Annex 4

Comments on Manual of Diagnostic Tests for Aquatic Animals from P.R. China

CHAPTER 2.3.9. INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

General comment: We suggest that the rainbow trout（*Oncorhynchus mykiss*）shall be listed as susceptible host species of spring viraemia of carp. In the meantime, to move rainbow trout into the list of susceptible host species (Aquatic Manual, chapter 2.3.9, section 2.2.1) from the section of species with incomplete evidence for susceptibility (Aquatic Manual, chapter 2.3.9, section 2.2.2).

Rational： 1 Rainbow trout fulfills the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5 of the Aquatic Animal Health Code (stage 1-3), the reasons as follows:

1.1 i) The virus, SVCV, was isolated from rainbow trout (Asl *et al*., 2008; Jeremić *et al*., 2006; Stone *et al*., 2003), these reports indicate rainbow trout is infected with SVCV in wild or farmed populations, which fulfill the criteria of point No.1, Article 1.5.4 of Chapter1.5 in Aquatic Code.

ii）The result of challenge test show that the mortalities of rainbow trout are 40% and 15% by injection and immersion, respectively (Emmenegger *et al*., 2016), so rainbow trout is sensitive to SVCV under experimental conditions, which fulfill the criteria of No.2 and 3, Article 1.5.4 of chapter1.5 in Aquatic Code.

So, the i) and ii) would meet the requirements of stage 1 in Article 1.5.3 of Chapter1.5 in Aquatic Code.

1.2 The SVCV were isolated and identified from symptomatic rainbow trout by virus isolation and RT-PCR (Stone *et al*., 2003; Jeremić *et al*. 2006). The results fulfill the criteria to determine whether the pathogenic agent has been adequately identified in Article 1.5.5 of chapter1.5 in Aquatic Code (stage 2).

1.3 i) Emmenegger （Emmenegger *et al*.,2016）reported that the majority of rainbow and steelhead trout that died during challenge tested positive for virus (n= 10/11) with the lowest titer of 1.5 X10^2 PFU/g from a fish that died on day 3 post-exposure and the highest titer (1.0 X 10^7 PFU/g) in a fish that died on day 16. The results indicate SVCV can grow or replicate in rainbow trout, and developing stages of the SVCV are present in the rainbow trout. This meets the requirements of point A in Article 1.5.6., Chapter1.5 in Aquatic Code.

ii) the clinic signs was observed from sick rainbow trout in both farmed population (Jeremić *et al*.

2006) and challenge test (Emmenegger *et al*.,2016), and SVCV was isolated from both farmed and challenged rainbow trout. This fulfills the rules of point B, C and D in Article 1.5.6., Chapter1.5 in Aquatic Code.

2 Reference

[1] Asl, A.H.K., Azizzadeh, M., Bandehpour, M., Sharifnia, Z., Kazemi, B., 2008a. The first report of SVC from Indian carp species by PCR and histopathologic methods in Iran. Pakistan J. Biological Sci. 11, 2675-2678.

[2] Emmenegger E J, Sanders G E, Conway C M, *et al*. Experimental infection of six North American fish species with the North Carolina strain of spring Viremia of Carp Virus. Aquaculture, 2016, 450: 273-282.

[3] Jeremić, S., Ivetić, V., Radosavljević, V., 2006. Rhabdovirus carpio as a causative agent of disease in rainbow trout (*Oncorhynchus mykiss*-Walbaum). Acta veterinaria, 56, 553-558.

[4] Stone, D.M., Ahne, W., Denham, K.L., Dixon, P.F., Liu, C.T., Sheppard, A.M., Taylor, G.R., Way, K., 2003. Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups.Dis. Aquat. Org. 53, 203-210.

Text as presented:

4. Diagnostic methods

……

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												
Histopathology[3]												
Cytopathology[3]												
Cell or artificial media culture		++	++	1		++	++	1		++	++	1
Real-time PCR												
Conventional PCR		++	++	1		++	++	1		++	++	1
Amplicon sequencing[4]										+++	+++	1
***In-situ* hybridisation**												
Immunohistochemistry						++	++	1				
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA						++	++	1				
IFAT ~~Other antigen detection methods~~						++	++	1				

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; 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 2.2.4.
[3]Histopathology and cytopathology can be validated if the results from different operators have been statistically compared. [4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose.

Proposed alternative text:

4. Diagnostic methods

……

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												
Histopathology[3]												
Cytopathology[3]												
Cell or artificial media culture		++	++	~~1~~3		++	++	~~1~~3		++	++	~~1~~3
Real-time PCR												
Conventional PCR		++	++	1		++	++	1		++	++	1
Amplicon sequencing[4]										+++	+++	1
***In-situ* hybridisation**												
Immunohistochemistry						++	++	1				
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA						++	++	1				
IFAT ~~Other antigen detection methods~~						++	++	1				

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; 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 2.2.4
[3]Histopathology and cytopathology can be validated if the results from different operators have been statistically compared. [4]Sequencing of the PCR product.
Shading indicates the test is inappropriate or should not be used for this purpose.

Rationale: The note at the bottom of table 4.1 indicated that "LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2)". In the validation pathway of Figure 1 (chapter 1.1.2 of the *Aquatic Manual*), stage 1 is analytical characteristics. Stage 2 is diagnostic characteristics and the stage 3 is reproducibility. Virus isolation in cell culture is a "gold" method, and the isolation of SVCV by cell culture is a traditional method which has been tested for many years. It is the most credible method and should be classified as the level 3.

Text as presented:

4.3. Cell or artificial media culture for isolation

.....

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.

Proposed alternative text:

4.3. Cell or artificial media culture for isolation

.....

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 occurs in re-culture, the test may be declared negative finally.

Rationale: In the text above, it is recommended to use RT-PCR to reconfirm negative resulted of samples of virus isolation. This is actually to use a low-confidence detection method (RT-PCR) to confirm high-confidence detection methods (virus isolation in cell culture--a kind of "gold standard"). It is expected that a large number of false positive results would occur. It will be very difficult to deal with these samples and the farms where they are located.

4.4.2. Conventional PCR

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Reverse-transcription polymerase chain reaction (RT-PCR) (confirmation of virus identity)

Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutna *et al*., 2003; Shimahara *et al*., 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al*., 2014). With the exception of the conventional PCR assay

developed by Shimahara *et al*. (2016) the other assays were not sufficiently ~~fully~~ validated against representatives from each of the recognised SVCV genogroups and they may fail to detect the full range of SVCV genotypes.

A summary of the Shimahara *et al*. (2016) RT-PCR method follows. Amplification of a 369 bp fragment of SVCV glycoprotein gene is performed using primers as follows: SVCV-G1: 5'-TGA-AGA-YTG-TGT-CAA-TCA-AGTC-3' and SVCV-G2: 5'-GCG-ART-GCA-GAG-AAA-AAG-TG-3'. Preparation of RNA template is the same as nested RT-PCR above. Reverse transcription of SVCV RNA and amplification of cDNA are carried out using SuperScript III one-step RT-PCR with PlatinumR Taq (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction mixture contained 10 pmol of each primer, 12.5 µl of 2× reaction mix, 1 µl of SuperScript III RT/Platinum *Taq* Mix and 2.5 µl template. After reverse transcription at 50°C for 30 minutes and 94°C for 2 minutes, 40 amplification cycles of 94°C for 15 seconds, 56°C for 30 seconds and 68°C for 1 minute followed by a final extension step at 68°C for 7 minutes is performed. All amplified products are confirmed as SVCV in origin by sequencing.

Proposed alternative text:

4.4.2. ~~Conventional~~ Nested RT-PCR

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~~*Reverse-transcription polymerase chain reaction (RT-PCR) (confirmation of virus identity)*~~

~~Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutna *et al*., 2003; Shimahara *et al*., 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al*., 2014). With the exception of the conventional PCR assay developed by Shimahara *et al*. (2016) the other assays were not sufficiently fully validated against representatives from each of the recognised SVCV genogroups and they may fail to detect the full range of SVCV genotypes.~~

~~A summary of the Shimahara *et al*. (2016) RT-PCR method follows. Amplification of a 369 bp fragment of SVCV glycoprotein gene is performed using primers as follows: SVCV-G1: 5'-TGA-AGA-YTG-TGT-CAA-TCA-AGTC-3' and SVCV-G2: 5'-GCG-ART-GCA-GAG-AAA-AAG-TG-3'. Preparation of RNA template is the same as nested RT-PCR above. Reverse transcription of SVCV RNA and amplification of cDNA are carried out using SuperScript III one-step RT-PCR with PlatinumR Taq (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction mixture contained 10 pmol of each primer, 12.5 µl of 2× reaction mix, 1 µl of SuperScript III RT/Platinum *Taq* Mix and 2.5 µl template. After reverse transcription at 50°C for 30 minutes and 94°C for 2 minutes, 40 amplification cycles of 94°C for 15 seconds, 56°C for 30 seconds and 68°C for 1 minute followed by a final extension step at 68°C for 7 minutes is performed. All amplified products are confirmed as SVCV in origin by sequencing.~~

Rational: The conventional PCR (Shimahara et al. , 2016) was added into Chapter 2.3.9 of Aquatic Manual. However, the assay performance was not validated according to the requirements in chapter 1.1.2, Aquatic Manual. Even we do not know the diagnostic sensitivity and specificity of this method. So we suggest to remove the conventional PCR (Shimahara et al. , 2016) from chapter 1.1.2. Maybe this method will be ready to list in chapter 1.1.2 of Aquatic Manual when it is validated completely. As all the other texts in article 4.4.2 are about Nested RT-PCR,so we suggest to change the title of article 4.4.2 to "Nested RT-PCR".

CHAPTER 2.1.X.
INFECTION WITH
BATRACHOCHYTRIUM SALAMANDRIVORANS

Text as presented:

4.9. 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*., 2017 ~~2016~~). The sensitivity of this test is likely to be lower than that of the real-time ~~q~~PCR (Dillon et al., 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using real-time ~~q~~PCR. This would make this technique most useful in animals with high infection loads. Such techniques may be useful for point-of-care testing if specificity is increased and provided thorough validation.

Proposed alternative text:

4.9. 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*., 2017 2016). The sensitivity of this test is likely to be lower than that of the real-time qPCR (Dillon et al., 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using real-time 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.~~
None validated.

~~Rationale: Because of the cross reaction between BSAL monoclonal antibody and other pathogens, LFA method has low specificity. This method is not been verified and not recommended in table 4.1 of chapter 2.1. X. So it is suggested to delete this~~

~~paragraph or change it to "none validated".~~

Text as presented:

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:

……

ii) Positive result by real-time PCR on at least one swab or skin tissue.

……

Proposed alternative text:

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:

……

~~ii) Positive result by real-time PCR on at least one swab or skin tissue.~~

……

Rationale: It is not different between the criteria ii) in Section 6.2.1 and the criteria i) in Section 6.2.2. The criteria “positive result by real-time PCR” cannot be used twice in suspect case and confirmed case at the same time. Once the result of real-time PCR is positive, the animal is confirmed (rather than suspicious) as positive. Therefore, the criteria ii) in Section 6.2.1 should be deleted.

CHAPTER 2.3.4.
INFECTION WITH INFECTIOUS
HAEMATOPOIETIC NECROSIS VIRUS

Text as presented:

6.1.2. Definition of confirmed case in apparently healthy animals

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iii) Isolation of virus in cell culture confirmed by IFAT or Ag-ELISA or by a neutralisation test and conventional PCR and amplicon sequencing;

iv) Isolation of virus in cell culture confirmed by conventional PCR and amplicon sequencing.

Proposed alternative text:

6.1.2. Definition of confirmed case in apparently healthy animals

……

iii) Isolation of virus in cell culture confirmed by IFAT or Ag-ELISA or by a neutralisation test ~~and conventional PCR and amplicon sequencing~~;

iv) Isolation of virus in cell culture confirmed by conventional PCR and

amplicon sequencing.

OR

6.1.2. Definition of confirmed case in apparently healthy animals

……

iii) Isolation of virus in cell culture confirmed by IFAT or Ag-ELISA or by a neutralisation test-and conventional PCR and amplicon sequencing;

~~iv) Isolation of virus in cell culture confirmed by conventional PCR and amplicon sequencing.~~

Rationale: The combination iii) contains the entire content of combination iv). The combination iv) is only part of it. If the combination iv) is considered to have enough the condition for positive confirmation already, the combination iii) are superfluous and should be shortened.

Conversely, if the combination iii) is considered to meet the conditions for confirming positive, then the combination iv) lacks sufficient conditions and should be deleted. The same is true of article 6.2.2 in Annex 12, so it is suggested to revise it too.

CHAPTER 2.3.10.
INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

Text as presented:

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

6.1.2. Definition of confirmed case in apparently healthy animals

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

i) VHSV isolation in cell culture followed by virus identification by conventional RT-PCR, and by sequencing of the amplicon;

ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;

iii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;

iv) Detection of VHSV in tissue preparations by real-time RT-PCR, and by conventional RT-PCR and sequencing of the amplicon.

Proposed alternative text:

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

6.1.2. Definition of confirmed case in apparently healthy animals

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

i) VHSV isolation in cell culture followed by virus identification by conventional RT-PCR followed~~, and~~ by sequencing of the amplicon, or real-time RT-PCR, or Ag-ELISA, or IFAT;

ii) Detection of VHSV in tissue preparations by conventional RT-PCR followed by~~–~~ sequencing of the amplicon and real-time RT-PCR;

~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;~~

~~iii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;~~

~~iv) Detection of VHSV in tissue preparations by real-time RT-PCR, and by conventional RT-PCR and sequencing of the amplicon.~~

Rationale: There are only two cases here, one using cell isolation and identification on the cell culture， another using tissue preparations and identification. To revise like above makes it more clear and easy to understand. The same is true of article 6.6.2 , it is suggested to revise it too.