evidence for susceptibility

[under study]

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

Bsal is a pathogenic agent that mainly affects urodeles. Evidence from experimental infections and disease outbreaks in the wild and in captivity show that at least most, if not all, species of the family Salamandridae, as well as species of the family Hynobiidae are likely to become infected when exposed to Bsal. However, differences in susceptibility to infection between species do exist: for example, for fire salamanders (*Salamandra salamandra*), the infectious dose of Bsal was determined to be a theoretical one zoospore, whereas a significantly higher dose was necessary to infect Alpine newts (*Ichthyosaura alpestris*; Stegen *et al*., 2017) and one western Palearctic species (*Lissotriton helveticus*) may be more resistant to infection (Martel *et al*., 2014). For the largest family of salamanders (Plethodontidae), little information is currently available; at least one European species (*Speleomantes strinatii*) can be infected but other, North American species (*Gyrinophilus porphyriticus*, *Plethodon glutinosus,* Ambystomatidae) seem less susceptible to infection (Martel *et al*., 2014). Susceptibility of the family of Cryptobranchidae is not clear, with a single infection found in a farmed Chinese giant salamander (*Andrias davidianus*, Zhiyong *et al*., 2018). No information is available on the urodele families *Proteidae, Rhyacotritonidae* and *Amphiumidae*. Bsal infection in anurans has only been detected in two species, in captivity, the wild and in lab trials (Nguyen *et al*., 2017; Stegen *et al*., 2017).

Thus far, infections with Bsal have been demonstrated only in amphibians post-metamorphosis. In one experimental infection trial, larvae of fire salamanders were exposed to Bsal, but were not infected (Van Rooij *et al*., 2015). The extent to which factors like age and sex affect susceptibility to infection post metamorphosis is unknown.

In Europe, Bsal has been detected in captive collections of urodeles (Fitzpatrick *et al*., 2018, Sabino-Pinto *et al*., 2015) and the pet trade in salamanders and newts has been hypothesized to play a central role in the distribution of this fungus (Fitzpatrick *et al*., 2018; Yap *et al*., 2015; Zhiyong *et al*., 2018). Hence, urodeles that directly (co-housing, contact of wild animals with released or captive animals) or indirectly (via materials, contaminated water or soil) come in contact with traded urodeles, may be more likely to contract Bsal infection.

2.2.4. Distribution of the pathogen in the host

Bsal only infects the skin, where it remains limited to the epidermis.

2.2.5. Persistent infection

A large number of salamanders, mainly belonging to the families Salamandridae and Hynobiidae, may survive episodes of infection (for example Alpine newts) or be considered tolerant, resulting in persistent subclinical infections. Although persistent infection has not been demonstrated for all species, in the native Bsal range in east Asia, Bsal infection and disease dynamics appear to be consistent for all species examined and appear capable of long-term persistent infections (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018).

In its invasive range, persistent infections (e.g. in Alpine newts) have been implicated in the extirpation of a highly susceptible species (fire salamanders). It is currently not clear which of the species, mentioned in 2.2.1 may sustain persistent infections in the invasive Bsal range. At least some species (the best-known example is the fire salamander) are highly susceptible and invariably die briefly after exposure (Stegen *et al*., 2017; Martel *et al*., 2014), which would make them unlikely to sustain persistent infections.

It is not known whether other, biotic reservoirs of Bsal exist.

2.2.6. Vectors

There is evidence that zoospores attached to the feet of birds (Stegen *et al*., 2017), which may thus act as vectors for Bsal.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In its native range in east Asia, Bsal has been demonstrated to be present in the wild at a prevalence of between 2 and 4% on average (data from China [People's Rep. of], Japan, Thailand, and Vietnam; Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al*., 2018), but in the absence of any observed morbidity or mortality under natural conditions. In some populations (*Paramesotriton hongkongensis*), prevalence may reach 50% (Zhiyong *et al*., 2018). In its invasive range in Europe, Bsal was present in a population of fire salamanders at a prevalence of between 25 and 63% (Stegen *et al*., 2017). In captive collections of urodeles in Europe, Bsal occurrence and associated mortality were detected in Germany (1), the United Kingdom (4), Belgium (1), the Netherlands (2) and Spain (1) (number in brackets indicates number of collections). When left untreated, morbidity and mortality can reach 100%, at least in members of the genus *Salamandra*.

Morbidity, mortality and minimum infectious dose vary considerably between species (Martel *et al*., 2014; Stegen *et al*., 2017). Based on natural outbreaks in captivity and in the wild and on infection trials, case morbidity and case mortality rate in fire salamanders can reach 100%, independent of the initial level of Bsal exposure. This has resulted in loss of over 99.9% of the fire salamander population at the Bsal index outbreak site in the Netherlands (Spitzen-van der Sluijs *et al*., 2016). All tested western Palearctic urodeles, except for *Lissotriton helveticus* and *Salamandrella keyserlingii*, showed 100% morbidity and mortality when exposed to a single, high dose of Bsal (Martel *et al*., 2014). However, at least for Alpine newts, case morbidity and case fatality rates depend on the Bsal dose the animal is exposed to: a high dose resulting in the highest mortality, while a low dose does not necessarily result in morbidity or mortality.

It is important to mention that morbidity and mortality depend on environmental temperature. For the Bsal type strain: temperatures above 20°C temper infection and temperatures above 25°C eventually result in killing of Bsal and elimination of infection (Blooi *et al*., 2015a). Exposure of infected animals to conditions that inhibit Bsal growth may thus result in non-clinical or sub-clinical infections in susceptible species.

2.3.2. Clinical signs, including behavioural changes

Chytridiomycosis caused by Bsal may be accompanied by a combination of the following signs: epidermal ulcerations (ranging from tiny to extensive), excessive skin shedding, skin haemorrhages and/or fluid loss, anorexia, apathy, abnormal body postures, convulsions and death) (Martel *et al.,* 2013).

2.3.3 Gross pathology

Skin anomalies (haemorrhages, ulcerations, presence of sloughed skin) are the main pathological findings (Martel et al., 2013).

2.3.4. Modes of transmission and life cycle

Colonial or monocentric thalli of this fungus develop inside host epidermal cells and produce motile zoospores or walled, encysted spores, both of which are infectious stages. Zoospores are released through one or several discharge tubes. While motile spores actively swim towards a suitable substrate (e.g. a host), the encysted spores float at the water–air interface and passively adhere to a passing host (Stegen *et al.,* 2017). *In vitro*, developing thalli form fine rhizoids. Mature thalli *in vitro* are between 16 and 50 µm in diameter, *in vivo* between 7 and 17 µm; zoospores are approximately 5 µm in diameter. Motile zoospores are roughly spherical, the nucleus is located outside of the ribosomal mass, with aggregated ribosomes, multiple mitochondria and numerous lipid globules. The position of the non-flagellated centriole in free swimming zoospores varies from angled to parallel to the kinetosome (Martel *et al.,* 2013).

There are no indications of vertical transmission. However, this cannot be excluded in species giving birth to metamorphosed offspring (e.g. *Salamandra atra*, *Salamandra lanzai*, *Lyciasalamandra helverseni*). Horizontal transmission occurs through direct contact or contact with contaminated soil or water (Stegen *et al*., 2017). Infectious stages include the motile zoospore and the environmentally resistant encysted spores (Stegen *et al*., 2017). Infections can be reproduced under experimental conditions by topically applying a Bsal inoculum on the dorsum of amphibians and housing the exposed animals at 15°C (Martel *et al*., 2013; 2014; Stegen *et al*., 2017). This inoculum can either contain motile zoospores or the immobile, encysted spores.

Pathways of Bsal dispersal within Europe are poorly understood but may be anthropogenic (e.g. through contaminated material). Zoospores attach to bird feet, suggesting birds may spread Bsal over larger distances (Stegen *et al*., 2017). Direct animal-to-animal contact is necessary for transmission of Bsal: salamanders only separated by 1 cm from infected conspecifics were not infected in laboratory trials, in contrast to co-housed animals (Spitzen-van der Sluijs *et al*., 2018). Overall, dispersal ability of Bsal in Europe currently seems limited: Bsal was found not to be transmitted to a neighbouring site in the Netherlands, despite being downstream of a small stream, and the current distribution of Bsal in Europe is probably not continuous (Spitzen-van der Sluijs *et al*., 2018).

Although Bsal dispersal between populations is now hypothesised to be mainly human mediated, other factors (e.g. wildlife, water) may play key roles and critical knowledge about Bsal dispersal is currently lacking.

2.3.5. Environmental and management factors

The Bsal type strain AMFP13/1 tolerates temperatures up to 25°C but is killed at higher temperatures (Blooi *et al*., 2015a). As Bsal infections have been demonstrated in aquatic newts at water temperatures above 25°C (Laking *et al*., 2017; Zhiyong *et al*., 2018), it is likely, however, that thermal tolerance may be Bsal lineage dependent. A temperature of 4°C results in slower build-up of infection but does not reduce morbidity or mortality (Stegen *et al*., 2017). Desiccation is likely to be poorly tolerated by Bsal, although data are currently lacking, and the encysted spore may be resistant to drying (Stegen *et al*., 2017; Van Rooij *et al*., 2015). It is not known to what extent Bsal tolerates freezing.

Co-occurrence of highly susceptible species such as fire salamanders with less susceptible species, such as Alpine newts may facilitate density independent disease dynamics that lead to the extirpation of the highly susceptible species (Stegen *et al*., 2017).

Barriers to pathogen dispersal, for example those preventing migration of infected hosts such as amphibian fences or roads, or those preventing transmission by potential Bsal vectors including humans, fomites and wildlife, may prevent transmission at small spatial scales (Spitzen-van der Sluijs *et al*., 2018).

2.3.6. Geographical distribution

Asia is currently considered the region of origin of Bsal (Martel *et al*., 2014), where the infection ~~Asia is currently considered the region of origin of Bsal (Martel *et al*., 2014), where the infection~~ appears to be endemic in amphibian communities across a wide taxonomic, geographical and environmental range, albeit at a low prevalence between 2-4% (Zhiyong *et al.,* 2018). In Asia, Bsal was shown to be widely present in urodele populations in China (People's Rep. of), Japan, Thailand and Vietnam. East Asia is presumed to be the native range of the fungus (Laking *et al*., 2017; Martel *et al*., 2014; Zhiyong *et al.,* 2018).

Europe is considered the invasive range of the fungus where Bsal was first identified during a mortality event in fire salamanders (*Salamandra salamandra*) in Bunderbos, the Netherlands (Martel *et al*., 2013). In Europe, Bsal was detected by surveys of wild susceptible species in Belgium, Germany and the Netherlands (Martel *et al*., 2014; Spitzen-van der Sluijs *et al*., 2016), and in captive urodele populations in Belgium, Germany, the Netherlands, Spain~~,~~ and the ~~and~~ United Kingdom, (Fitzpatrick *et al*., 2018; Sabino-Pinto *et al*., 2015).

Bsal has not been reported in Africa or the Americas.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Not available.

2.4.2. Chemotherapy including blocking agents

A combined treatment using Polymyxin E, voriconazole and a temperature regime of 20°C has been shown to be effective in eradicating Bsal from infected hosts (Blooi *et al*., 2015b). If the treatment is not performed properly and does not achieve eradication, low level carriers are created and the likelihood of Bsal detection, is reduced.

2.4.3. Immunostimulation

Not available.

2.4.4. Breeding resistant strains

Breeding resistant strains is one of the few options for long term sustainable disease mitigation.

2.4.5. Inactivation methods

Bsal is sensitive to a wide variety of disinfectants (Van Rooij *et al*., 2015). Inactivation using formalin has been shown to hamper DNA detection using qPCR. Bsal is killed within 30 seconds in 70% ethanol (Van Rooij *et al.,* 2017). Inactivation in 70% ethanol allows for subsequent molecular tests, yet is less suitable for histopathology. The Bsal type strain AMFP 13/1 is killed at temperatures exceeding 25°C consequently inactivation of this fungus can be achieved through heat treatment by autoclaving.

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

In captivity, pathogen detection is difficult due to low prevalence in sub-clinically infected animals, that often carry Bsal at low intensities (Martel *et al*., 2014; Zhiyong *et al*., 2018). These often belong (but are not restricted to) taxa of Asian urodeles. Highly susceptible species (such as fire salamanders) may serve a sentinel function. Temperature regimes in captivity may strongly interfere with pathogen detection. Temperatures higher than 20°C (and below 25°C) severely impairs pathogen proliferation in the host skin (Blooi *et al*., 2015a) and may result in infections that cannot be detected.

3. Specimen selection, sample collection, transportation and handling

This Section draws on information from Sections 2.2., 2.3. and 2.4. to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

In case of disease or mortality in urodeles in captivity, sampling should be focused primarily on diseased or moribund animals (i.e. those showing skin lesions and abnormal behaviour). In a population with ongoing disease and mortality, live but diseased animals are preferentially sampled. The second choice is dead animals. Only freshly dead animals should be sampled as detectability of Bsal deteriorates post-mortem (Thomas *et al*., 2018). However, in the absence of diseased or dead animals, apparently healthy animals can be sampled.

Similarly in wild populations diseased or moribund or freshly dead animals should preferentially be sampled, but as these may quickly be removed (i.e. through predation, scavenging) healthy animals may only be available. Populations which have declined or where dead animals have been observed should be targeted.

3.2. Selection of organs or tissues

The only relevant tissue is skin tissue and probably only from amphibians post metamorphosis. Both invasive (skin biopsies) and non-invasive (cotton tipped swabs) sampling are appropriate, given the apical shedding of Bsal spores. In dead animals, dorsal skin is the preferred tissue, given its slower post mortem decay (Thomas *et al*., 2018).

3.3. Samples or tissues not suitable for pathogen detection

Any other tissue than skin is not suitable for the detection of Bsal in amphibians.

3.4. Non-lethal sampling

Non-lethal sampling is possible, either by collecting skin biopsies (toeclips or tailclips) or by non-invasively collecting samples using cotton tipped swabs. The latter is preferred given its minimal impact on animal wellbeing. As Bsal is limited to the superficial skin layers of the amphibian host, non-lethal sampling results are equivalent to lethal sampling results. In the absence of other, Bsal specific diagnostic tests (other than the laborious isolation of the fungus), large numbers of animals can be sampled using skin swabs with minimal effects on animal welfare. Cotton tipped swabs should be rubbed firmly over the abdomen (10 times), the underside of a foot (10 times) and the ventral tail (10 times) using the tip of the swab. The use of disposable gloves for manipulating amphibians is highly recommended.

3.5. Preservation of samples for submission

3.5.1. Samples for pathogen isolation

Bsal isolation is a very laborious procedure, requiring up to two months for obtaining a pure culture from a clinical sample. Isolation from animals that died due to Bsal infection is hampered by bacterial overgrowth. The best sample for Bsal isolation is a diseased, living animal, which is euthanised just prior to an isolation attempt. Before sampling diseased animals should be kept at temperatures between 5 and 15°C to avoid clearance of infection (Blooi *et al*., 2015a).

3.5.2. Fixed samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Skin swabs should be stored dry and preferably frozen.

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

Skin samples for histopathology should be fixed immediately after collection. The recommended ratio of formalin (10%) to tissue is 10:1.

3.5.4. Fixed samples for electron microscopy

For transmission electron microscopy, skin samples can be fixed in glutaraldehyde in 0.05 M sodium cacodylate buffer and 1% osmium tetroxide post-fixation (Martel *et al*., 2013).

3.5.5. Samples for other tests

Not applicable.

3.6. Pooling of samples

Pooling of up to five skin swab samples appears to allow reliable detection of Bsal in clinically affected animals (Sabino-Pinto *et al*., 2018) but estimates on the impact on test characteristics have not been determined. Given low infection intensities in subclinically infected animals, sampling of individual animals is recommended.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

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

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

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

Method [amend or delete as relevant]	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts	+	+	+		+	+	+		+	+	+	
Histopathology[3]	+	+	+		++	++	++		++	++	++	
Cell or artificial media culture									++	++	++	
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Conventional PCR	+	+	+		+	+	+		+	+	+	
Amplicon sequencing[4]									+++	+++	+++	
In-situ hybridisation	+	+	+		+	+	+		+	+	+	
LAMP	+	+	+									
Lateral flow assay	+	+	+	1	++	++	++	1	++	++	++	1
Immunohistochemistry	+	+	+	1	+	+	+	1				

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2.); PCR = polymerase chain reaction.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Early and juvenile life stages have been defined in Section 2.2.3.
[3]Cytopathology and histopathology can be validated if the results from different operators has been statistically compared. [4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Wet mounts of skin scraping or pieces of shed skin can be examined at magnification 10× using light microscopy. The presence of motile spores of approximately 5 µm are indicative of amphibian chytrid infection.

4.2. Cyto- and histopathology

No reports are available on the use of cytology. Histopathology of skin in amphibian post metamorphosis may provide strong indications of Bsal infection. In a haematoxylin/eosin staining of skin sections, histopathological evidence suggestive of Bsal infections is multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular chytrid-type fungal thalli (Martel *et al*., 2013; White *et al*., 2016). Using immunohistochemistry, Bsal thalli can be stained, which aids in detecting low level infections (Thomas *et al*., 2018). Histopathology is highly indicative, yet does not allow specific identification of Bsal, which needs further confirmation. In randomly collected skin samples from experimentally infected salamanders, histopathology was capable of detecting Bsal in only a minority of the samples (Thomas *et al*., 2018). In dead animals, post-mortem decay of the epidermis may mask the lesions (Thomas *et al*., 2018). Lesions can be so extensive, that the epidermis is entirely eroded and no fungal thalli can be observed. Mild infections can be missed due to the multifocal and small lesions (Thomas *et al*., 2018). For asymptomatically infected animals, sensitivity should be rated low. Sensitivity and specificity of histopathology and immunohistochemistry have not been quantified.

4.3. Cell or artificial media culture for isolation

Bsal can be isolated and cultured on artificial media, yet this is a laborious and difficult procedure, typically requiring between 4 weeks and 2 months. There is a significant probability of bacterial overgrowth, which hampers fungal isolation, resulting in poor sensitivity. The protocol of Fisher *et al*. (2018) can be used. Small (approximately 1 mm^2) pieces of skin from an infected, diseased animal should first be thoroughly cleaned by wiping through agar plates. The cleaned pieces of skin can then each be transferred to a well of a 96-well plate, containing tryptone-gelatin hydrolysate lactose broth (TGhL) containing peniciilin / streptomycin (200 mg/litre) and incubated at 15°C. Wells showing chytrid growth without bacterial contamination can be used for subculturing (Martel *et al*., 2013). Chytrid growth can be visualized by examining the wells under an inverted microscope (10–40 × magnification).

Given the difficulties to isolate Bsal from infected animals and the high uncertainty to obtain a viable culture, this method is not appropriate as first diagnostic approach, but (in rare cases) to confirm infection and for obtaining isolates for research (for example for epidemiological tracing).

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

The following information is derived from Blooi *et al*. (2013), Thomas *et al*. (2018) and Sabino Pinto *et al*. (2018). DNA from skin swabs can be extracted in 100 µl Prepman Ultra Reagent (Applied Biosystems, Foster City, CA) or by using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The latter follows the animal tissues protocol (Qiagen DNeasy Blood and Tissue kit) with pre-treatment for Gram-positive bacteria and expanded initial incubation for 1 hour. DNA from skin tissue can be extracted using proteinase K digestion or DNA Easy Tissue Kit. Extracted DNA is diluted tenfold to minimise possible PCR inhibition. Controls should be run with each assay: at least a negative extraction control and a positive control; preferably, an internal PCR control is included. Positive control consists of DNA extracts of a tenfold dilution series of Bsal zoospores from 1 to 100.000 to allow quantification.

A TaqMan PCR has been validated (Thomas *et al*., 2018). SYBR green real-time PCR, may be used as well but needs further validation to determine specificity and sensitivity (Martel *et al*., 2013). The TaqMan PCR can either be used as simplex PCR or in combination with primers to detect *B. dendrobatidis* in a duplex PCR (Blooi *et al*., 2013) and uses the forward primer STerF (5'-TGC-TCC-ATC-TCC-CCC-TCT-TCA-3'), reverse primer STerR (5'-TGA-ACG-CAC-ATT-GCA-CTC-TAC-3') and

Cy5 labelled probe STerC (5'-ACA-AGA-AAA-TAC-TAT-TGA-TTC-TCA-AAC-AGG-CA-3') to detect the presence of the 5.8S rRNA gene of Bsal. Intra- and interassay efficiency were 94 and 99%, respectively (Blooi *et al*., 2013). This TaqMan duplex PCR does not decrease detectability of both Bd and Bsal, except in case of mixed infections (Thomas *et al*., 2018). The use of simplex Bsal-specific PCR is therefore recommended in case Bd has been detected in the sample. The sensitivity of this qPCR is between 96 and 100% and diagnostic specificity 100% (95% CI: 73–100%; Thomas *et al*., 2018) when used in clinically affected animals. Although DNA quantities as low as 0.1 genomic equivalent can be detected (Blooi *et al*., 2013), Thomas *et al*. (2018) recommend a threshold of 1 genomic equivalent per reaction to reduce the likelihood of false positive results. Borderline results (≤ 1 GE per reaction) should be classified as suspect and need confirmation by sequencing (or isolation).

Samples are preferably run in duplicate. A sample is considered positive based on the combination of (1) the shape of the amplification curves (2) positive results in both duplications, (3) returning GE values above the detection threshold (1 GE per reaction) (4) low variability between duplicates (< 0.3 Ct value).

4.4.2. Conventional PCR (PCR)

The use of real-time PCR is recommended. No conventional PCR protocol has been validated.

4.4.3. Other nucleic acid amplification methods

None validated.

4.5. Amplicon sequencing

For confirmation of suspect samples, amplified products can be sequenced with the primers as described in 4.4.1.

4.6. *In-situ* hybridisation (and histoimmunochemistry)

In situ hybridisation: no validated protocols available.

Immunohistochemistry is currently not Bsal specific, due to the lack of Bsal specific antibodies (Dillon *et al*., 2017; Thomas *et al*., 2018). Sensitivity of immunohistochemistry in diseased or dead animals can be estimated to be high if clinically affected skin regions have been selected.

4.7. Bioassay

Not available.

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

A lateral flow assay (LFA) using an IgM monoclonal antibody (mAb) was developed to detect infection in amphibian skin samples. This mAb does not discriminate between *B. salamandrivorans, B. dendrobatidis* and *Homolaphlyctis polyrhiza* (Dillon et al., 2016). The sensitivity of this test is likely to be lower than that of the qPCR (Dillon *et al.*, 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using qPCR. This would make this technique most useful in animals with high infection loads. Such techniques may be useful for point-of-care testing if specificity is increased and provided thorough validation.

4.9. Other serological methods

Not available.

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

The use of real-time PCR on skin swabs is recommended for surveillance.

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status[4]

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

Such surveys typically consist of non-invasive sampling using skin swabs that are examined for the presence of Bsal using real-time PCR. When applied to animals in the wild, confirmation by using a complementary technique, other than sequencing the PCR product, is often not feasible.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection 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) Positive result by real-time PCR.

ii) Histopathological changes (including immunohistochemistry) consistent with the presence of the pathogen or the disease.

iii) The presence of motile spores, compatible with chytrid zoospores, in wet mount of urodele skin.

iv) Positive result from lateral flow assay (LFA).

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection is confirmed if positive results have been obtained on at least one animal from two tests used in the following combination:

i) Positive result by real-time PCR on skin swab or skin tissue, and by histopathology or immunohistochemistry on skin tissue.

ii) Positive result by real-time PCR on skin swab or skin tissue, and pathogenic agent isolation from the skin and confirmation by real-time 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 shall be suspected if at least one of the following criteria are met:

i) Clinical signs (haemorrhages, ulcerations, presence of sloughed skin, see Section 2.3.2.), notably the presence of skin ulcers and / or disecdysis.

[4] For example transboundary commodities.

ii) Positive result by real-time PCR on at least one swab or skin tissue.

ii) Histopathological changes consistent with the presence of the pathogenic agent or the disease.

iv) Visual observation (by microscopy) of motile spores, compatible with amphibian chytrid zoospores, in a wet mount of the skin of at least one diseased urodele.

v) Positive result of antigen detection technique such as LFA.

iv) Positive result from immunohistochemistry.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection is confirmed if positive results have been obtained on at least one animal from two tests used in the following combination:

i) Positive result by real-time PCR on skin swab or skin tissue and by histopathology.

ii) Positive result by real-time PCR on skin swab or skin tissue, and pathogenic agent isolation from the skin and confirmation by real-time PCR.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

7. References

BLOOI M., MARTEL A., VERCAMMEN F., HAESEBROUCK F. & PASMANS F. (2015a). Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **5**, 8037.

BLOOI M., PASMANS F., LONGCORE J.E., SPITZEN-VAN DER SLUIJS A., VERCAMMEN F. & MARTEL A. (2013). Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *J. Clin. Microbiol*., **51**, 4173–4177.

BLOOI M., PASMANS F., ROUFFAER L., HAESEBROUCK F., VERCAMMEN F. & MARTEL A. (2015b). Successful treatment of *Batrachochytrium salamandrivorans* infections in salamanders requires synergy between voriconazole, polymyxin E and temperature. *Scientific Reports*, **5**, 11788.

DILLON M.J., BOWKETT A.E., BUNGARD M.J., BECKMAN K.M., O'BRIEN M.F., BATES K., FISHER M.C., STEVENS J.R. & THORNTON C.R. (2017). Tracking the amphibian pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* using a highly specific monoclonal antibody and lateral-flow technology. *Microb.l Biotechnol.*, **10**, 381–394.

FARRER R.A., MARTEL A., VERBRUGGHE E., ABOUELLEIL A., DUCATELLE R., LONGCORE J.E., JAMES T.Y., PASMANS F., FISHER M.C. & CUOMA C.A. (2017). Evolutionary innovations underpin niche-specific infection strategies across emerging pathogenic chytrid fungi. *Nature Communications*, **8**: 14742 DOI: 10.1038/ncomms14742.

FISHER M.C., GHOSH P., SHELTON J.M.G., BATES K., BROOKES L., WIERZBICKI C., ROSA G.M., FARRER R.A., AANENSEN D.M., ALVARADO-RYBAK M., BATAILLE A., BERGER L., BÖLL S., BOSCH J., CLARE F.C., COURTOIS E., CROTTINI A., CUNNINGHAM A.A., DOHERTY-BONE T.M., GEBRESENBET F., GOWER D.J., HÖGLUND J., JAMES T.Y., JENKINSON T.S., KOSCH T.A., LAMBERTINI C., LAURILA A., LIN C.F., LOYAU A., MARTEL A., MEURLING S., MIAUD C., MINTING P., NDRIANTSOA S., O'HANLON S., PASMANS F., RAKOTONANAHARY T., RABEMANANJARA F.C.E., RIBEIRO L.P., SCHMELLDER D.S., SCHMIDT B.R., SKERRATT L., SMITH F., SOTO-AZAT C., TESSA G., TOLEDO L.F., VALENZUELA-SÁNCHEZ A., VERSTER R., VÖRÖS J., WADMAN B., WEBB R.J., WELDON C., WOMBWELL E., ZAMUDIO K.R., LONGCORE J.E. & GARNER T.W.J. (2018). Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, **8** (1), 7772. doi: 10.1038/s41598-018-24472-2.

FITZPATRICK L., PASMANS F., MARTEL A. & CUNNINGHAM A.A. (2018). Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. Scientific Reports, **8**, 13845.

Annex 22 (contd)

LAKING A.E., NGO H.N., PASMANS F., MARTEL A. & NGUYEN T.T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, **7**, 44443 DOI: 10.1038/srep44443

MARTEL A., BLOOI M., ADRIAENSEN C., VAN ROOIJ P., BEUKEMA W., FISHER M.C., FARRER R.A., SCHMIDT B.R., TOBLER U., GOKA K., LIPS K.R., MULETZ C., ZAMUDIO K., BOSCH J., LÖTTERS S., WOMBWELL E., GARNER T.W.J., SPITZEN-VAN DER SLUIJS A., SALVIDIO S., DUCATELLE R., NISHIKAWA K., NGUYEN T.T., VAN BOCXLAER I., BOSSUYT F. & PASMANS F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, **346** (6209), 630–631.

MARTEL A., SPITZEN-VAN DER SLUIJS A., BLOOI M., BERT W., DUCATELLE R., FISHER M.C., WOELTJES A., BOSMAN W., CHIERS K., BOSSUYT F. & PASMANS F. (2013). *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. *Proc. Natl Acad. Sci. USA,* **110**, 15325–15329.

NGUYEN T.T., NGUYEN T.V., ZIEGLER T., PASMANS F. & MARTEL A. (2017). Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphibia Reptilia*, **38**, 554–556 DOI: 10.1163/15685381-00003125

SABINO-PINTO J., BLETZ M., HENDRIX R., PERL R.G.B., MARTEL A., PASMANS F., LÖTTERS S., MUTSCHMANN F., SCHMELLER D.S., SCHMIDT B.R., VEITH M., WAGNER N., VENCES M. & STEINFARTZ S. (2015). First detection of the emerging fungal pathogen *Batrachochytrium salamandrivorans* in Germany. *Amphibia Reptilia*, **36**, 411–416.

SABINO-PINTO J., TOBIAS KRAUSE E., BLETZ M.C., MARTEL A., PASMANS F., STEINFARTZ S. & VENCES M. (2018). Detectability vs. time and costs in pooled DNA extraction of cutaneous swabs: a study on the amphibian chytrid fungi. *Amphibia Reptilia*, **40**, 1–11.

SPITZEN-VAN DER SLUIJS A., MARTEL A., ASSELBERGHS J., BALES E.K., BEUKEMA W., BLETZ M.C., DALBECK L., GOVERSE E., KERRES A., KINET T., KIRST K., LAUDELOUT A., MARIN D.A., FONTE L.F., NÖLLERT A., OHLHOFF D., SABINO-PINTO J., SCHMIDT B.R., SPEYBROECK J., SPIKMANS F., STEINFARTZ S., VEITH M., VENCES M., WAGNER N., PASMANS F. & LÖTTERS S. (2016). Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerg. Infect. Dis*., **22**, 1286–1288.

SPITZEN-VAN DER SLUIJS A., STEGEN G., BOGAERTS S., CANESSA S., STEINFARTZ S., JANSSEN N., BOSMAN W., PASMANS F. & MARTEL A. (2018). Post-epizootic salamander persistence in a disease-free refugium suggests poor dispersal ability of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **8**, 3800.

STEGEN G., PASMANS F., SCHMIDT B.R., ROUFFAER L.O., VAN PRAET S., SCHAUB M., CANESSA S., LAUDELOUT A., KINET T., ADRIAENSEN C., HAESEBROUCK F., BERT W., BOSSUYT F. & MARTEL A. (2017). Drivers of *Batrachochytrium salamandrivorans* mediated salamander extirpation. *Nature*, **544** (7650): 353+ DOI: 10.1038/nature22059

THOMAS V., BLOOI M., VAN ROOIJ P., VAN PRAET S., VERBRUGGHE E., GRASSELLI E., LUKAC M., SMITH S., PASMANS F. & MARTEL A. (2018). Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. *Transbound. Emerg. Dis*., **65**, e478-e488.

VAN ROOIJ P., MARTEL A., HAESEBROUCK F. & PASMANS F. (2015). Amphibian chytridiomycosis: a review with focus on fungus-host interactions. *Vet. Res*., **46**, 137.

VAN ROOIJ P., PASMANS F., COEN Y. & MARTEL A. (2017). Efficacy of chemical disinfectants for the containment of the salamander chytrid fungus *Batrachochytrium salamandrivorans*. *PLoS One*, 12, e0186269 DOI: 10.1371/journal.pone.0186269

WHITE L.W., FORZÁN M.J., PESSIER A.P., ALLENDER M.C., BALLARD J.R., CATENAZZI A., FENTON H., MARTEL A., PASMANS F., MILLER D.L., OSSIBOFF R.J., RICHGELS K.L.D. & KERBY J.L. (2016). Amphibian: a case definition and diagnostic criteria for *Batrachochytrium salamandrivorans* chytridiomycosis. *Herpetological Review*, **47**, 207.

YAP T.A., KOO M.S., AMBROSE R.F., WAKE D.B. & VREDENBURG V.T. (2015). BIODIVERSITY: Averting a North American biodiversity crisis. *Science*, **349** (6247), 481–482.

ZHIYONG Y., MARTEL A., WU J., VAN PRAET S., CANESSA S. & PASMANS F. (2018). Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conservation Letters*, 11: e12436.

*

* *

NB: There are currently no OIE Reference Laboratories for infection with *Batrachochytrium salamandrivorans*

NB: First adopted in 20XX.

Annex 23

Organisation Mondiale de la Santé Animale

World Organisation for Animal Health

Organización Mundial de Sanidad Animal

Original: English
September 2018–January 2019

REPORT OF THE OIE ELECTRONIC *AD HOC* GROUP ON TILAPIA LAKE VIRUS

September 2018–January 2019

Activities, achievements and next steps

This interim report covers activities and achievement of the *ad hoc* group September 2018 – January 2019. In 2018 several TiLV endemic countries were approached by the Director general of the OIE, Dr Monique Eloit, to provide positive TiLV material, for example China, Israel, Malaysia, Peru, Philippines and Thailand.

Material has been obtained from Peru (8 x tissue samples) and Thailand (1 x virus isolate from red tilapia cultured in the SSN-1 cell line). Three of the most promising samples from Peru and the cell culture isolate from Thailand were assessed for infectivity by passaging on the E11 cell line. No isolates were obtained from the material from Peru, however, cytopathic effect was observed after passage of the isolate from Thailand.

The Thailand isolate (designated 18-03492) was confirmed as TiLV after testing with the TiLV RT-nPCR assay described by Dong et al. (2017), with modifications to be consistent with AFDL test protocols, and sequence analysis of the 415bp gel purified RT-PCR amplicon. By BLAST search through the NCBI database, the 375 bp primer-trimmed amplicon shared the highest nucleotide identity of 94.7% with two isolates from Israel; Tilapia lake virus isolate Til-4-2011 segment 3 (KU751816.1) and Tilapia lake virus clone 7450 (KJ605629.1). The isolate tested negative after adventitious agent testing for KHV, NNV and ISKNV/RSIV.

A preliminary evaluation has been conducted with Thai isolate 18-03492 using the 3 real-time PCR assays (described below) to compare limits of detection (LOD) using nucleic acid extracted from the sample. Results are still to be analysed. Included in this evaluation was an assessment of two master mixes for use in the SYBR assay. The analytical sensitivity (ASe) for the Hong Liu assay, determined using a positive control plasmid diluted in water, is 2 plasmid copies per reaction. As plasmids have not been prepared for the other real-time assays, their ASe has not been determined.

Real-time PCR – probe based

1. Hong Liu – China, personal communication to the *ad hoc* Group.

2. Waiyamitra *et al.*, 2018.

Real-time PCR – SYBR

1. Tattiyapong *et al.*, 2017.

Isolate 18-03492 has been amplified in E11 cells and 100mL of clarified supernatant has been gamma-irradiated at 50kGy. This gamma-irradiated material will be tested by real-time RT-PCR to assess the effect of the gamma-irradiation on the viral genome. If results are as expected (i.e. minimal reduction in C_T value) the inter-laboratory comparability panels will be prepared as described in the "OIE *ad hoc* Group on tilapia lake virus (TiLV) November 2017-January 2018 report". After homogeneity and stability testing, it is anticipated the panels will be sent to collaborating laboratories in April 2019 for testing and reporting of results by July 2019. After analysis of results and discussion by *ad hoc* Group members, the *ad hoc* Group report of the inter-laboratory comparability testing and recommendations on test performance will be submitted to the OIE Aquatic Animal Health Standards AAHSC for its meeting in September 2019.

References:

Dong HT, Siriroob S, Meemetta W, Santimanawong W, Gangnonngiw W, Pirarat N, Khunrae P, Rattanarojpong T, Vanichviriyakit R, Senapin S (2017) Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection. Aquaculture 476, 111-118.

Tattiyapong P, Sirikanchana K, Surachetpong W (2017) Development and validation of a reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection in clinical samples and experimentally challenged fish. Journal of Fish Diseases 41, 255-261.

Waiyamitra P, Tattiyapong P, Sirikanchana K, Mongkolsuk S, Nicholson P, Surachetpong W (2018) A TaqMan RT-qPCR assay for tilapia lake virus (TiLV) detection in tilapia. Aquaculture 497, 184-188.

Annex 24

WORK PLAN OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION FOR 2018/2019

AQUATIC CODE			
Chapter/Subject	**Activity**	**Status at February 2019**	
		Number of times circulated for comments	*To be proposed for adoption May 2019 (A); For Member Country comments (C); For information (I);*
Definition of 'basic biosecurity conditions'	Amend the definition to make it more explicit in its application to compartments	3	A
Criteria for listing species as susceptible to infection with a specific pathogen (Chapter 1.5.)	Develop a new Article 1.5.9. to address diseases with a wide host range/review existing text	6	A
Infection with salmonid alphavirus (Chapter 10.5.)	Amend after consideration of the work of the *ad hoc* Group; horizontal changes	3	A
Infection with koi herpesvirus (Article 10.7.2.)	Amend after consideration of the work of the *ad hoc* Group; horizontal changes	3	A
Article 10.9.2. of Infection with spring viraemia of carp virus (Chapter 10.9.)	Amend after consideration of the work of the *ad hoc* Group; applied horizontal changes	3	A
Infection with Ranavirus (Chapter 8.3.)	Amend text to align with revised listed name, i.e. Infection with *Ranavirus* species	2	A
Acute hepatopancreatic necrosis disease (Chapter 9.1.)	Review use of AHPND and AHPND (VP) throughout the chapter	2	A
Articles 10.2.1. and 10.2.2. of Infection with *Aphanomyces invadans* (Chapter 10.2.)	Amend to ensure consistency with other amended fish disease-specific chapters; amend to remove the use of italics on Family names for fish	2	A
Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)	Amend after consideration of the work of the *ad hoc* Group; apply horizontal changes and change name of the pathogenic agent	2	A
Model Article X.X.8.	Review M 'lifelong holding' and address the safe disposal of dead aquatic animals or products derived thereof	2	A

Annex 24 (contd)

AQUATIC CODE (contd)			
Chapter/Subject	**Activity**	**Status at February 2019**	
		Number of times circulated for comments	*To be proposed for adoption May 2019 (A); For Member Country comments (C); For information (I);*
New draft chapter on Biosecurity for Aquaculture Establishments (Chapter 4.X.)	Develop draft chapter on Biosecurity for aquaculture establishments	2	C
Infection with shrimp haemocyte iridescent virus (SHIV); assessment for SIHV	Assessment of SHIV against the criteria for listing an aquatic animal disease in accordance with Chapter 1.2. Criteria for listing aquatic animal diseases	1	C
Approaches for determining periods required to demonstrate disease freedom	Develop approaches for determining periods required to demonstrate disease freedom	1	Under review by the Aquatic Animals Commission
Model Article 10.X.13.		1	C
Article 10.6.13. Infection with infectious haematopoietic necrosis virus (Chapter 10.6.)		1	C

AQUATIC MANUAL			
Chapter/Subject	**Activity**	**Status at February 2019**	
		Number of times circulated for comments	*To be proposed for adoption May 2019 (A); For Member Country comments (C); For information (I);*
Infection with yellow head virus genotype 1 (Chapter 2.2.9.)	Sort lists of species in the Sections in alphabetic order	3	A
Infectious haematopoietic necrosis (Chapter 2.3.4.)	Amend after consideration of the work of the *ad hoc* Group	2	A
Infection with salmonid alphavirus (Chapter 2.3.6.)	Amend after consideration of the work of the *ad hoc* Group; update information about the real-time reverse-transcription polymerase chain reaction; amend the ranking of histopathology for both presumptive and confirmatory diagnosis	3	A
Koi herpesvirus disease (Chapter 2.3.7.)	Amend after consideration of the work of the *ad hoc* Group	3	A

AQUATIC MANUAL (contd)			
Chapter/Subject	**Activity**	**Status at February 2019**	**Chapter/Subject**
		Number of times circulated for comments	*To be proposed for adoption May 2019 (A); For Member Country comments (C); For information (I);*
Spring viraemia of carp virus (Chapter 2.3.9).	Revise and reformat the chapter using the new disease template:	1	C
New draft Chapter 2.1.X. Infection with *Batrachochytrium salamandrivorans*		1	C
Ongoing *AD HOC* GROUPS			
Topic	**Last report**	**Next meeting**	
***Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases (to complete the assessment of infection with spring viraemia of carp virus [VHSV], infection with red sea bream iridovirus [RSIV] and infection with kidney necrosis virus [ISKNV])**	November 2018	2020	
Electronic *ad hoc* Group on Tilapia lake virus	February 2019	Work electronically and report to the AAHSC September 2019 meeting	
***Ad hoc* Group on the new *Aquatic Manual* template chapter**		Continuous process to commence work on applying the new chapter template to disease-specific chapters	
UNDER CONSIDERATION FOR FUTURE WORK			
Chapter/Subject	**Activity**	**Status**	
Restructure of Section 4 *Disease prevention and control* of the *Aquatic Code*	Ongoing process.		
Draft new chapter on emergency disease preparedness		Yet to be prioritised	
Draft new chapter on disease outbreak management	Support articles in each disease-specific chapter on returning to freedom following an outbreak	Yet to be prioritised	
Draft new chapter on application of zoning	Chapter specific to the application of zoning to provide clearer guidance on establishing zones for trade and disease control purposes	Yet to be prioritised	

Annex 24 (contd)

UNDER CONSIDERATION FOR FUTURE WORK		
Chapter/Subject	**Activity**	**Status**
Review structure and application for different trade purposes of articles in disease-specific chapters	Re-structure disease-specific articles for aquatic animal products and live aquatic animals not for human consumption, including ornamentals	Yet to be prioritised
Safe commodities		Yet to be prioritised
Susceptibility of amphibian species to infection with OIE listed diseases		Yet to be prioritised
Work to apply Article 1.5.9. to OIE listed diseases (VHSV, WSSV, Crayfish plague, EUS)		VHSV as part of the ongoing work of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases. WSSV, Crayfish plague and EUS yet to prioritized.
Standards concerning broodstock and the safe production and trade with genetic products		Yet to be prioritised