Annex 2

Annex 17

CHAPTER 4.X.

BIOSECURITY FOR AQUACULTURE ESTABLISHMENTS

EU comment

The EU thanks the OIE and can support most of the changes proposed to the chapter, except for those proposed in Articles 4.X4., 4.X.6. and 4.X.8.

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.

EU comment

The EU suggests expanding point 6) to include record keeping in the General Principles for Biosecurity to support the principle of review and audit, as follows:

"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. To facilitate effective review and audit, and to provide evidence and assurance for the implementation of biosecurity measures, appropriate records and documentation should be kept. Record keeping, audit and review are described in Article 4.X.8."

Justification: Accurate records and documentation are important and necessary for effective audit and review, and provide evidence for the implementation of Biosecurity Measures.

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.

EU comment

The EU suggests including the following either at the end of 1.g) or as an additional point in its own right, as follows:

"Suspicion of notifiable disease and unexplained or unusual mortalities in aquatic animals must be reported immediately to the Competent/ Veterinary Authority, investigation and diagnosis of the cause of mortality must be undertaken."

Justification: The prompt reporting on suspicion of notifiable disease and of unexplained or unusual mortalities in aquatic animals to the Competent/ Veterinary Authority facilitates early application of official disease control measures, investigation and diagnosis, and is an important mitigation measure to reduce the risk of further spread of pathogenic agent.

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.

EU comment

The EU suggests that this section includes the following statement at the end of the second paragraph above, as follows:

"Movement of aquatic animal waste into aquaculture establishments should be kept to an absolute minimum and avoided where possible. Waste should be stored, transported, disposed of and treated following the guidance in OIE Aquatic Code Chapter 4.7 Handling, Disposal and Treatment of Aquatic Animal Waste."

Justification: given the risk associated with aquatic animal products and waste, this is suggested to emphasise best practice and refer to the related guidance in the OIE Aquatic Code for management of risk associated with waste.

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.

EU comment

The EU suggests the addition of a line to the above paragraph to highlight the risk of pathogen introduction through flooding, as follows:

"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, the risk of introduction through flooding should also be considered and flood risk assessed. Exposure to transport water and ballast water should be considered."

Justification: Flooding can increase the risk of disease introduction and spread; therefore, flood risk should also be assessed and appropriate mitigation measures applied. For example, this would be a particular risk to sites that draw from a protected water source but may also be at risk of water incursion through flooding.

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*.

EU comment

The EU suggests an additional point to highlight the risk associated with inappropriate storage and management of feed on site and measures to mitigate the risk, as follows:

"<u>d) are managed and handled on site in such a way that ensures there is no risk of subsequent contamination. Food must be stored in a secure, dry location, containers with lids kept on and protected from risk of splashing/ contamination by water. During feeding - feed must not be delivered in a way that risks contamination or subsequent spread across the site.</u>"

Justification: The risk measures listed above are important for ensuring effective management of risk associated with food prior to being used on site, in addition we suggest that consideration is also given to risks that could contaminate feed through poor practice on site. For example, small scale sites may feed by hand which risks contamination of the feed container through wet hands/ equipment/ splashing, and facilitate spread of disease within a site.

5. <u>Fomites</u>

Equipment, *vehicles*, clothing, <u>footwear,</u> 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 <u>should be managed; possible mitigation measures include the following</u> ~~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.

EU comment

The EU suggests the expansion of risk mitigation measures for fomites to emphasise and cover the risk associated with equipment used across multiple sites. We suggest doing this expanding point b), as follows:

"b) Assigning dedicated equipment, protective clothing and footwear for sole use in the aquaculture establishment. Where this cannot be avoided, movements of equipment between establishments must be kept to an absolute minimum, 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."

Justification: Point c) refers to dedicated equipment for use in production units of different health status, however, consideration should also be given to the use of equipment across multiple sites – which does occur in industry, and for this reason we suggest this risk mitigation is also specifically highlighted.

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~~ production loss in ~~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;

EU comment

The EU suggests slightly expanding points f) and g) with extra information included in the guidance, as follows:

"*f)* emergency procedures in the event of a *biosecurity* failure; this may include measures to eradicate *pathogenic agents* such as stock culling and site disinfection, also reference Code chapters 4.3 and 7.4.

***g)* internal and external communication procedures, and roles and responsibilities of personnel and essential contact information e.g. for personnel, the Competent Authority and Veterinarian;"**

Justification: Both f) and g) are really important points to ensure effective and prompt response to stock health issues/ biosecurity failure, however, emergency procedures could include more severe and large-scale measures for pathogen eradication such as stock cull and site disinfection so we would suggest considering adding these as specific examples in relation to point f) to make the scope of the point clear, and then to reference the related OIE Code chapters for ease.

Also, with ref to point g) it is advisable to include contacts along with documented communication procedures, roles and responsibilities, this facilitates prompt action and effective comms. and we therefore request the OIE AAC considers also including this point.

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

~~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.

EU comment

The EU suggests consider removing the word 'may' from the examples of documentation in point b), as follows:

"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."

Justification: All the documents and records listed here are good practice and, in many cases, are essential records for which there is often a legal requirement that they are kept (eg movement records, mortality records, medicine records). Given that the records are listed here as examples only, and include some that are essential, we suggest this minor adjustment is considered to make the wording more definite.

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 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.

Annex 18A (contd)

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

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Annex 18B

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

EU comment

The EU supports the proposed changes to Article 1.3.3.

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 Taura syndrome virus

– Infection with white spot syndrome virus

– Infection with yellow head virus genotype 1

– Infection with shrimp haemocyte iridescent virus.

Annex 19

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)

EU comment

The EU in general supports the proposed changes to this article and has one comment included in the text below.

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

EU comment

The inclusion of the possibility to follow the Competent Authoritiy's recommendations for the disinfection of eggs could undermine the OIE standards. Indeed, as drafted in point a) above, any recommendations of an importing country would need to be followed by the exporting country, even if these were not based on science. On the other hand, general equivalence principles are already layed out elsewhere in the Aquatic Code. For these reason swe suggest deleting the words "or those specified by the *Competent Authority* of the *importing country*".

   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.

Annex 20

CHAPTER 10.6.

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

EU comment

The EU supports the propoposed changes to this chapter.

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.

Annex 21A

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

EU comment

The EU can in general support most of the changes proposed to this chapter, however we have a few comments in different sections and a number of editorial suggestions, mainly those related to wrong cross references. We have checked throughout, we have found several that are incorrect but there may well be others that we have missed. We have also added two new comments in section 2.2.1 and table 4.1.

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*).

EU comment

We suggest including Rainbow trout (*Oncorhynchus mykiss*) as a susceptible host species in section 2.2.1 (Jeremíc *et al.* 2006 and Asl *et al.* 2008)

~~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).

EU comment

We suggest considering the addition of the common catfish (*Ameirius spp.*) as a susceptible species in 2.2.1 or at least in section 2.2.2 below as "Species with incomplete evidence for susceptibility).

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.

EU comment

We have some queries regarding the last paragraph in section 3.1. There is potentially confusing use of the word 'group' (used with two different meanings), suggest replacing 'age groups' with 'ages', as follows:

"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~~ classes 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."

In addition, we request that the context for the sample collection described by this paragraph be clarified – we assume this is for the purpose of surveillance in clinically healthy animals where it would be appropriate to select a representative sample of all susceptible species on site, rather than for disease investigation where clinically affected animals are targeted (and as described in the first paragraph).

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.

EU comment

We believe that target organs and tissues for subclinical/ healthy fish also needs to be included here.

In addition, this is using terminology commonly associated with salmonids (alevin), suggest instead:

"For clinically affected fish: whole fry ~~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.

EU comment

To avoid confusion, the EU suggests only referring to the chapter that contains general guidance for fish sample preservation and not referencing the chapter for crustaceans

and molluscs (also to note if the intention here was to reference all general information chapters, the amphibian chapter 2.1.0 is missing).

~~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.

EU comment

In relation to RT-PCR we acknowledge the limitations because of the partial validation of these methods. However, we believe that RT-PCR should be scored in Table 4.1 at least with similar scores as the LAMP method.

In the same table 4.1 we suggest renaming "Real-time PCR" to "Real-time RT-qPCR" and "Conventional PCR" to "Coventional RT-PCR".

The footnotes of table 4.1 should also be changed to: "RT-PCR = reverse transcriptase-polymerase chain reaction; RT-qPCR = real time RT-PCR" as this is an RNA virus.

Footnote 4 should also be amended as followes: "Sequencing of the RT-PCR product".

⇒ 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.

EU comment

We suggests reviewing and correcting the cross references, for example above:

"Virus extraction: Use the procedure described in Chapter 2.3.0., Section ~~A.2.2.2~~ 2.2.2."

This also applies to subsequent sections making the same reference throughout.

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~~).

EU comment

The above reference to section 4.6.2. needs double-checking – there is no section 4.6.2., and section 4.6. is for immunohistochemistry, which is not a recommended method in this case.

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 spriviviruse*s (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).

Annex 21A (contd)

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.~~

Annex 21A (contd)

~~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 (*).~~

Annex 21A (contd)

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 <u>demonstrate</u> ~~declare~~ freedom ~~from spring viraemia of carp~~ <u>in apparently healthy populations</u>

The method for surveillance of susceptible fish populations for declaration of freedom from <u>infection with</u> SVC<u>V</u> is inoculation of cell culture with tissue extracts (as described in Section 4.5.) to demonstrate absence of the virus.

EU comment

We believe that the above paragraph reference to section 4.5 is incorrect, the cell culture method is section 4.3.

6. Corroborative diagnostic criteria

<u>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.</u>

EU comment

The section references in the above paragraph need double-checking, as they appear to be the wrong way round…

Section 6.1 below is for apparently healthy animals, without absence of clinical signs

Section 6.2 below is for clinically affected animals, in the presence of clinical signs.

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[1]

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

[1] For example transboundary commodities.

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

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Annex 21A (contd)

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

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 20xx.

Annex 21B

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.

Annex 21B (contd)

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.

Annex 21B (contd)

⇒ 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).

Annex 21B (contd)

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.

Annex 21B (contd)

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[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)

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

[2] 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

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* *

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

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 20xx.

Annex 22

CHAPTER 2.1.X.

INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

EU comment

The EU in general supports this proposed new chapter, however we have some suggestions to substantiate the text including extra references and some comments that have been inserted in the text below.

The European Food Safety Authority (EFSA) has produced three scientific opinions on Bsal which address all the sections covered by this draft OIE Manual chapter and we would thus suggest referencing those EFSA scientific opinions in this chapter.

EFSA, 2017. Scientific and technical assistance concerning the survival, establishment and spread of Batrachochytrium salamandrivorans (Bsal) in the EU, EFSA Journal 2017;15(2):4739 (https://www.efsa.europa.eu/en/efsajournal/pub/4739).

EFSA AHAW Panel, 2017. Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): Batrachochytrium salamandrivorans (Bsal), EFSA Journal 2017;15(11):5071 (https://www.efsa.europa.eu/en/efsajournal/pub/5071).

EFSA AHAW Panel, 2018. Risk of survival, establishment and spread of Batrachochytrium salamandrivorans (Bsal) in the EU, EFSA Journal 2018;16(4):5259 (https://www.efsa.europa.eu/en/efsajournal/pub/5259).

In addition, we have some concerns about the lack of an OIE reference laboratory for Bsal and Bd. We have therefore encouraged EU Member States to enquire among their experts whether they could consider applying for OIE reference laboratory status.

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.

EU comment

In relation to the effectof dessication, these fungi are dependent on water and desiccation is fatal to all life stages *(Johnson et al., 2003; Van Rooij* et al., *2015).*

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).

EU comment

According to EFSA scientific opinions, data on host specied susceptibility should be interpreted with caution, as they are based on results from a small number of study animals, which mostly originated from the same source populations. In addition, the species that tested negative cannot be considered resistant.

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.

EU comment

Bsal has been detected in 5 populations in captivity (EFSA AHAW Panel, 2017)

In relation to the last sentence "Hence, urodeles that…", we consider that this statement may be too strong and it could be rephrased including the measures that could be put in place when trading salamanders in order to reduce the risk of contracting Bsal infection (EFSA AHAW Panel, 2018, section 3.2.3 and following).

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.

EU comment

With reference to EFSA AHAW Panel, 2018, we suggest adding a last sentence as follows:

"The knowledge on wild frog species as Bsal carriers is limited to a single Asian species, *Bombina microdeladigitora,* a species that is not used in the food trade (Nguyen *et al*., 2017)".

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

EU comment

In addition to EFSA's work (EFSA, 2017, and EFSA AHAW Panel, 2017), we suggest adding the following reference:

Zhu et al, 2014: Zhu W, Xu F, Bai C, Liu X, Wang S, Gao X, Yan S, Li X, Liu Z and Li Y, 2014. A survey for *Batrachochytrium salamandrivorans* in Chinese amphibians. Current Zoology, 60, 729–735.

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.

EU comment

We suggest mentioning that heat treatment can be applied to the thermo-tolerant species of salamanders (EFSA AHAW Panel, 2018 , section 3.2.1.2).

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, 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.

EU comment

For species that could act as sentinels and in the scenario of 'close populations', the use of a sentinel, a susceptible individual to be cohoused during the quarantine, has been considered as an alternative to Bsal testing of all the salamanders at the end of the quarantine. Possible sentinel species are *Salamandra salamandra* and other hyper susceptible species (see Martel et al., 2014 and AHAW Panel, 2018).

3. Specimen selection, sample collection, transportation and handling

This Section draws on information from Sections 2.2., 2.3. and 2.4. to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

In 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.

EU comment

This table scores all laboratory methods; however, those methods have not been mentioned in the body of the chapter. This seems to be odd when compared to how other OIE *Aquatic Manual* chapters are drafted.The table could also benefit from an extra column with 'references' which may motivate/substantiate scoring (+, ++, +++) provided.

Annex 22 (contd)

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).

EU comment

The EU invites the OIE to reappraise the validation process of the TaqMan PCR and to confirm whether validation has truly been performed in accordance with Chapter 1.1.2. of the Aquatic Manual. Indeed, as indicated in Table 4.1., that test was only partially validated up to level 3. Furthermore, the intended purpose for which the test was validated and for which it would be fit is not stated in the referenced publication by Thomas *et al*. 2018. We would thus suggest amending the text of the first sentence of the paragraph above as follows:

"A TaqMan PCR has been partially validated up to level 3 however without stating its intended purpose (Thomas *et al*., 2018)."

Furthermore, the EU suggests inserting the following sentence in the paragraph above after "[...] when used in clinically affected animals.":

"However, for trade or field purposes a more conservative lower confidence bound of 80,4% sensitivity should be used (EFSA AHAW Panel, 2017)."

Indeed, this would be important for trade or field purposes, as opposed to laboratory experimental purposes. Reference is made to a Scientific Opinion of EFSA on the subject, in particular to table 3 on page 18 (EFSA AHAW Panel, 2017).

Finally, we note that the entire section 4.4.1. heavily refers to trademarked reagents and commercially available laboratory kits. Even if reference is made to the scientific publications from where this infomeation is derived, this seems very unusual for an OIE Standard and should preferably be avoided.

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.

EU comment

In general Section 4 describes the diagnostic assays that can be used in (i) surveillance of apparently healthy populations, (ii) presumptive and (iii) confirmatory diagnostic purposes.

In the table Real-time PCR is indicated with +++ in all columns whereas in the paper of Thomas *at al*., (sensitivity between 96-100% and specificity between 73-100%) it is clearly mentioned in the narrative text that the real time PCR was used/validated in clinically infected/affected animals.

Therefore it would be good to be consistent in the terminology used in Table 4.1 with relation to the validation stage and its purpose . For what purpose should the test be used and is the diagnostic assay validated accordingly (fit for purpose principle)?

These principles are explained in the OIE Aquatic Manual Chapter 1.1.6 . From that chapter: the first step in assay development is to define the purpose of the assay, because this guides all subsequent steps in the validation process. Assay validation criteria are the characterising traits of an assay that represent decisive factors, measures or standards upon which a judgment or decision may be based. By considering the variables that affect an assay's performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into categories such as: (a) the sample – individual or pooled, matrix composition, and host/organism interactions affecting the target analyte quantitatively or qualitatively; (b) the assay system – physical, chemical, biological and operator-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result interpretation – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the purpose for which the assay is applied.

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[3]

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:

EU comment

The word "shall" should be replaced by the word "should" throughout the chapter, as is common practice in OIE Standards (except in Chapter 1.1. of the Codes).

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.

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.

[3] For example transboundary commodities.

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.

EU comment

As there currently is no OIE reference laboratory for Bsal, the paragraph above should preferably be deleted.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

7. References

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NB: There are currently no OIE Reference Laboratories for infection with *Batrachochytrium salamandrivorans*

NB: First adopted in 20XX.